21°C is the emerging ideal temperature for kidney preservation in the presence of hydrogen sulfide

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology
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Abstract

Kidney transplantation is the treatment of choice for patients with end-stage kidney disease. However, the shortage of transplantable kidneys has led to ever-increasing waiting lists. As a result, kidneys obtained via donation after cardiac death (DCD) are being used more frequently for transplantation. However, they exhibit poorer outcomes due to ischemia-reperfusion injury (IRI) and cold preservation methods, such as static cold storage (SCS). Supplementing cold preservation solutions with hydrogen sulfide donors, such as AP39, has been shown to improve renal IRI and graft outcomes, but the injury associated with cold preservation remains. This thesis evaluates the effect of subnormothermic kidney preservation at 21°C with AP39. In an in vitro model of renal IRI, adding AP39 to University of Wisconsin (UW) solution improved its viability for subnormothermic preservation at 21°C in a dose-dependent manner. Additionally, subnormothermic storage of DCD porcine kidneys in AP39-supplemented UW solution reduced necrosis compared to SCS in UW alone. Since oxygenated perfusion is the norm for normothermic (35-37°C) and subnormothermic (20-34°C) kidney preservation research, AP39-supplemented autologous blood was used for the ex vivo perfusion of DCD porcine kidneys at 21°C. The treatment group exhibited higher urine output, lower tissue injury and pro-survival gene expression patterns compared to subnormothermic perfusion with blood alone and SCS. Since the use of blood would complicate the clinical translation of our approach due to national shortages and logistical complexities, we investigated the effect of adding AP39 to Hemopure, a hemoglobin-based oxygen carrier. Subnormothermic perfusion of DCD porcine kidneys with AP39-supplemented Hemopure improved renal graft function and reduced tissue injury in a similar manner as in the previous model. Several important conclusions emerged. Adding 200 nM AP39 to preservation
solutions, blood and blood substitutes improved renal outcomes. Importantly, oxygenated subnormothermic perfusion with AP39-supplemented perfusates improved DCD kidney outcomes compared to SCS, the clinical standard of care. Additionally, subnormothermic preservation with AP39 reduced renal apoptosis and induced pro-survival gene expression. Overall, we transformed a novel idea into a viable kidney preservation approach that is pending patent approval. If clinically approved, this approach could facilitate the increased use of DCD kidneys for kidney transplantation.

Keywords

Hydrogen sulfide, AP39, Kidney Preservation, Subnormothermic, Ischemia Reperfusion Injury, Apoptosis, Hypoxia, Porcine, Kidney Perfusion, Organ Procurement, Donation after Cardiac Death, Renal Transplantation.
Summary for Lay Audience

Kidney transplantation is the treatment of choice for patients with end-stage kidney disease. However, the shortage of transplantable kidneys has led to ever-increasing waiting lists. Kidneys from deceased donors are being used more frequently to meet the demand. Unfortunately, these kidneys typically exhibit poorer outcomes than kidneys from living donors and thousands of these kidneys are discarded each year due to logistical limitations and strict selection criteria. As such, strategies that can improve the outcomes and facilitate the use of more deceased donor kidneys are of interest. One factor that contributes to the poor outcomes of these kidneys is cold preservation from the time of retrieval until transplantation. Our research has shown that adding hydrogen sulfide donors to cold preservation solutions improves kidney graft outcomes. However, the premise of cold preservation still causes tissue damage. Thus, the aim of this thesis is to evaluate the impact of kidney preservation around room temperature (21°C) with the use of a hydrogen sulfide donor called AP39. We used a combination of cellular and pig kidney perfusion models to investigate the effect of adding AP39 during kidney preservation at 21°C. We found that adding AP39 to preservation solutions, blood and blood substitutes improved their suitability for kidney preservation at 21°C. Importantly, kidney preservation at 21°C improved renal graft function and reduced kidney injury compared to cold storage, the clinical standard of care, in our pig kidney perfusion models. Additionally, AP39 reduced kidney injury and induced pro-survival gene expression in this context. Overall, we transformed a novel yet obscure idea into a viable and effective kidney preservation approach that is pending patent approval. If clinically approved upon testing with discarded human kidneys, this approach could facilitate the increased use of deceased donor kidneys for kidney transplantation.
Co-Authorship Statement

The detailed information of co-authorship for each chapter is given below:


Juriasingani S conducted the bulk of the research for this literature review and wrote the manuscript. Akbari M, Luke P and Sener A provided suggestions on studies to include and edited the manuscript.


