Exploration of Repositioned Drugs in the Management of Renal Ischemia Reperfusion Injury.

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology and Laboratory Medicine

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Abstract

Recent evidence demonstrates that oxygenated perfusion at 22°C is superior to static cold storage for preserving pre-transplanted kidneys; nonetheless, there is opportunity for improvement. Therefore, we aim to repurpose existing off-patent drugs in combination with oxygenated perfusion to further reduce organ damage caused by ischemia reperfusion injury. Through the development and characterization of both cold and room temperature injury models, the room temperature-based model demonstrated decreased pro-inflammatory cytokines and increased cell viability compared to previously developed cold IRI model. These findings provide evidence that the room temperature-based model can not only mitigate the risk associated with cold IRI, but also serve as a viable platform for conducting large-scale drug repositioning studies. Piloting this model for a small-scale drug screening, we identified several candidates with potential anti-inflammatory properties applicable to mitigating IRI. Consequently, the development of a novel preservation system can increase the availability of healthy donor kidneys for transplant and enhance patient long-term outcomes.

Keywords

Transplantation, kidney, ischemia reperfusion injury, drug therapy, drug repositioning.
Summary for Lay Audience

Approximately 1.2 million people globally die from kidney failure each year. While kidney transplantation is better for patients than dialysis, there are not enough organs available for transplant to meet the ever-increasing demand. This is because there is an entire category of organs not eligible for transplantation, known as donation after circulatory death (DCD) kidneys. DCD kidneys are too damaged for transplant because blood has stopped flowing within the organ for a prolonged period of time. A well-known phenomenon called ischemia reperfusion injury, is the process of an organ temporarily losing oxygen, followed by the return of oxygen, which results in harm to the organ and can have detrimental effects for organ recipients down the road. Cold storage, the current standard for preserving a kidney during transplantation, would not prevent further damage. As a result, research has proven that preservation at room temperature using a machine pump (that can circulate blood through the organ) can protect the organ long enough to be transplanted. While machine preservation is known to reduce injury in organs during preservation, there is still a considerable gap. Therefore, our goal is to discover drugs that can protect the organ from harm during storage and transport. Drugs that are already on the market for a specific purpose, may have additional effects that are not yet known, and their secondary uses can be just as helpful in clinical practice. This thesis has implemented a laboratory-created simulation using kidney cells that mimics the events that take place during transplantation and machine pump preservation. Using this simulated cell-based model, a small-scale drug screening was conducted, where several drugs were identified as having some protective effects against the damage relating to IRI. By developing this novel preservation system, we can create better preservation methods to protect at-risk kidneys for more long-term organ function, which can ultimately lead to better quality of life and improved survival rates.
Acknowledgements

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>7-AAD</td>
<td>7-Aminoactinomycin D</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage-associated molecular patterns</td>
</tr>
<tr>
<td>DBD</td>
<td>Donation after brain death</td>
</tr>
<tr>
<td>DCD</td>
<td>Donation after cardiac death</td>
</tr>
<tr>
<td>DGF</td>
<td>Delayed graft function</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia-inducible factor 1</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IRI</td>
<td>Ischemia reperfusion injury</td>
</tr>
<tr>
<td>ITS</td>
<td>Insulin-transferrin-selenium</td>
</tr>
<tr>
<td>KRT</td>
<td>Kidney replacement therapy</td>
</tr>
<tr>
<td>MAPK/ERK1/2</td>
<td>Mitogen-activated protein kinase/extracellular signal–regulated kinase 1/2</td>
</tr>
<tr>
<td>mPTP</td>
<td>Mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa-light-chain enhancer of activated B cell</td>
</tr>
<tr>
<td>NGAL</td>
<td>Neutrophil gelatinase associated lipocalin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PI3K/AKT</td>
<td>Phosphatidylinositol 3-kinase/protein kinase B</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RIPK1</td>
<td>Receptor-interaction serine/threonine kinase 1</td>
</tr>
<tr>
<td>RIPK3</td>
<td>Receptor-interaction serine/threonine kinase 3</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature (22°C)</td>
</tr>
<tr>
<td>SCS</td>
<td>Static cold storage</td>
</tr>
<tr>
<td>SMP</td>
<td>Subnormothermic machine perfusion</td>
</tr>
<tr>
<td>sIRI</td>
<td>Simulated IRI</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>UW</td>
<td>University of Wisconsin</td>
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Chapter 1

1 Introduction

1.1 End Stage Renal Disease

End-stage renal disease (ESRD) is the final stage of chronic kidney disease (CKD) where the kidney has sustained irreversible damage and is no longer capable of performing its normal functions, such as removing wastes from the body (Hall & Chertow, 2007). ESRD is fatal if untreated. Treatment options include dialysis or, receiving a kidney transplant from a donor, which is the more effective option.

According to the most recent results of a 2019 cross-sectional study, 13.4% of individuals globally have CKD, and at least 4 million Canadians suffer from this condition (Bello et al., 2019). However, in 2010, while it was projected that 4.9–9.7 million people needed kidney replacement therapy (KRT), it was estimated that only 2.6 million individuals received a transplant globally (Thurlow et al., 2021). This suggests that only around half of those needing KRT had access to it, while 2.3 million people likely perished because of a lack of access to lifesaving treatments (Thurlow et al., 2021). While the topic of treatment availability is multifaceted – significantly influenced by the prevalence of disease within a region, cultural belief systems, and socioeconomics – a key determinant of ESRD outcomes remain a continuing shortage of kidneys that are appropriate for transplant. Given that the number of individuals who will require KRT is expected to climb to 5.4 million by 2030, and that effective preventative therapies will not yet be available in the near future, the burden of CKD and ESRD is approaching unsustainable levels for health care systems (Kitzler & Chun, 2023).
1.2 Dialysis vs. Transplantation

The significant demand for kidney transplantation stems from its substantial survival advantage over long-term dialysis as a treatment for ESRD. According to a systematic evaluation of over 100 trials comparing clinically relevant outcomes between KRT and dialysis, kidney transplantation is associated with a lower risk of death and cardiovascular events, as well as a higher quality of life compared to therapy with chronic dialysis (Tonelli et al., 2011); patients who receive a transplant have more flexibility and independence as they are no longer subject to lifestyle restrictions or to rigid dialysis schedules. Based on a study from 2017, the 5-year mortality risk was assessed to be 47% lower than that of patients on the waiting list and receiving dialysis, despite the risk of death being higher during the first year post-transplantation due to surgical risks and other transplant-related complications (Kaballo et al., 2018).

1.2.1 Types of Kidney Transplants

While KRT is currently the best option for individuals with ESRD, there are different types of transplants that patients may receive. Those who willingly donate one of their kidneys while still alive are known as living donors. The best results for both recipient outcomes and organ performance are possible with living organ donation because simultaneous operations decrease the time the kidney is without blood flow and prevents the need for organ preservation, resulting in the least amount of damage to the kidney. While this is the best option for transplantation, not everyone is willing to give up a kidney and therefore, there is a need to resort to deceased donors. The next best option is donation after brain death (DBD) donors. DBD donors are those who have been deemed to be brain dead, which means that all brain function has been irreversibly lost while oxygen circulation has been artificially maintained (L. Kumar, 2016). In this case, blood flow
within the organ can be maintained right until the point of procurement. This enables the organ to remain inside the body for an extended duration, while also ensuring that it is preserved in its own natural physiological conditions. The best outcomes come from both living and DBD donors since they both provide heart-beating organs (heart is still pumping blood to kidney during procurement), which dramatically reduces the level of harm that can occur when an organ is removed from the body. However, according to a 2020 report from the Canadian Institute for Health Information (CIHI), only 50% of those on a waiting list received a kidney transplant. While most of those that received a transplant received either a living or DBD donation, there remains only 20% of recipients who received a donation after circulatory death (DCD) organ (Canadian Institute for Health Information, 2021). According to the CIHI, there is a significant discrepancy between the number of donor organs that are available and the number of organs that are ultimately transplanted, with about 2000 ESRD patients still currently waiting for a donation. The reason being is that most DCD organs are ineligible for transplantation. DCD donors are those who have experienced irreversible cardiac arrest and a decision had been made to stop receiving life-saving treatment, thereby allowing the opportunity for their organs to be donated (Dunne & Doherty, 2011). With DCD organs (especially uncontrolled or unanticipated deaths) come unique difficulties, such as enduring a longer period of warm ischemia (without blood flow) between cardiac arrest and organ removal (Boyarsky et al., 2020). Moreover, the damage associated with the ischemia, as well as the processes involved in preserving (cold ischemia) and transplanting the organ into a recipient (also known as reperfusion), results in a phenomenon called ischemia reperfusion injury. The damage associated with this type of injury limits the viability and availability of the kidney for transplantation. In fact, the 1- and 5-year patient survival rates for living donor renal transplantation were 93% and 83.3%, respectively, but these rates were
significantly lower for deceased donor renal transplantation, at 79.1% and 74.5%, respectively (Kute et al., 2014). Hence, the challenges associated with the use of DCD donors is one of the main reasons behind the chronic shortage of organs.

Figure 1-1 summarizes the different types of organ donations. However, as described later in this paper, our focus is on mitigating or reversing damage often coinciding with DCD organs.

![Organ Donation Diagram](image)

**Figure 1-1. Summary of organ donor types.**

There are two main categories: deceased donors and living donors. Deceased donors can be further classified into donation after brain death (DBD) and donation after circulatory death (DCD). Our work focuses on improving the quality and quantity of DCD organs (dark blue highlight).

1.3 Ischemia Reperfusion Injury

The primary mechanism of harm that develops following kidney transplantation is ischemia reperfusion injury (IRI), which has several clinical ramifications. Ischemia, which starts at the time of the donor's death and lasts during the storage and transportation of the organ, is the process by which blood flow and oxygen are ceased within the kidney. Reperfusion refers to the event in which oxygen and blood flow is restored in the kidney when it is transplanted into the recipient.
Increased metabolic and histologic lesions form in DCD organs as a result of their propensity for extended ischemia times, which are further exacerbated by reperfusion (Nieuwenhuijs-Moeke et al., 2020). The mechanisms underlying IRI are multifaceted and complex, however a brief overview of relevant events that take place are described below.

### 1.3.1 Mechanisms and Pathophysiology

#### Ischemia

During ischemia, a switch from aerobic to anaerobic metabolism occurs due to the lack of oxygen supply, resulting in reduced adenosine triphosphate (ATP) production and a build-up of lactate (Malek & Nematbakhsh, 2015). The increased lactate levels create an acidic environment within the mitochondria, triggering the release of lysosomal enzymes (Nieuwenhuijs-Moeke et al., 2020). ATP depletion results in the inhibition of ATP dependant Na⁺/H⁺ pumps, eventually causing intracellular accumulation of Na⁺ and Ca²⁺ (Malek & Nematbakhsh, 2015). In short, this process leads to the eventual accumulation of calpains (a family of proteolytic enzymes which remain inactive until reperfusion) and reactive oxygen species (ROS) (Nieuwenhuijs-Moeke et al., 2020).

#### Reperfusion

During reperfusion, the restoration of oxygen results in a burst in ATP production, which causes an even larger generation of ROS (Nieuwenhuijs-Moeke et al., 2020). Moreover, with oxygen being restored, pH levels return to normal, resulting in further accumulation of Ca²⁺ and the activation of the calpains (Nieuwenhuijs-Moeke et al., 2020). An accumulation of these events triggers the opening of mitochondrial permeability transition pores (mPTP) and the release of
various substances, inducing a chain of events that lead to injury of the mitochondria, cytoskeleton, and cellular membrane.

1.3.2 Activation of Cell Death Programs

The accumulation of ROS, combined with the activation of calpains and proteases, activate cell death programs, including necrosis, apoptosis, necroptosis, and autophagy.

Necrosis is the uncontrolled form of cell death that leads to an unregulated release of cellular fragments into the extracellular environment. As described in more detail in following sections, these fragments function as damage-associated molecular patterns (DAMPs), which trigger the innate and adaptive immune response, leading to necroinflammation (Nieuwenhuijs-Moeke et al., 2020).

Apoptosis is a highly regulated process, controlled by the activation of the caspase (a family of proteases) signalling cascade (Tonnus et al., 2019). Through this regulated process, intracellular contents are cleaved and released, serving as DAMPs as well (Nieuwenhuijs-Moeke et al., 2020).

One of the caspases implicated in apoptosis is caspase-8 (Tonnus et al., 2019). Necroptosis, a type of cell death that combines elements of apoptosis and necrosis, occurs in the absence of activated caspase-8 (Nieuwenhuijs-Moeke et al., 2020). Receptor-interacting protein kinases (RIPK) are the key players to note, where RIPK1 and RIPK3 combine to create a complex called a necrosome (Tonnus et al., 2019). Following are a series of events that induce the release of cellular contents, which also act as DAMPs (Nieuwenhuijs-Moeke et al., 2020).

The last cell death pathway to note is autophagy. Autophagy is an important homeostatic defence mechanism in which cellular components are broken down and recycled by lysosomes to
prevent cellular death and maintain metabolic function (Alirezaei et al., 2010). Mammalian target of rapamycin complex 1 (mTORC1) and adenosine monophosphate-activated protein kinase (AMPK) are two kinases that control the initial phase of autophagy (Parzych & Klionsky, 2014). mTORC1 inhibits autophagy, which is mediated by the phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (AKT) pathway or the mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase 1/2 (ERK1/2) signaling pathway (Parzych & Klionsky, 2014). On the other hand, autophagy is activated by the inactivation of AMPK (Parzych & Klionsky, 2014). A series of subsequent events lead to the formation of an autolysosome (a membrane-bound organelle containing cellular components and a lysosome) (Zhou et al., 2022). Cellular components that would have launched a series of inflammasomal reactions are degraded and its by-products are recycled for future use (Zhou et al., 2022).

Overall, these cell death mechanisms contribute to the emergence and proliferation of IRI. It is necessary to have a full grasp of the underlying mechanisms of these processes in order to develop strategies to lessen the negative impacts associated with IRI. Drug therapy can be used to modulate the aforementioned proteins and enzymes that are involved in cell death.

1.3.3 Activation of the Innate Immune Response

The innate immune response serves as a rapid defense mechanism against pathogens, hypoxia, or other types of injury, and is comprised of two main components – the toll-like receptor signalling pathways and the complement system (Hovland et al., 2015; Marshall et al., 2018).

Toll-like Receptor Signalling Pathways
Several different cell types feature a class of proteins called toll-like receptors (TLRs), which are essential for the innate immune response. Particularly TLR2 and TLR4, which are elevated in ischemic conditions and act as key players in IRI (Nieuwenhuijs-Moeke et al., 2020). TLR2 and TLR4 are activated by DAMPs, and this activation results in a cascade of subsequent events that ultimately result in the nuclear translocation and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a crucial protein that triggers the production of proinflammatory genes and, in turn, inflammatory cytokines and chemokines such as interleukin-6 (IL-6), tumour necrosis factor-alpha (TNFα), and interferon-gamma (IFNγ) (Nieuwenhuijs-Moeke et al., 2020). Moreover, activation of TLR4 plays a significant role in the activation of leukocytes (immune cells) and induction of renal fibrosis (further detailed in this paper) (Nieuwenhuijs-Moeke et al., 2020).

Complement System

The complement system, also activated by DAMPs, is another key player to note in the context of renal IRI (Danobeitia et al., 2014). In brief, the activation of all 3 complement pathways - classical, alternative pathway, and lectin - results in the amplification of the inflammatory response, leukocyte recruitment and activation, and direct tubular cell death and injury associated with IRI damage in donor kidneys (Danobeitia et al., 2014).

In short, DAMPs are released as a result of cellular stress and damage associated with IRI. Pattern recognition receptors (PRRs) are expressed on innate immune cells and are able to identify these DAMPs (Sosa et al., 2020). The production of pro-inflammatory cytokines and the attraction of immune cells to the damaged region are both triggered by the activation of PRRs (Sosa et al.,
This immunological response increases oxidative stress and inflammation, which further exacerbates tissue damage and dysfunction (Sosa et al., 2020). With that being said, activation of the innate immune response is closely associated with the pathophysiology of IRI. As a result, addressing the innate immune response has been a treatment focus to reduce the adverse effects of IRI.

1.3.4 Activation of Hypoxia Inducible Factor

An innate protective mechanism against ischemia injury exists as a result of evolution (Semenza, 2012). Hypoxia Inducible Factor (HIF) is a transcription factor that is a critical regulator of cellular response to low oxygen levels, found largely in tubular epithelial cells (Conde et al., 2017). Under hypoxic conditions, subunits HIFα and HIFβ (which are normally unbound) bind to create its active heterodimeric form, which then promotes transcription of various genes (Semenza, 2012).

One such upregulated gene is vascular endothelial growth factors (VEGF). The role of VEGF is multifaceted, depending on the stressor and its interaction with other cells and molecules. In fact, there is evidence suggesting a dual role in the context of inflammation. On one hand, VEGF is important in cell proliferation and differentiation, mediates endothelium-dependent vasodilation, triggers microvascular hyperpermeability, and participates in interstitial matrix remodelling (Schrijvers et al., 2004). However, VEGF can also function as a proinflammatory cytokine by increasing endothelial cell permeability, triggering the production of endothelial cell adhesion molecules, and by acting as a chemoattractant for monocytes (Reinders et al., 2003). The modulation of VEGF has drawn interest in several clinical scenarios, but little is known about its function in the context of kidney IRI. Understanding the impact of VEGF downregulation in
relation to renal IRI can help explain its function. A deeper comprehension of VEGF's role in the pathophysiology of kidney IRI can be attained by analyzing the effects of controlling VEGF production. This could lead to the discovery of innovative therapeutic approaches and shed light on the complex mechanisms behind renal IRI.

1.3.5 Cellular and Tissue Damage

As was previously indicated, the initial dysregulation of Na⁺/H⁺ antiporters brought on by ischemia and reperfusion causes swelling of the endothelial cells (edema), consequently impairing endothelial cell-cell contact and increasing vascular permeability (Nieuwenhuijs-Moeke et al., 2020). Overall, this results in a loss of cellular structural integrity. It is important to highlight that this mechanism upregulates transforming growth factor-β (TGF-β) and NF-κB, both of which contribute to renal fibrosis (Danobeitia et al., 2014; Nogueira et al., 2017). Additionally, the TLR and complement pathways' recruitment and activation of leukocytes further exacerbate progression of fibrosis (Danobeitia et al., 2014; Nieuwenhuijs-Moeke et al., 2020).

In summary, the descriptions given above offer a condensed picture of the many interconnected and intricate systems that take place in the context of renal IRI. Nonetheless, this paper has emphasized important aspects that will be pertinent in elucidating the mechanism of action for various drugs moving forward. Figure 1-2 provides a simplified illustration of the pathways induced by ischemia reperfusion.
Figure 1-2. A simplified schematic depicting the events that occur during IRI.

In summary, IRI is characterized by uncontrolled ROS production during reperfusion and loss of mitochondrial integrity, which results in the opening of mPTP and the release of DAMPs in the intra- and extracellular space. A cascade of events is activated from here, including cell death
programs, endothelial dysfunction leading to greater vasoconstriction upon reperfusion, phenotypic changes of endothelial cells, and leukocyte transmigration into the interstitial space. The innate immune system will be activated by binding of DAMPs to TLRs and complement system receptors, resulting in additional graft injury, alongside the onset of fibrosis implicated in chronic graft failure. Pathways that cause inflammation and harm are shown in red, while pathways that prevent inflammation are shown in green. As this is a simplified representation, not all players are depicted, but those that are relevant to the project are.