Juriasingani S executed experiments, analyzed and interpreted the data, wrote the manuscript and handled revisions. Akbari M helped with technical guidance and manuscript editing. Chan JYH provided preliminary data that guided experiment design. Whiteman M provided the AP39. Sener A designed the study, edited the manuscript and secured funding from Lawson Research Institute.

Juriasingani S executed experiments, analyzed and interpreted the data, wrote the manuscript and handled revisions. Ruthirakanthan A, Richard-Mohamed M and Akbari M helped with experiment execution and data acquisition. Aquil S, Patel S and Al-Ogaili R performed the animal surgeries. Whiteman M provided the AP39. Luke PPW helped with study design. Sener A designed the study, edited the manuscript and secured funding from Physicians Services Incorporated Foundation and the Lawson Research Institute.


Juriasingani S executed experiments, analyzed and interpreted the data and wrote the manuscript. Jackson A, Zhang M and Ruthirakanthan A helped with experiment execution and data acquisition. Sogutdelen E, Levine M and Mandurah M performed the animal surgeries. Whiteman M provided the AP39. Luke PPW helped with study design. Sener A designed the study, edited the manuscript and secured funding from Physicians Services Incorporated Foundation and the Kidney Foundation of Canada.
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<tr>
<td>AJUBA</td>
<td>Lim-domain containing protein AJUBA</td>
</tr>
<tr>
<td>ATN</td>
<td>Acute tubular necrosis</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAG3</td>
<td>BCL2-associated anthanogene (BAG) family chaperone regulator 3</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
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<tr>
<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BCL10</td>
<td>B-cell lymphoma/leukemia 10</td>
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<tr>
<td>DBD</td>
<td>Donation after brain death</td>
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<tr>
<td>DCD</td>
<td>Donation after cardiac death</td>
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<tr>
<td>DDIT3</td>
<td>DNA damage -inducible transcript 3</td>
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<tr>
<td>DGF</td>
<td>Delayed graft function</td>
</tr>
<tr>
<td>ECD</td>
<td>Extended criteria donors</td>
</tr>
<tr>
<td>EGRI</td>
<td>Early growth response protein 1</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>H$_2$S</td>
<td>Hydrogen sulfide</td>
</tr>
<tr>
<td>HTK</td>
<td>Histidine-Tyrosine-Ketoglutarate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>HMP</td>
<td>Hypothermic machine perfusion</td>
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<tr>
<td>HOXD8/10</td>
<td>Homeobox protein Hox-D8/10</td>
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<td>HSPA1A</td>
<td>Heat shock 70kDa protein 1A</td>
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<td>HSPD1</td>
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<td>IL-1β</td>
<td>Interleukin-1β</td>
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<td>IRI</td>
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<td>MAPK7</td>
<td>Mitogen-activated protein kinase 7</td>
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<tr>
<td>MPTP</td>
<td>Mitochondrial permeability transition pore</td>
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<td>Na2S</td>
<td>Sodium sulfide</td>
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<tr>
<td>NaHS</td>
<td>Sodium hydrosulfide</td>
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<tr>
<td>NDD</td>
<td>Neurologically determined death</td>
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<td>NMP</td>
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<tr>
<td>NRROS</td>
<td>Negative regulator of reactive oxygen species</td>
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<td>PCK1</td>
<td>Phosphoenolpyruvate carboxykinase, cytosolic</td>
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<td>PDK3</td>
<td>Pyruvate dehydrogenase kinase, mitochondrial</td>
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<td>RBF</td>
<td>Renal blood flow</td>
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<tr>
<td>RGCC</td>
<td>Regulator of cell cycle</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SCS</td>
<td>Static cold storage</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<tr>
<td>SMAD3</td>
<td>Mothers against decapentaplegic homolog 3</td>
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<td>Transforming growth factor β</td>
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<td>Tumour necrosis factor-α</td>
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<td>UW</td>
<td>University of Wisconsin</td>
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Chapter 1

1 Introduction

Sections 1.6.1 and 1.6.2 have been adapted from:


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1.1 End-stage renal disease

Chronic kidney disease refers to a persistent and progressive reduction in kidney function, which is primarily measured using the estimated glomerular filtration rate. This reduction in kidney function can be caused by a multitude of factors including diabetes, hypertension and hereditary conditions.¹ The final and most severe phase of chronic kidney disease is referred to as end-stage renal disease (ESRD), where both kidneys have failed irreversibly and kidney function has decreased to less than 10% of normal.² ESRD is a significant global health concern because no cure exists for this condition. It is estimated that 1.2 million people died from kidney failure worldwide in 2015, an increase of 32% since 2005.³ Additionally, the global incidence of ESRD is rising and this trend is evident in Canada because the number of ESRD patients has increased by 33% between 2010 and 2019.⁴

ESRD patients require renal replacement therapy to live. The two prevalent forms of renal replacement therapy are dialysis and kidney transplantation. Dialysis refers to the mechanical filtration of blood to eliminate waste and toxins in place of the kidneys. In Canada, 56.8% of ESRD patients were receiving some form of dialysis as of the end of 2019.⁴ Dialysis takes a toll on patients due to its impact on their quality of life, ability to work and long-term survival. It also places an enormous burden on the Canadian healthcare system as the annual cost of dialysis for one patient is nearly $100,000. As a result, approximately $2.5 billion dollars are spent on the care of Canadians receiving dialysis each year and this amount increases to $40 billion when considering the costs associated with CKD broadly.⁵ While dialysis is the primary treatment modality for ESRD patients in
Canada, the balance is shifting towards renal transplantation. The reasons for this shift are discussed in the next section.

1.2 Renal Transplantation

Renal transplantation refers to the surgical implantation of a functional donor kidney into a recipient with kidney failure. In 2019, 1,648 adult kidney transplants were performed in Canada. Between 2010 and 2019, the total number of kidney transplants performed in Canada has increased by 41%. This increase can be attributed to the superiority of renal transplantation over dialysis in terms of recipient outcomes. A robust systematic review looking at 110 studies that encompass a total of 1,922,300 patients has found that most transplant centers report lower mortality, higher 5-year survival rates, lower risk of cardiovascular events and improved quality of life among transplant recipients compared to patients on dialysis. The Canadian Organ Replacement Register reports that the unadjusted 5-year graft survival rate is ~ 47% for patients on dialysis (depending on type of dialysis) vs. ~ 85% for kidney transplant recipients (depending on type of organ donor). Additionally, several studies have shown that transplants are more cost-effective in the long run than dialysis. Furthermore, it has been estimated that increasing the kidney transplant rate in Canada by just 10% could significantly decrease the burden of CKD on disability payments from public health and pension plans.

1.2.1 Shortage of organs and increased use of deceased donor kidneys

One persisting problem that limits the rates of kidney transplantation is the shortage of donor kidneys that occurs at a regional level across the globe. Despite the 1,648 adult kidney transplants performed in 2019, 3,261 Canadians were still on the waiting list at the
end of the year. Additionally, 72 patients died in 2019 while waiting for a kidney transplant. Traditionally, kidneys from living donors (related or unrelated) are preferable in order to maximize recipient outcomes. However, physicians at many transplant centers around the world also use marginal/sub-optimal organs from deceased donors to bridge the gap. Of the 1,648 kidney transplants performed in Canada in 2019, 505 kidneys were procured from living donors and 1,143 kidneys were procured from deceased donors.

There are several types of deceased donors and these types have important implications for post-transplant graft and recipient outcomes. Donation after brain death or neurologically determined death (DBD or NDD) refers to donors who experienced brain death but their blood circulation and respiration remained intact by measures such as ventilation. Donation after cardiac death (DCD) refers to donors who experienced a cardiac standstill or cessation of cardiac function before the organs were procured. DCD donation can occur as a result of unforeseen tragedies leading to death in a hospital (uncontrolled DCD) or death resulting from withdrawal of life support (controlled DCD). Organs from DCD donors can be considered as marginal because they experience ischemic injury due to the immediate or gradual reduction in blood flow as cardiac death occurs. Clinically, DCD kidneys lead to poorer post-transplant outcomes than living donor kidneys due to the ischemic injury that occurs during cardiac death. DCD and NDD donors can also be classified as expanded criteria donors (ECD) if they are ≥ 60 years old or aged 50-59 with any two of the following three criteria: (1) death resulting from a stroke, (2) a history of hypertension, and (3) terminal level of creatinine (a waste product produced by muscles) in the serum of >1.5 mg/dL. Organs from ECD donors, especially ECD/DCD donors, can also be considered as marginal due to the presence of comorbidities. However, appropriate
donor and recipient screening can mitigate the risks associated with transplanting organs from DCD, ECD or ECD/DCD donors.