1.3.6 Clinical Manifestations of IRI

IRI during kidney transplantation leads to various consequences that contribute to unfavorable clinical outcomes. Among these, delayed graft function (DGF) is a prominent concern. DGF refers to the occurrence of acute kidney injury within the first week of transplantation, requiring dialysis intervention (Siedlecki et al., 2011). It is associated with increased rates of acute cellular rejection and reduced graft survival in the long-term (Mannon, 2018). The incidence of DGF is influenced by both the duration of cold ischemia and the extent of warm ischemic injury (Mannon, 2018). In the case of DCD, the nature of the induction of cardiac death typically leads to a longer warm ischemic period, further exacerbating the risk of DGF (Mannon, 2018). Existing preservation techniques (described in the next section) are insufficient in tackling the unique challenges presented by DCD kidneys, necessitating the development of improved preservation strategies that would enhance outcomes and ensure the long-term survival of the transplanted kidney.
1.4 Current Preservation Techniques

1.4.1 Static Cold Storage

The gold standard for organ preservation between the time an organ is removed from the donor and when it is transplanted into the recipient has been static cold storage (SCS). To do this, the kidney must be flushed to eliminate donor’s blood, cooled with a preservation solution at 4°C, and then kept in ice until the recipient receives the organ. Damage caused by ischemia is greatest at higher temperatures (referred to as warm ischemia), as the hypoxic conditions cannot meet the energy demands of a normal functioning organ. SCS has proven to be a cost-effective and simple approach for preserving organs, as the cold temperatures work by lowering the metabolic rate of the organ to about 5% of its normal function (Hosgood et al., 2023). However, the prolonged cold ischemia caused by this preservation technique, the anoxic environment that encourages anaerobic metabolism, combined with the burden on the kidney during reperfusion, are a cocktail of factors that cause significant organ damage (Hosgood et al., 2023). Consequently, SCS is associated with higher incidences of DGF and acute rejection, as well as relatively poor long-term graft survival rates (Hosgood et al., 2023).

1.4.2 Machine Perfusion

Machine perfusion has emerged as a method for organ preservation to not only fulfill the steadily rising demand for kidney transplants, but also to enhance the quality of the graft prior to transplant. Despite being the more expensive alternative, machine perfusion enables preservation of the organ in more physiologically accurate settings (Hosgood et al., 2023); this includes preserving organs at temperatures and oxygen levels that are comparable to the human body. The system, which consists of a pulsatile pump and tubing, enables the circulation of blood, blood-like
products, or other preservation solutions, through the organ. Additional capabilities include the capacity to monitor and control several variables, including oxygenation, pressure, flow rate, and temperature, that can simulate the natural environment of the human body (Vaziri et al., 2011). A visualization of a machine perfusate system is illustrated in Figure 1-2. Additionally, there is evidence that organs can be preserved using machine perfusion at several different temperature points, particularly at subnormothermic, normothermic, and hypothermic temperatures. Our lab, in particular, has demonstrated using an *ex vivo* porcine model that subnormothermic machine perfusion (22°C; SMP) results in superior outcomes compared to SCS (Bhattacharjee et al., 2019). This includes a notable reduction in acute tubular necrosis, apoptosis, and kidney damage markers, as well as an increase in urine production post-reperfusion (Bhattacharjee et al., 2019). Moreover, recent data from a systematic review and meta-analysis of 13 randomized clinical trials revealed that machine perfusion increased graft survival at 3 years and reduced the incidence of DGF when compared to SCS (Peng et al., 2019).
Figure 1-3. Simplified schematic of machine perfusate system.
The machine perfusion system has a peristaltic pump that simulates the beating of the heart, a heating system that can be tuned to the appropriate temperature, and an oxygenator that can deliver a steady stream of controlled oxygen to the organ. A monitor ensures all parameters are close to physiological conditions.

However, the difficulty is that while evidence suggests that machine perfusion reduces tissue damage and inflammation compared to SCS, clinical outcomes post-transplant appear to be a source of disagreement in the scientific and medical communities. Although machine production dramatically decreased the risk of DGF, the effect on overall 1- and 5-year survival rates were negligible (Kruszyna & Richter, 2021). Additionally, while machine perfusion is superior to SCS in terms of DGF occurrence rate (21.8% vs. 42.6%, respectively), it still is not comparable to success attainable by using living donor organs (DGF occurrence rate of less than 5%) (Jahn et al., 2021). Therefore, we must find additional ways to further enhance the preservation techniques used in kidney transplantation.
1.5 Potential Solutions – Therapeutic Targeting of IRI

Given the level of urgency required to respond and contain the recent SARS-COV-2 virus, this occurrence highlighted the amount of time and resources needed to develop, test, and mandate new therapies. For this reason, researchers had to turn to alternative strategies, such as drug repositioning, to mitigate the effects of COVID-19 within a quick timeframe. Drug repositioning refers to the usage of medications for the treatment of medical conditions other than its original indication by leveraging its secondary therapeutic effects (Jourdan et al., 2020). These drugs have already undergone extensive safety testing and are approved for clinical use, thereby enabling a quicker transition of its secondary use to the clinic. A prime example of a successful repositioned drug is aspirin. While its initial indication was an analgesic for treating mild to moderate pain, it has since been studied extensively to uncover a variety of other applications, including treatment for coronary artery disease, diabetes, colorectal cancer, Alzheimer’s disease, and arthritis (Li et al., 2020). Our objective, which is based on the same idea, is to employ post-market drugs as a method of reducing the effects of IRI during kidney transplantation. To accomplish this, we want to undertake a drug screening to investigate any potential secondary properties of these drugs. By doing this, we hope to find medications that can effectively counteract the negative effects or IRI and enhance kidney transplant procedure outcomes.

1.5.1 Drug Selection

Proper procedure would involve random drug selection, as our intention is to eventually test an entire drug library. However, the unique circumstances of this project, including its time and financial constraints, necessitated the selection of drugs that have already undergone prior testing by other research groups and exhibit potential anti-inflammatory properties in other disease
contexts. Given that inflammatory pathways are largely conserved across different organ systems, drug selection focused on compounds exhibiting established anti-inflammatory properties in the treatment of other diseases, including but not limited to COVID-19, rheumatoid arthritis, and ulcerative colitis (Chen et al., 2018). In other words, many other studies have been conducted with regard to drug repositioning and the targets for the drugs selected have been established to a certain degree. While there are noticeable differences in the broader context of inflammation and immunity, commonalities and similarities in targets exist across different systems. Therefore, we intend to explore potential effects in the context of renal IRI. The medications chosen are listed in Table 1-1 along with their initial intended use and any potential mechanisms of action that could lessen inflammation. While some anti-inflammatory properties are demonstrated in these drugs, we want to test their effects in the context of IRI and kidney transplantation specifically.

Table 1-1. Summary of candidate drugs and its potential mechanisms of action for the management of IRI in the context of kidney transplantation.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Therapeutic group</th>
<th>Mechanism of action</th>
<th>References</th>
</tr>
</thead>
</table>
| Prazosin  | Antihypertensive  | - Inhibition of TNFα  
- Preventing the release of hydrolytic and proteolytic enzymes via maintaining the integrity of the lysosomal membrane | (Qasim et al., 2021) |
| Melatonin | Miscellaneous anxiolytics, sedatives and hypnotics, | - ROS scavenger  
- Reduction of IL-6 and TNFα via blocking NF-κB binding to DNA | (Cho et al., 2021) |
<table>
<thead>
<tr>
<th>Nutraceutical products</th>
<th>Antihypertensive</th>
<th>Antihypertensive</th>
<th>Macrolide antibiotics</th>
<th>Natural supplement, antioxidant</th>
<th>Anti-diabetic</th>
<th>Supplement, antioxidant</th>
<th>Selective serotonin reuptake inhibitors (SSRI)</th>
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<tbody>
<tr>
<td><strong>Verapamil</strong></td>
<td></td>
<td>- Vasodilator, increase GFR</td>
<td>- Inhibition of TNFα induced activation of NF-κB signalling pathway</td>
<td>- Inhibition of IL-1 and VEGF</td>
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<tr>
<td><strong>Erythromycin</strong></td>
<td>Macrolide antibiotics</td>
<td></td>
<td>- Inhibit release of ROS and chemotactic factors (IL-8)</td>
<td>- ROS scavenger</td>
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<td><strong>N-acetyl cysteine</strong></td>
<td>Natural supplement, antioxidant</td>
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<tr>
<td><strong>Metformin</strong></td>
<td>Anti-diabetic</td>
<td></td>
<td>- Inhibition of nuclear translocation of NF-κB</td>
<td>- Suppression of IL-1β, IL-6, TNF-α</td>
<td>- Low phosphorylation of several MAPK genes</td>
<td>- ROS scavenger</td>
<td>- Activation of S1R, and subsequent activation of</td>
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<tr>
<td><strong>Alpha lipoic acid</strong></td>
<td>Supplement, antioxidant</td>
<td></td>
<td>- Inhibition of NF-κB and subsequent inflammatory cytokines</td>
<td>- prevents dysregulation of sodium transporters</td>
<td>- ROS scavenger</td>
<td>- Inhibition of NF-κB and subsequent inflammatory cytokines</td>
<td>- Activation of S1R, and subsequent activation of</td>
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<tr>
<td><strong>Amlodipine</strong></td>
<td>Antihypertensive</td>
<td></td>
<td>- Vasodilator, increase GFR</td>
<td>- Reduction in TGF-β protein</td>
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<tr>
<td><strong>Fluvoxamine</strong></td>
<td>Selective serotonin reuptake inhibitors (SSRI)</td>
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<tr>
<td>Drug</td>
<td>Category</td>
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<tr>
<td>Hydroxychloroquine</td>
<td>Antirheumatic</td>
<td>- AKT-mediated nitric oxide signalling</td>
<td>et al., 2021</td>
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<td></td>
<td></td>
<td>- NO-mediated vasodilation</td>
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<td></td>
<td></td>
<td>- TLR9 antagonist</td>
<td>(M Marsh et al., 2022)</td>
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<td>Simvastatin</td>
<td>Lipid-lowering agent</td>
<td>- Modulation of AKT/mTOR signalling</td>
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<td></td>
<td></td>
<td>- Suppression of NF-κB and TLR4 signalling pathways</td>
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<tr>
<td>Pioglitazone</td>
<td>Anti-diabetic</td>
<td>- Activation of AMPK signalling pathway</td>
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<td></td>
<td></td>
<td>- Inactivation of TLR4 expression</td>
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<td></td>
<td></td>
<td>- Reduction in subsequent pro-inflammatory cytokines</td>
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<td></td>
<td></td>
<td>- TNF-α, IL-1β, and IL-6</td>
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<tr>
<td>Fludrocortisone</td>
<td>Corticosteroid</td>
<td>- Reduce expression of CCL2, IL-6 and IL-8</td>
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<tr>
<td>Glyburide</td>
<td>Anti-diabetic</td>
<td>- Inhibits caspase-1 activation via NLPR3, secretion of IL-1β and IL-18</td>
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<tr>
<td>Fenofibrate</td>
<td>Antilipidemic agent</td>
<td>- Activation of PPAR and AMPK signalling, subsequent inhibition of NF-κB</td>
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<tr>
<td>Rivaroxaban</td>
<td>Anticoagulant</td>
<td>- Inhibition of factor Xa</td>
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<tr>
<td></td>
<td></td>
<td>- Suppression of NF-κB signalling</td>
<td></td>
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</tbody>
</table>
| **Nifedipine** | Anti-hypertensive | - ROS scavenger  
- Inhibition of NF-κB signalling | (Horiuchi, 2006; Santa-Helena et al., 2017) |
| **Infliximab** | Treatment of various autoimmune diseases | - Suppression of TNF-α and VEGF | (Danese, 2008) |
| **Rosuvastatin** | Lipid-lowering agent | - Modulation of AKT/mTOR signalling  
- Suppression of NF-κB and TLR4 signalling pathways | (Diamantis et al., 2017; Shawki et al., 2021) |
| **Roflumilast** | Reduction of COPD exacerbations | - Inhibition of TGF-β signalling pathway | (Patel et al., 2023) |

Figure 1-4 illustrates the various medications’ mechanisms of action in the pathways associated with renal IRI to further highlight the impact of the above-mentioned treatments.
Figure 1-4. An illustration of potential targets of the selected drugs.

Candidate drugs have been added into the previous schematic (Figure 1-2) in blue italics to better illustrate the drug targets outlined in Table 1-1. Some drugs inhibit (⊣) enzymes, while some drugs activate (→). Some drugs may have dual roles; thus, their targets are represented in multiple areas of the diagram.
1.6 The Use of *in vitro* Models

Given the large number of drugs available on the market, it is most appropriate to implement an *in vitro* model as a platform for initial drug screening.

Firstly, when developing an *in vitro* model, the cell type must be carefully considered, as it must be a close representation of the processes of a whole organ. While the kidney is composed of many cell types, renal proximal tubular epithelial cells have a special functional role within the kidney, including renal and whole-body homeostasis, detoxification processes, and expression of transport proteins that facilitate the movement of many vital components (e.g., electrolytes, glucose, amino acids, etc.) (Faucher et al., 2020). More importantly, renal proximal tubular epithelial cells are particularly susceptible to injury caused by ischemia and reperfusion, and are significant promoters of kidney inflammation, including the secretion of several inflammatory cytokines and expression of immune receptors (Chen et al., 2018; Faucher et al., 2020). The proximal tubule cells' susceptibility to hypoxia results from their position within the medulla's energy-intensive region within the kidney (Evans et al., 2020). This vulnerability is exacerbated by the particular configuration of blood vessel and tubular structures that limits oxygen delivery to this specific location within the kidney (Evans et al., 2020). It is important to note that cellular susceptibility to IRI and their respective responses varies significantly, reflecting the intricate nature of this phenomenon. Proximal epithelial cells hold a particularly significant position in the chain of events that lead to IRI-related harm. In this context, HK-2 cells serve as valuable surrogates, shedding light on the broader organ's IRI response.

In fact, since being identified and cultivated for the first time in 1984, HK-2 cells have attracted scientific attention due to their ability to retain many of their *in vivo* properties *in vitro* (Gildea et al., 2010). Over 170 papers have since mentioned using this cell line (Gildea et al.,
2010). As this cell line has been thoroughly investigated, its functional characteristics are well understood. While immortalized cell lines, such as HK-2 cells, may be maintained in long-term culture, higher passage cells have been known to become more resilient to ischemia and reperfusion-related stressors (Gildea et al., 2010; Son et al., 2020). The fact that immortalized cell lines have undergone genetic changes to avoid natural cellular senescence is another issue. These genetic alterations can affect the stability and behavior of the cells, potentially leading to changes in genetic and phenotypic characteristics over time, which may result in heterogeneity between experiments. (Audesirk, 2010).

Regardless, *in vitro* models can allow researchers to study specific mechanisms without other confounding variables, such as systemic components, affecting the results. Moreover, *in vitro* models replace and reduce the need for animal subjects. HK-2 cells, in particular, are human derived, increasing its efficacy in translating results from the bench to clinic.

Until recently, no reliable *in vitro* model that accurately depicts the events occurring during kidney transplantation existed. Therefore, a new *in vitro* model that mimics the stages of SCS during kidney transplantation recently established and characterized by our lab. In brief, the types of methods employed to induce hypoxia, different storage solutions, as well as the length of time cells are exposed to hypoxia and reoxygenation were previously considered in developing this model.

However, the purpose of this project is to be able to eventually implement drugs into our 22-degree perfusate system. Moreover, we have already established that preservation of kidneys *ex vivo* at 22°C is superior to cold temperatures. Therefore, we must develop a model that more closely mimics the events that take place during subnormothermic preservation and demonstrate
similar responses \textit{in vitro}. This project will expand on the current cold model and compare the inflammatory responses to preservation techniques carried out at room temperature.

### 1.7 Rationale

Through the implementation of pharmaceuticals that can reduce the harm caused by IRI after kidney transplantation, our research aims to advance the field of organ preservation. A promising strategy to discovering an effective therapy that can be quickly implemented into clinical use is drug repositioning through extensive screening of clinically approved drug candidates. There are currently no \textit{in vitro} models that can replicate the events that occur during pre-transplant storage. The use of large animals or human models, meanwhile, can be expensive and time-consuming when it comes to drug testing. As such, the development and characterization of an effective \textit{in vitro} model is a logical first step in this aim, as it will make it easier to screen and evaluate the effectiveness and mechanisms of action of drug candidates against IRI in kidney transplants.

### 1.8 Hypothesis Statement

We hypothesize that the room temperature or 22°C \textit{in vitro} model (sIRI-RT) will show decreased inflammation and cell death under hypoxia-anoxia-reoxygenation (HAR) injury conditions compared to the previously developed 4°C model (sIRI-cold). Repositioning of various approved drugs can be used to further ameliorate the effects of IRI within the sIRI-RT model.
1.9 Specific Aims

To test this hypothesis, this project aims to 1) Establish and contrast an *in vitro* hypoxia-anoxia-reoxygenation (HAR) injury model using human tubular epithelial cells at both 4°C and room temperature (22°C), 2) Characterize the levels of cell death, inflammation, and innate immune activity under conditions of HAR injury, and 3) Compare the expression of the aforementioned factors following treatment of 20 selected drugs using the *in vitro* HAR models.
Chapter 2

2 Materials and Methods

2.1 Cell Culture

Human tubular epithelial cells (HK-2) were obtained from the American Type Culture Collection (ATCC, VA, USA) and cultured in complete media, which contains Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; ThermoFisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS; ThermoFisher Scientific, MA, USA), 1% penicillin-streptomycin (Pen/Strep; Gibco, heat inactivated, CA, USA), 1.0 mg/mL recombinant human insulin, 0.55mg/mL human transferrin and 0.5 ug/mL sodium selenite (1X ITS; Sigma-Aldrich, MA, USA), and 5ng/mL epidermal growth factor (EGF; ThermoFisher Scientific, MA, USA). Cells were cultured at 37°C/5% CO₂/95% humidity in standard 100mm petri dishes (ThermoFisher Scientific, MA, USA).

HK-2 cells are adherent cells, therefore for passaging, cells were treated with 1mL 0.25% trypsin-EDTA (trypsin; Wisent, QC, CA) at 37°C for 1 min followed by a wash with 1X phosphate buffered saline (PBS; Wisent, QC, CA). Trypsin was then inactivated by diluting with 3mL of complete media. Cells were collected and pelleted by centrifugation at 500rpm for 5 minutes. Cells were resuspended in 2mL of complete media and plated for a passage ratio of 1:4 for continued growth. Cells were discarded by the 20th passage.