In an attempt to meet the increasing global demand for kidney transplants, kidneys from deceased donors are being used more frequently. In Canada, the deceased donor rate has increased 42% between 2009 and 2018. Moreover, the number of organs procured from DCD donors procured across Canada has increased by 76% between 2015 and 2019. However, kidneys from deceased donors are often discarded after being declined for transplantation due to various reasons including stringent selection criteria and systemic allocation issues. It is estimated that around 2,700 kidneys are declined annually in the US. Most of these organs are incinerated, but some end up being redirected for research use. Since these kidneys represent an untapped pool of organs that could help meet the demand for kidney transplants, strategies that could facilitate their use by mitigating IRI and improving outcomes are of interest.

1.2.2 Clinical parameters used to evaluate post-transplant graft function

Renal graft dysfunction is a major clinical concern and several types of complications can contribute to this condition. Surgical complications include urinary leaks, renal vascular thrombosis and pseudoaneurysms due to a renal puncture. Medical complications include graft rejection, drug nephrotoxicity and acute tubular necrosis (ATN). Various clinical parameters are used to evaluate post-transplant graft function. The immediacy, quantity and frequency of urine output are important measures of kidney function. Additionally, ultrasounds are used to determine the renal resistive index and detect edema. Importantly, blood analysis is critical for determining serum creatinine
levels and whether a patient is hyperkalemic, acidotic or hypervolemic. Depending on the patient’s outcomes, dialysis may be required in the first week following the transplant surgery, which is referred to as delayed graft function (DGF). The exact criteria for the diagnosis of DGF varies by physician, transplant center, region and country. However, it is undeniable that DGF is a major clinical concern as the incidence of DGF in kidneys from deceased donors is around 20% across various transplant centers in North America. Additionally, DGF leads to decreased long-term graft survival. Thus, strategies to mitigate DGF in marginal kidneys are of interest, especially since such organs are being used more frequently.

1.3 Ischemia-reperfusion injury

One major factor that contributes to DGF is ischemia-reperfusion injury (IRI). IRI is a result of the interruption in renal blood flow (RBF) that occur as a kidney is procured, preserved and transplanted. Ischemia refers to the loss of RBF following nephrectomy and during preservation. This is followed by reperfusion, which refers to the reintroduction of RBF during transplant surgery. Together, this leads to a series of cellular and physiological events that damage the kidney graft. IRI is an inevitable aspect of kidney transplantation; however, it is particularly exacerbated in kidneys from deceased donors. For example, DCD kidneys experience an additional period of ischemia prior to nephrectomy as cardiac death occurs and blood circulation decreases. Additionally, kidneys from deceased donors can experience longer durations of ischemia as they are transported between transplant centers across the country or continent, which contributes to the increased rates of DGF observed following the transplantation of these organs. Thus, optimizing organ
preservation and minimizing IRI are critical for reducing DGF and facilitating the increased use of marginal grafts from deceased donors.

When considering strategies to mitigate IRI, it is important to consider the cellular mechanisms underlying the clinical manifestations of IRI. Ischemia is characterized by a cessation of blood flow that results in hypoxia. Hypoxia decreases electron transport chain activity and causes cells to switch to anaerobic respiration. The resulting decrease in adenosine triphosphate (ATP) production inhibits the activity of membrane-bound ATPases, such as the Na+/K+ ATPase, which causes accumulation of intracellular Na+ followed by an influx of water leading to cellular edema. The accumulation of Na+ ions impairs other transporters, which causes the accumulation of intracellular Ca2+. This Ca2+ overload has implications for the mitochondria, which are key sites of IRI-induced damage. The accumulation of Ca2+ in the cytoplasm and mitochondrial matrix has been shown to increase mitochondrial permeability and cause the production of reactive oxygen species (ROS), both of which are exacerbated during reperfusion.