Depending on the experiment, cells were seeded into either 6-well (Corning, NY, USA), 12-well (VWR, PA, USA), 24-well (Corning, NY, USA) or clear, flat bottom 96-well plates (VWR, PA, USA) and grown to 90% confluency.
2.2 Simulation of Ischemia Reperfusion Injury

Cold Model

Based on previous work, an *in vitro* IRI model mimicking cold storage conditions was established (Figure 2-1). HK-2 cells were grown to 90% confluency, where then were subjected to 1 hour of hypoxia (0.5% oxygen) at 37 ºC using a hypoxia chamber (Whitley H85 Hypoxystation, YSW, UK) in 1X Hank’s Balances Salt Solution (HBSS; Gibco, CA, USA). Next, cells were subjected to 24 hours of anoxic (0% oxygen) storage at 4 ºC using an anaerobe gas generating pouch system with an O₂ indicator (GasPak; VWR, PA, USA) in University of Wisconsin solution (UW; Bridge to Life, IL, USA), where solution was replaced, and plates were placed in the GasPak within the hypoxia chamber to maintain hypoxic conditions. Following 24 hours of storage in the GasPak, 75% of the UW solution was replaced by complete media, where cells are then subjected to 24 hours of reoxygenation (20% oxygen) at 37 ºC in a humidified incubator.

Room Temperature Model

To mimic the 22ºC perfusate model, methods are as previously described, however storage was at 22ºC in normoxic conditions (Figure 2-1). HK-2 cells were grown to 90% confluency, where then were subjected to 1 hour of hypoxia (0.5% oxygen) at 37 ºC using a hypoxia chamber in 1X HBSS. Next, cells were subjected to 24 hours of normoxia (20% oxygen) at 22 ºC using a BioSpa 8 Automated Incubator (Agilent BioTek, CA, USA) in UW solution. Lastly, 75% of the UW solution from each well was replaced by complete media, where cells are then subjected to 24 hours of reoxygenation (20% oxygen) at 37 ºC in a humidified incubator.
**Control Group (no injury)**

The seeding density for the control group will be adjusted to account for the time needed for the cells to grow to confluence at the end of the two days since the sIRI model described above takes place over two days. In order for HK-2 cells to be fully confluent in time for the experiment and to avoid overcrowding, cells are plated at around 50% confluency.

**Figure 2-1. A schematic of the in vitro models of IRI in kidney transplantation.**
An overview of the experimental conditions that were applied in later screening tests to verify the model and to evaluate potential drugs.

### 2.3 Drug Treatment

20 drugs were selected based on existing literature, where evidence suggested drugs may have secondary anti-inflammatory properties. The 20 drugs were obtained from the Inpatient Prescription Centre at LHSC, and solid drugs were prepared in house based on their individual
solubility properties. Some drugs are only soluble in DMSO, which in high doses, can have cytotoxic effects. Based on the literature, final concentrations of DMSO were kept under 0.1% of the volume required to incubate cells (Moskot et al., 2019).

In a 96-well plate, HK-2 cells were plated, grown to about 50% confluency or 25000 cells, and subjected to warm hypoxia as previously described. Cells were treated with selected drugs for the full duration of normoxia at 22 °C in UW solution. Dose-response curves were established at concentrations between 5mM to 0.00001mM. After reoxygenation, supernatants were collected for an initial cell death analysis using an LDH cytotoxicity assay. Further analysis using flow cytometry, and quantification of various pro-inflammatory cytokines and damage markers were conducted on short-listed drugs.

2.4 Cell Death Assays

2.4.1 Real-Time Quantification of Cell Death and Imaging via Cytation 5

In a 96-well plate, cells are subjected to warm hypoxia and cold storage/room temperature storage, as previously described. During the 24 hours of reoxygenation, live cell imaging analysis of cell death was conducted using a Cytation 5 (Agilent BioTek, CA, USA) in real time. Cell culture plates were transferred by the BioSpa 8 to the Cytation 5 cell imaging multimode reader every two hours, where environmental conditions were maintained throughout the imaging steps. Images were captured at 4x in the Texas Red and Brightfield channels. Immediately prior to reoxygenation, 1μg/mL of propidium iodide (PI; ThermoFisher Scientific, MA, USA) was added to each well, to work as a reporter of late apoptotic or necrotic cells. Red objects detected by Cytation 5 are PI positive staining of DNA, indicating dying or dead cells. Red objects per well
were quantified by the Cytation 5 software at each imaging time point to produce a cell death versus time curve.

### 2.4.2 Quantification of Apoptosis and Necrosis via Flow Cytometry

Following experiments in 6-well plates containing cells at 90% confluency, cell viability and cell death were quantified. Cell culture supernatants from each well were collected into labeled 15mL conical tubes to collect floating cells. Adherent cells were washed with 500μL of 1X PBS (which were then collected into their respective tubes) and detached by the addition of 500μL of trypsin to each well and incubated at 37°C for 2 minutes. Next, 1.5mL of complete media were added to each well to inactivate the trypsin. The media containing the detached cells were collected and added into their respective conical tubes. Tubes were centrifuged at 500rpm for 5 minutes to pellet cells. Supernatant was decanted and pelleted cells were then resuspended in 200μL of 1x Annexin-V Binding Buffer (BioLegend, CA, USA). After resuspension, 2.5μL of FITC-conjugated Annexin-V (Annexin-V; BD BioScience, NJ, USA) and 5μL of 7-Aminoactinomycin (7-AAD; eBioScience, CA, USA) were added to a 100μL aliquoted sample to stain for apoptosis and necrosis, respectively. Samples were incubated with stains in the dark for 30 minutes prior to analysis. Samples were analyzed using the CytoFLEX S (Beckman Coulter, CA, USA) using the following channels and gains: 150 FSC, 150 SSC, 50 FITC, 50 PerCP-A. Prior to experiment, a compensation matrix was produced using 1) unstained live cells, 2) heat-killed cells (at 90 °C for 10 minutes) 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.
2.4.3 LDH Cytotoxicity Assay

The release of LDH occurs following damage to the plasma membrane, which was used as a quick method to quantify cell death for initial drug screening. LDH activity was measured using the CyQUANT™ LDH Cytotoxicity Assay Kit (ThermoFisher Scientific, MA, USA) as per the manufacturer protocol. Immediately following experiment in 96-well plates, cells were lysed using 10µL of the lysate buffer provided by the assay kit for 45 min at 37 °C prior to collection of lysed cells (50µL) from each sample. The LDH assay was run on the supernatants containing lysate samples. Absorbance was measured at 490-nm (LDH activity) using an iMark microplate reader (Bio-Rad, CA, USA). To determine optimum cell number for the assay, an initial assay consisting of a serial dilution of cells (0-40,000) was conducted. For HK-2 cells, about 50% confluency or 2.5x10⁴ cells/well in a 96-well plate was determined to be the optimum cell number, of which were used for any subsequent experiments involving an end-point analysis using an LDH assay.

2.5 NF-κB p65 Transcription Factor Assay

The levels of transcription factor nuclear factor kappa B (NF-κB) were quantified as a representation of immune, acute phase and inflammatory responses following IRI injury. An NF-κB p65 Transcription Factor Assay (NF-κB assay; abcam, MA, USA) was used to quantify levels of NF-κB, which is an ELISA-based method for detecting specific transcription factor DNA binding activity in nuclear extracts. Cells were grown to confluency in 100mm plates, where then underwent one of three conditions: control, sIRI-cold or sIRI-RT. Immediately following reoxygenation, nuclear extracts were obtained using a Nuclear Extract Kit (Active Motif, CA, USA). The Nuclear Extract Kit was used in accordance with the manufacturer’s instructions. In brief, media was aspirated, and cells were washed with ice-cold PBS. Unlike passaging, cells were
lifted and collected through gentle scraping using cell scraper so as not to inactivate any signal transduction pathways with the use of trypsin. Cells were then collected, centrifuged, and lysed to eventually obtain nuclear fractions. Immediately following nuclear extraction, the NF-κB assay was conducted in accordance with the manufacturer’s instructions. In general, the assay involves NF-κB to be sandwiched between dsDNA (pre-coated in the well) and an enzyme labelled antibody. A color developing reagent then binds to the antibody and is then inactivated to develop a final coloured product. Developed samples were then read at 450nm using an iMark microplate reader. As there were no standard curves available for this assay, results were represented as strength of absorbance being proportional to the level of NF-κB binding.

### 2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

For cell culture supernatant collection, cells were plated in 24-well plates. The volume of any preservation solutions or complete media used throughout the experiments were 500μL/well. Cell culture supernatants were centrifuged at 3000xg for 10 mins prior to performing assay or storage at -80°C. ELISAs (MyBioSource, CA, USA) were used to assess the quantities of kidney injury marker, NGAL, and inflammatory markers, IL-6 and TNFα, secreted in HK-2 cell culture media from control, IRI-induced, and drug-treated cells following reoxygenation. ELISA kits were used in accordance with the manufacturer’s instructions. In general, an ELISA involves the protein of interest to be sandwiched between the capture antibody (pre-coated in the well) and an enzyme labelled antibody. A substrate reagent then binds to the antibody and is then inactivated to develop a final coloured product. Developed samples were then read at 450nm using an iMark microplate reader. Standard curves were produced for each experiment, to which were used to calculate the concentrations of the proteins of interest in the samples.
2.7 Multiplex ELISA via Eve Technologies

For cell culture supernatant collection, cells were plated in 24-well plates. The volume of any preservation solutions or complete media used throughout the experiments were 500µL/well. Post-reperfusion, cell culture supernatants only from experiments comparing control, sIRI-cold, and sIRI-RT conditions were collected. At 4°C, samples were centrifuged at 3000g for 10 minutes. Samples were aliquoted immediately after centrifugation and kept at -80°C. Supernatant samples were then sent to Eve Technologies (Alberta, Canada), where Luminex xMAP® technology was used to simultaneously detect various cytokine levels like IL-6, interleukin-8 (IL-8), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and more.

2.8 Statistical Analysis

GraphPad Prism 9 and Microsoft Excel were used for statistical analyses and figures. The student’s t-test was used to evaluate statistical differences between two groups, and one-way analysis of variance (ANOVA) was used alongside a post-hoc Tukey HSD test to evaluate statistical differences between three or more groups. Unless otherwise specified, data is reported as the mean ± SEM of at least three independent experiments (n=3). The threshold for statistical significance was set at p<0.05 with a confidence interval of 95%.
Chapter 3

3 Results

3.1 Development of *in vitro* IRI model at room temperature (22°C)

As mentioned above, prior work comprising of a simulation of IRI in an *in vitro* model under cold conditions was conducted, referred to as simulated IRI (sIRI). This involved subjecting HK-2 cells with different combinations of storage solutions for each of the injury stages, confirming that hypoxic conditions are necessary for injury and that hypoxic conditions were achieved under sIRI conditions. As a result, previous work determined the optimal conditions that elicited the greatest level of injury and cell death, which would similarly be seen in DCD organ transplantation: 1 hour of warm (37°C) hypoxia in D-Hank’s solution, 24 hours of cold (4°C) anoxia in UW solution, and then finally 24 hours of reoxygenation in 25% UW solution and 75% complete media at 37°C. The results, labelled as sIRI-cold in subsequent sections, are consistent with results obtained by previous findings.

Now, consistent with the stages of transplantation during oxygenated machine perfusion at 22°C, two aspects of the sIRI model were altered: the temperature – 4°C to 22°C, and oxygenation state during the preservation stage – anoxic to normoxic. In other words, the *in vitro* model mimicking the stages of transplantation using machine perfusion is as follows: 1 hour of warm (37°C) hypoxia in D-Hank’s solution, 24 hours of RT (22°C) normoxia in UW solution, and then finally 24 hours of reoxygenation in 25% UW solution and 75% complete media at 37°C. This injury condition will be referred to as sIRI-RT in subsequent sections.

All other variables are conserved to demonstrate that room temperature preservation is superior to that of cold storage, consistent with the literature involving *ex vivo* work. Flow
cytometry results (Figure 3-1A) revealed that room temperature preservation outperformed cold preservation in terms of cell death parameters. After reoxygenation, cell death was detected by identifying cells that were positively stained for Annexin V and 7-AAD. Phosphatidylserine, a component of the plasma membrane that moves from the inside of the cell to the outside during the initial stages of apoptosis, is detected by Annexin V. Similarly, 7-AAD binds to DNA, which is only made possible by the deterioration of the cell membrane, making this approach a reliable means to detect advanced cell death.

Analysis using flow cytometry revealed a significant improvement in cell viability (Figure 3-1B) and decreased levels of apoptosis (Figure 3-1C), yet still not comparable to fully healthy cells as indicated by the control conditions.
Figure 3-1. sIRI-RT offers better preservation conditions compared to cold.

HK-2 cells were exposed to warm hypoxia for 1 hour at 37°C, followed by 24 hours of cold storage at 4 °C (or room temperature storage at 22 °C), and finally 24 hours of reoxygenation. Control groups are cells not subjected to any injury conditions. End-point analysis after reoxygenation was carried out with flow cytometry. (A) Annexin V and 7-AAD double staining results demonstrate an increase in early (bottom right quadrant) and late (top right quadrant) apoptotic cell populations in sIRI-cold compared to control. There is a decrease in the aforementioned populations in the sIRI-RT model compared to cold. Percentages from each quadrant were extracted to represent percent (B) Cell viability (n=5) and (C) apoptosis (n=5). Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and post-hoc Tukey HSD test. **** p<0.000001.

3.2 Hypoxic conditions contribute to IRI

As two variables were altered to develop the sIRI-RT model, both hypoxic (0.5% oxygen) and normoxic (21% oxygen) states were tested to determine the most optimal preservation conditions. Figure 3-2A findings confirmed that the hypoxic conditions contribute more to IRI and consequently, cell death. Therefore, similar to events during machine perfusion, the sIRI-RT model that provides better preservation conditions are storing cells in oxygenated conditions at 22°C. Analysis using flow cytometry revealed noteworthy improvement in cell viability (Figure 3-2B) when HK-2 cells are stored in normoxic conditions at 22°C.
Figure 3-2. Oxygenated storage conditions are required for cell viability during room temperature preservation.

Following 1 hour of warm hypoxia, HK-2 cells were subjected to either hypoxic or normoxic conditions during the 24 hours of room temperature preservation at 22°C. End-point analysis after reoxygenation was carried out with (A) flow cytometry using Annexin V and 7-AAD staining. (B) Cell viability (n=4) was quantified using flow cytometry results. Data is represented as the mean ± SEM. Means were analyzed using Student’s t-test, *p<0.05.

3.3 Further validation of sIRI models of injury

By labelling HK-2 cells with the DNA stain, propidium iodide, and monitoring with live cell imaging, it was possible to discover evidence of cell death during the reoxygenation stage, further supporting the models’ predictions. Cells exposed to sIRI-cold conditions showed the greatest propidium iodide staining, followed by sIRI-RT, and then control (Figure 3-3). Propidium iodide binds to DNA, which is only made possible by the deterioration of the cell membrane during
apoptosis, making this approach a reliable means to detect cell death. Propidium iodide would be unable to penetrate intact cell membranes of viable cells.
Figure 3-3. Further validation of sIRI-cold and sIRI-RT models using live-cell imaging.

HK-2 cells were exposed to one of three experimental groups: control (no IRI damage), sIRI-cold, or sIRI-RT conditions. Cells were stained with propidium iodide (PI) immediately prior to reoxygenation, and they were followed using live cell imaging for 24 hours through the duration of the reoxygenation stage. (A) During reoxygenation, sIRI-cold and sIRI-RT cells both experienced a linear rise in cell death, as seen by PI-positive cells, compared to control cells, which experienced a much smaller increase, n=4. A two-way analysis of variance (ANOVA) was conducted to assess the effects of time and experimental group type on the number of PI positive stained cells over a 24-hour period. There was a significant interaction effect between time and experimental group type on the number of PI positive stained cells (F (22, 99) = 34.41, ****p < 0.0001). This interaction suggests that the impact of experiment group on the stained cell counts varied significantly across different time points. (B) A visual representation of PI-positive cells over a 24-hour period in 8-hour increments demonstrates more red staining in sIRI-cold cells compared to sIRI-RT cells. Data is represented as the mean ± SEM.
3.4 Comparison of pro-inflammatory cytokines

As mentioned previously, the harm caused by IRI following kidney transplantation is exacerbated by the inflammatory response, characterized by the release of various cytokines and chemokines. To examine the difference in pro-inflammatory cytokine production that is known to be associated with IRI conditions, cell culture supernatants were analyzed for various cytokines using Multiplex ELISA. In comparison to sIRI-RT, we found that several pro-inflammatory cytokines had significantly higher levels in sIRI-cold supernatants (Figure 3-4). The control group, which represents healthy cells and should have very low cytokine levels, is as expected. This shows that, with respect to the inflammatory response seen during renal IRI, the sIRI-RT model elicits a reduced IRI response compared to sIRI-cold model.

Figure 3-4. Pro-inflammatory cytokines are produced during sIRI.
HK-2 cells were subjected to control (no IRI injury), sIRI-cold or sIRI-RT conditions. Cell culture supernatants post-reoxygenation were collected and analyzed for pro-inflammatory cytokines (A) IL-6, (B) IL-8, (C) GM-CSF, (D) IFNγ and (E) TNFα using Multiplex ELISA, n=5. Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and post-hoc Tukey HSD test. ns p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.000001.
3.5 Comparison of cell activation and damage

Another crucial aspect of IRI during kidney transplantation is the release of cellular material and proteins as a result of cell death and damage, which can act as injury indicators or signals to further stimulate the innate immune response. NGAL is a protein produced in the kidney in reaction to damage or inflammation, and it has long been used as a diagnostic tool for kidney injury. NF-κB is a transcription factor that regulates the expression of genes involved in inflammation and immune response and is activated in response to several stimuli, including oxidative stress. In the kidney, NF-κB activation promotes the release of pro-inflammatory cytokines and chemokines, exacerbating the damage produced by IRI. We assessed the release of these damage markers after subjecting cells to sIRI injury at both temperatures to further validate whether our model accurately matches the conditions of IRI following DCD kidney transplantation. ELISA analysis of cell culture supernatants revealed that the sIRI-cold group had higher levels of both NF-κB (Figure 3-5A) and NGAL (Figure 3-5B), while the sIRI-RT group had a decrease in levels. Our sIRI-RT model, as expected, is not completely perfect, as damage marker levels remain elevated when compared to the control.
Figure 3-5. Relative differences of relevant activity indicative of cell damage between conditions.

HK-2 cells were subjected to control (no IRI injury), sIRI-cold or sIRI-RT conditions. Levels of (A) Nuclear factor kappa B (NF-κB) from nuclear extracts and (B) neutrophil gelatinase-associated lipocalin (NGAL) in cell culture supernatants were measured by ELISA, n=5. Cold model demonstrates an increase in NF-κB and NGAL levels compared to RT, suggesting RT induces a reduced IRI response. Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and post-hoc Tukey HSD test. **p<0.01, ***p<0.001, ****p<0.000001.

3.6 Small scale drug screening using the sIRI-RT model

A smaller-scale drug screening was undertaken to ensure this model provided a legitimate and efficient platform for drug testing as the primary purpose of the developed IRI model is to eventually conduct large-scale drug screening. To test this model, we selected several candidate drugs based on studies being conducted by other research groups. As inflammatory pathways are largely conserved across multiple systems, the studies that influenced the selection of the drugs
were centered around reducing COVID-19-related damage or the discovery of methods for protecting other organs during transplantation.

During the preservation stage of the sIRI-RT model, HK-2 cells were treated with one of 20 medications at a starting dose of 1mM. This is consistent with the notion of administering medications into the machine perfusion system during the preservation and storage stage of a transplant. Initially, LDH levels were measured as a simple and affordable technique to evaluate cell damage (Figure 3-6). Drugs to note are n-acetyl cysteine, fluvoxamine, fludrocortisone, fenofibrate, and nifedipine, which were able to considerably decrease LDH levels.

Dose-response curves are required to establish the dose range at which a medication achieves the desired therapeutic effect while avoiding the negative effects of overdose. While the starting dose was at 1mM, all drugs may not be effective at the same dose. Therefore, dose response curves were conducted for all drugs at 5mM, 1mM, 0.1mM, 0.01mM, 0.001mM, 0.0001mM, and 0.00001mM (Appendix A). While dose responses were conducted, only n-acetyl cysteine, fluvoxamine, fludrocortisone, fenofibrate, and nifedipine consistently showed a reduction in LDH levels from the baseline (sIRI + no drug treatment), therefore dose response curves are shown in Figure 3-7 for only these short-listed drugs. From this, we were able to determine optimal doses for subsequent experiments. Optimal doses were selected based on the lowest LDH activity and/or greatest reduction compared to baseline.

HK-2 cells were treated with one of five short-listed drugs at the identified optimal doses during the preservation stage of sIRI-RT treatment. Flow cytometry was used to assess the medication treatments' efficacy to minimize cell mortality (Figure 3-8A). Among the five medications tested, n-acetyl cysteine increased cell viability the most, followed by nifedipine,
fludrocortisone, and fluvoxamine (Figure 3-8B). Surprisingly, there was no significant increase in cell viability as compared to untreated cells (sIRI-RT + no treatment).

Lastly, cell culture supernatants were tested for inflammatory cytokines, IL-6 and TNFα, as well as the damage marker NGAL, as the reduction of inflammation is key in minimizing IRI damage. When compared to sIRI-RT treatment alone, all five short-listed medications significantly reduced IL-6, TNFα, and NGAL levels (Figure 3-9). Interestingly, findings indicate that fluvoxamine had the most promising anti-inflammatory properties of the five short-listed drugs studied.
Figure 3-6. Drug treatment further reduces cell death in sIRI-RT model.
Consistent with the sIRI-RT model, HK-2 cells were subjected to 1 hour of warm hypoxia. Cells were treated with one of 20 candidate drugs during the course of the 24-hour preservation period at 22°C, starting with a concentration of 1mM (0.001mM for fludrocortisone). Finally, cells were subjected to 24 hours reoxygenation 37°C as usual. Post reoxygenation, quantification of lactate dehydrogenase (LDH) levels was conducted as a marker for cell death, n=4. A reduction in LDH activity was detected in n-acetyl cysteine, fluvoxamine, fludrocortisone, fenofibrate and nifedipine. Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and post-hoc Tukey HSD test. *p<0.05, **p<0.01.
Figure 3-7. Dose-response curves were conducted to determine the most optimal dose for treatment.

HK-2 cells were treated with one of the 20 candidate drugs during the preservation period under sIRI-RT conditions at doses of 5mM, 1mM, 0.1mM, 0.01mM, 0.001mM, 0.0001mM, and 0.00001mM, of which, short-listed drugs of n-acetyl cysteine, fluvoxamine, fludrocortisone, fenofibrate, and nifedipine are presented above. Post reoxygenation, quantification of lactate dehydrogenase (LDH) levels were conducted as a marker for cell death, n=4. Optimal doses are 1mM for n-acetyl cysteine, fluvoxamine, fenofibrate, and nifedipine, and 0.001mM for fludrocortisone. Data is represented as the mean ± SEM.
Figure 3-8. Drug therapy further improves cell viability under sIRI-RT conditions.

Consistent with the sIRI-RT model, HK-2 cells were subjected to 1 hour of warm hypoxia. Cells were treated with 5 short-listed drugs during the course of the 24-hour preservation period at 22°C at a concentration of 1 mM (0.001 mM for fludrocortisone). Finally, cells were subjected to 24 hours reoxygenation 37°C as usual. End-point analysis following reoxygenation was carried out with (A) flow cytometry using Annexin V and 7-AAD staining. (B) Cell viability (n=4) and (C) apoptosis (n=4) were quantified using flow cytometry results. Overall, there is increased cell viability in all treated groups compared to control (sIRI-RT + no treatment). Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and post-hoc Tukey HSD test. ns p>0.05, *p<0.05, **p<0.01, ***p<0.001.
**Figure 3-9. Drug therapy further reduced inflammation under sIRI-RT conditions.**

Consistent with the sIRI-RT model, HK-2 cells were subjected to 1 hour of warm hypoxia. Cells were treated with 5 short-listed drugs during the course of the 24-hour preservation period at 22°C at a concentration of 1 mM (0.001 mM for fludrocortisone). Finally, cells were subjected to 24 hours reoxygenation 37°C as usual. Following reoxygenation, cell culture supernatants were collected and analyzed for pro-inflammatory cytokines (A) IL-6 and (B) TNFα, and damage marker (C) NGAL through ELISA, n=4. A reduction in inflammatory markers were observed for all drug treatments, suggesting a further reduction in inflammation relative to the control (sIRI-RT + no treatment). Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and post-hoc Tukey HSD test. ns p>0.05, *p<0.05, **p<0.01, ***p<0.001.
Chapter 4

4 Discussion

IRI, a risk factor for acute renal damage, delayed graft function, and acute and chronic rejection, occurs during kidney transplantation (Hameed et al., 2020). Static cold storage is currently the industry standard for organ preservation. The grafts nevertheless sustain damage from cold ischemia despite the method's success in lowering metabolic demand and boosting cell survival prior to transplant (Hosgood et al., 2023). More importantly, there is a need to increase the number of qualified donors to address the transplant shortage. DCD donors have an increased susceptibility to damage as this type of donor often experience extended periods of warm ischemia, thereby making them ineligible for transplant (Urbanelliis et al., 2021). Recent transplantation research has concentrated on developing more physiological storage settings for organs, as well as strategies to mitigate or reverse the effects of IRI in order to improve preservation. As a result, ongoing research and technological developments emphasize the use of oxygenated perfusion at normothermic or subnormothermic temperatures. Notably, our research group has already demonstrated the superior outcomes of oxygenated subnormothermic perfusion compared to cold storage. Nevertheless, despite these developments, IRI-related harm continues to be a source of concern. Our goal is to incorporate existing drugs that target the damage caused by IRI directly into the perfusate system. However, screening of drug candidates using animal models raises ethical concerns as this would entail blind testing. As an alternative, in vitro studies are a great way to elucidate potential properties of the drug candidates, allowing for a reasonable degree of suspicion for success. Currently no models exist to allow for such testing, and therefore it is essential to first develop an in vitro model that accurately replicates the damage induced by renal
IRI. For this project, we designed and characterized an *in vitro* model of IRI that mimics the stages of oxygenated subnormothermic preservation of DCD kidney transplantation and compared it to a previously established *in vitro* model of cold preservation. We also conducted a small-scale drug screening to study the therapeutic potential of various drug candidates.

Our first goal was to gain a thorough grasp of how cell death and viability were affected by cold storage and oxygenated subnormothermic preservation in an *in vitro* kidney transplantation model. We used a variety of methodologies, such as live cell imaging and flow cytometry, to analyze and contrast the results of these preservation procedures. Our findings showed a notable distinction between the two preservation settings in terms of cell death; compared to cells stored in cold storage, cells treated to oxygenated subnormothermic preservation showed a considerable reduction in cell death as demonstrated using 7-AAD and PI staining, which are frequently used as markers for cell death. In the oxygenated subnormothermic preservation group, the levels of staining were reduced, indicating higher cell viability and lower rates of cell death. Our findings concerning cell death are supported by the results of our *ex vivo* study, which used TUNEL (terminal deoxynucleotidyl transferase dUTP nick end-labeling) staining and H&E (hematoxylin and eosin) staining of histologic sections of tubular epithelial cells. TUNEL staining allowed us to quantify the level of apoptosis, while H&E staining provided insights into necrosis. Our findings from the *ex vivo* study was consistent with those from these *in vitro* models, demonstrating that the oxygenated subnormothermic preservation group had a lower incidence of apoptosis and necrosis (Bhattacharjee et al., 2019). Excessive cell death sets off a chain reaction of adverse effects such inflammation and activation of the innate immune system, which contribute to post-transplantation functional impairment (Priante et al., 2019). The observed similarities in cell death
patterns between the *in vitro* and *ex vivo* models therefore provide additional evidence for the applicability and relevance of our *in vitro* model in simulating clinical kidney transplantation.

Damage brought on by ischemia and reperfusion is the greatest concern with respect to transplantation success. Many current preservation methods, including those employed by other research groups, rely on non-oxygenated perfusion. However, our group went a step further by using a hemoglobin oxygen carrier that allows for the direct supply of oxygen to the organ and therefore more accurately simulates physiological conditions (Bhattacharjee et al., 2019). Our goal was to facilitate aerobic respiration and stop the buildup of ROS and lactate, which are known to cause tissue damage (Bhargava & Schnellmann, 2017). We examined the results of oxygenated vs. non-oxygenated conditions being applied to our existing IRI model at room temperature to evaluate the efficacy of oxygenation. The decrease in cell death under oxygenated conditions supports the positive effects of oxygen supplementation. By simulating physiological conditions and preventing the detrimental effects associated with oxygen deprivation, oxygenated conditions offer promising avenues for improving the preservation and viability of organs in clinical applications.

Numerous cytokines play important roles in the inflammatory response and tissue damage in renal IRI. IL-6 is a pro-inflammatory cytokine that starts the acute phase response and encourages additional immunological responses, such as helping to draw in and activate neutrophils and macrophages (Tanaka et al., 2014). IL-8 is a chemokine that draws neutrophils to the site of injury and triggers the release of ROS and proteases, which intensifies the inflammatory cascade (Kwiatkowska et al., 2017). GM-CSF promotes granulocyte and macrophage production and activation, which is known to contribute to tissue injury (Huen et al., 2015). TNF-α, which is produced by a variety of immune cells, increases inflammation by promoting immune cell
recruitment, adhesion molecule expression, and the production of other pro-inflammatory cytokines (Taguchi et al., 2021). IFN$\gamma$, primarily produced by activated T cells and natural killer cells, modulates immune responses, and promotes inflammation (Day et al., 2006). The aforementioned cytokines contribute to the tissue damage and inflammatory response seen in kidney IRI, making them prospective targets for therapeutic strategies aimed at reducing kidney injury. The levels of several cytokines and chemokines that are stimulated under cold storage settings have been successfully defined in earlier studies. This foundation served as the basis for our investigation, which was to investigate how oxygenated subnormothermic settings affected the inflammatory response. We noticed a considerable drop in the levels of these inflammatory markers as compared to cold storage, which indicates successful mitigation of IRI-induced inflammation. This finding emphasizes how crucial preservation at near physiological conditions can be in reducing the inflammatory cascade brought on by ischemia-reperfusion damage.

We measured and compared the levels of NF-$\kappa$B and NGAL during cold storage and subnormothermic storage to strengthen the validity and robustness of our in vitro model. NF-$\kappa$B is an essential transcription factor that controls several immunological and inflammatory responses, and NGAL is a biomarker linked to kidney damage (Park & Hong, 2016; Törnblom et al., 2020). We analyzed the inflammatory response and damage progression in our model by measuring the levels of these markers. According to our findings, subnormothermic storage settings produced better results than cold storage conditions, as seen by lower levels of NF-$\kappa$B and NGAL. These inflammatory markers declined, which points to a potential amelioration of the inflammatory cascade and a decrease in kidney injury. Nevertheless, it is crucial to remember that in contrast to the control group, there was still a sizable degree of inflammation present despite the improvements shown in the subnormothermic groups. The control group is equivalent to organs
taken from living or DBD donors and reflects the healthiest state. Therefore, to improve the viability and success of kidney transplantation, our future goal is to further reduce damage and inflammation by aiming for even better results.

The innate immune response is essential in defending against numerous stressors and foreign substances that enter the body. We chose 20 drugs for a small-scale drug screening using our recently developed in vitro model, taking advantage of the nonspecific nature of the innate immune response. This decision was made under the presumption that these medications, while not being direct anti-inflammatory agents themselves, would have some degree of anti-inflammatory properties as they are currently being used to treat various inflammation-related disorders. We sought to find possible candidates that would provide therapeutic advantages in the context of our model and possibly extend their applicability to inflammatory disorders associated with the kidney through common pathways and mechanisms via the innate immune system (Marshall et al., 2018).

To evaluate the panel of drugs for their therapeutic potential in reducing cellular harm and cell death, we used the LDH cytotoxicity assay. Broken plasma membranes are a hallmark of apoptosis, necrosis, and other types of cellular injury and LDH is a marker that is quickly released into the extracellular space when the plasma membrane is damaged (P. Kumar et al., 2018). Given the quantity of medications to be tested and the evaluation of their therapeutic advantages via dose-response curves, using this assay was beneficial due to its decreased labour intensity. Reduced LDH released into the medium during the initial screening would suggest reduced cell death and damage. Of note, n-acetyl cysteine, fluvoxamine, fenofibrate, nifedipine, and fludrocortisone showed some degree of protection against damage brought on IRI, as shown by LDH assay values ranging from 5mM to 0.00001mM. We used flow cytometry to identify cell death and assessed some of the same inflammatory and damage indicators as earlier to further validate these results.
Additional evidence of the preventive effects of these medications against IRI-induced damage was provided by this investigation, which showed a significant decrease in IL-6 and NGAL levels, as well as a sizable reduction in TNF-α. All three markers measured post-treatment – IL-6, NGAL and TNF-α – are produced predominantly through activation of NF-κB (Buonafine et al., 2018; Tanaka et al., 2014). Therefore, further investigation will be required to determine the underlying reason for the variable influence on marker level reduction.

While LDH assays offer a practical and affordable way to evaluate therapeutic efficacy, it is necessary to consider any potential confounding factors, such as cell number, that may affect the precision and interpretation of the data (Smith et al., 2011). Additionally, the utility of some medications in an in vitro setting might not be the same as how they behave in vivo. For instance, VEGF stimulates the growth of new blood vessels, a process known as angiogenesis (Shibuya, 2011). The relevance of VEGF-related effects, however, might be constrained in an in vitro model where blood vessel development is not possible. To fully assess the potential of medications, additional testing is required, including in vivo studies and the use of more cutting-edge technology for the assessment of inflammation and cell death.

4.1 Limitations

In vitro models are a useful tool for conducting research as in vivo studies are financially and ethically impractical for high throughput research such as drug screening. Nevertheless, in vitro models are artificial representations of the physiological environment, as it is an oversimplification of the complex processes that occur in the human body. Cells in in vivo systems are influenced by several factors, including interactions between different cell types, tissues, organs, and organ systems, as well as systemic factors including hormones, immune responses,
circulation, and neural signaling (Ghallab, 2013). Given that in vitro models usually consist of single cell types, they often fail to accurately represent the true in vivo response.

The use of normoxia (20% oxygen) in in vitro models is another limitation of this project. Despite the fact that room air typically contains 20% oxygen, it is important to remember that the majority of mammalian tissue cells experience oxygen partial pressures between 1-6% oxygen in their natural in vivo environment (Stuart et al., 2018). This difference between physiological oxygen levels in vivo and in vitro normoxia generates potential discrepancies that could affect the model's accuracy and applicability. Our interpretations and conclusions may be affected by variations in cellular responses caused by the differences in oxygen availability between in vitro and in vivo environments.

Last but not least, HK-2 cells have the benefit of being cloned from a single cell, creating a homogenous cellular population to enable repeatable outcomes (Kaur & Dufour, 2012). These models, which include the use of cloned cell lines, might fall short of fully capturing the complex biological diversity and individual heterogeneity seen among recipients of human kidney transplants. The interaction of patients' genetic make-ups, pre-existing illnesses, and varying immunological responses adds to the complexity of patient outcomes. Therefore, while in vitro studies offer insightful information about prospective treatment measures, careful interpretation is needed when applying these results to the wide range of patient populations found in clinical settings.

4.2 Future Directions

The potential for drug repositioning in the transplantation field can be substantial. Firstly, combination therapy will undoubtedly be a key feature in how we approach transplantation in the
future. To avoid graft rejection and minimize complications, transplant recipients may need to take a multitude of medications. It may be possible to explore pharmacological combinations that display synergistic effects or target multiple mechanisms implicated in transplant rejection by understanding all the mechanistic properties of the drug library. A consequence of this strategy is improved outcomes while reducing adverse side effects.

More importantly, as there are many different organ systems covered by transplantation (such as liver, lung, etc.), drug repositioning can offer therapeutic benefits for a range of transplant types. This approach can encourage the implementation of *in vitro* models for other cell types involved in IRI.

Finally, our *in vitro* model can eventually offer a distinctive framework for investigating the modalities of drug candidates in the context of renal IRI following DCD kidney transplantation. This model can be utilized for large-scale drug screening by employing a library of over 770 clinically authorized drug candidates. With the use of pre-prepared drugs and cutting-edge technology that enables the simultaneous treatment of cells, it will be possible to discover drugs with potential therapeutic effects quickly and effectively.

### 4.3 Conclusions

In conclusion, *ex vivo* research often utilize *in vitro* models due the many advantages they provide. This includes reducing the need for using animals or people as subjects, offering a simplified and controlled representation of the complex *in vivo* environment, and enabling researchers to conduct preliminary research to screen a significant number of treatments before proceeding with more expensive *ex vivo* and *in vivo* studies. Therefore, this room temperature *in vitro* model developed as part of this project can act as the foundation for future *ex vivo* and *in vivo*
studies related to kidney preservation given the accuracy of its IRI response. The response to IRI is accurately simulated by this model, making it appropriate for use in subsequent *ex vivo* and *in vivo* studies. Several methods were used to evaluate the model's efficacy, including flow cytometry and live cell imaging as assessments of cell viability and death, and the measurement of several inflammatory markers. Importantly, the model's IRI response's agreement with *ex vivo* results further verifies its applicability and dependability. Moreover, we have piloted the capability of this model to identify therapeutic agents that, when added to a machine perfusion organ preservation system, can lessen the harm caused by IRI; this work has identified several candidate agents that show promise for future study. Additionally, the quick transition of pharmaceuticals to clinical use will be made possible by repositioning already clinically approved drugs. Consequently, the improvement of storage conditions will present an opportunity for DCD organ preservation and restoration. As such, increasing the amount and quality of organs available for transplantation can help us reach our goals to improve the quality of life for individuals with ESRD.
Appendix A. Not all drugs showed decreased LDH levels post-treatment at concentrations 5mM, 1mM, 0.1mM, 0.01mM, 0.001mM, and 0.0001mM.
While not shown in prior sections of t, HK-2 cells were treated with all candidate drugs for 24 hours at 22°C during the preservation period. Since the optimal dose of action would differ for each agent, all medicines were titrated at concentrations of 5mM, 1mM, 0.1mM, 0.01mM, 0.001mM, and 0.0001mM. End-point analysis of LDH levels were quantified post reperfusion. The drugs that failed to show a discernible reduction in LDH levels in comparison to the control condition (not treatment) were excluded from the main figures of this paper. Data is represented as the mean ± SEM. Means were analyzed using one-way ANOVA and post-hoc Tukey HSD test. *p<0.05, **p<0.01.
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