Prolonged ischemia also causes other mitochondrial events that exacerbate oxidative damage during reperfusion. Prolonged ischemia can impair the activity of mitochondrial antioxidant enzymes and reduce the activity of the electron transport chain. The subsequent electron leak contributes to an increase in ROS production during reperfusion. Reperfusion is characterized by the influx of oxygen upon re-establishment of blood flow, which paradoxically further damages the ischemic tissues rather than reversing the damage. The leaked electrons reduce the newly present oxygen, which leads to an excess of superoxide radicals that cannot be cleared by the impaired antioxidant system. The ROS that accumulate during reperfusion can damage mitochondrial proteins and...
lipids, which leads to more pronounced impairment of mitochondrial function and eventual cell death.$^{25}$ Increased ROS-mediated damage to the electron transport chain leads to more pronounced ATP depletion, which can cause apoptosis and necrosis.$^{25}$ ROS-mediated damage to mitochondrial membrane lipids along with Ca$^{2+}$-induced mitochondrial swelling causes the formation of mitochondrial permeability transition pores (MPTP) in the inner mitochondrial membrane. Additionally, BCL-2-associated X protein (BAX) oligomerizes and translocates from the cytosol to form pores in the outer mitochondrial membrane. Due to the increased mitochondrial membrane permeability, cytochrome c is released into the cytosol where it activates the caspases that carry out apoptosis (Fig. 1-1).$^{24,25,30}$
Figure 1-1 Activation of the intrinsic apoptotic pathway during IRI. The accumulation of Ca$^{2+}$ and production of ROS causes mitochondrial swelling and impairs mitochondrial function, which leads to increased mitochondrial permeability. Mediators of the intrinsic apoptotic pathway, such as cytochrome C, are released which activates caspases that carry out apoptosis.

In addition to causing oxidative stress and cell death, IRI induces inflammation and causes microvascular dysfunction, which leads to tissue damage and reduces graft function. Hypoxia-induced endothelial damage reduces the production of vasodilators, such as nitric oxide, and increases the production of vasoconstrictors, such as endothelin-1 and platelet-derived growth factor-B. Disruption of endothelial cell-cell junctions causes interstitial edema, which contributes to vascular permeability and reduces RBF. In addition, leukocyte adherence and extravasation leads to renal microvascular congestion, which also decreases RBF, and these processes are facilitated by the
upregulation of endothelial adhesion molecules during IRI.\textsuperscript{33} The infiltration of neutrophils and natural killer cells during reperfusion leads to renal tubular damage and the secretion of ROS and pro-inflammatory cytokines.\textsuperscript{19,34,35} Damaged tubular epithelial cells release danger-associated molecular proteins and contribute to the secretion of pro-inflammatory cytokines, which potentiates inflammation within the kidney.\textsuperscript{19,36} In particular, interleukin 6 (IL-6) and tumour necrosis factor-α (TNF-α), are key mediators of kidney injury as they have been shown to modulate leukocytes and renal tubular cells.\textsuperscript{36,37} Both of these cytokines have been linked to acute renal failure in animal models of renal IRI and knocking down these genes ameliorated renal injury.\textsuperscript{36,38,39} IRI-mediated microvascular dysfunction and inflammation are particularly important as they contribute to fibrosis, graft dysfunction and allograft rejection.\textsuperscript{19}

One of the major pathological consequences of IRI is acute tubular necrosis (ATN), which can cause DGF in transplant recipients.\textsuperscript{40–42} Morphologically, ATN manifests as apoptosis, loss of cells, gaps in the renal tubular architecture and luminal obstruction due to cell debris. It can be seen throughout the kidney (cortex and medulla) and it is associated with interstitial damage and endothelial injury.\textsuperscript{41} Clinically, ATN is linked to a decrease in glomerular filtration rate, which contributes to DGF.\textsuperscript{41,42} Another consequence of IRI is reduced RBF,\textsuperscript{33,43} which also contributes to lower glomerular filtration, and a 40-50% reduction in RBF has been observed in poorly functioning renal allografts.\textsuperscript{44} Overall, the mechanisms underlying IRI are as complicated as the resulting renal graft function and tissue injury. While the diverse range of effector mechanisms offers a multitude of potential targets for mitigating renal IRI, it is evident that any therapeutic strategy would need extensive testing due to the complexity of IRI.
1.4 Hypothermic preservation of renal grafts and cold IRI

Optimal organ preservation is essential for mitigating the effects of IRI, especially with DCD kidneys and marginal grafts. Current organ preservation methods typically involve hypothermic conditions to lower metabolic demands in order to improve graft viability in hypoxic storage conditions.\(^{45,46}\) However, prior to the advent of hypothermic storage, preliminary perfusion pumps and heparinized blood were used to maintain graft viability and transplants were treated as emergency procedures to minimize graft injury.\(^{45,47}\) In 1963, Calne et al. conducted one of the earliest investigations into the effect of cooling kidneys by comparing surface cooling to cooling via perfusion with cold heparinized blood.\(^{48}\) While the latter method was more effective, the use of cold heparinized blood caused problems such as reduced RBF in the post-transplant period. Therefore, the need for acellular organ preservation solutions became apparent.\(^{45}\)

Pioneering work by Collins et al. in 1969 led to the development of an acellular solution (Collins solution) that mimicked intracellular ion concentrations. Canine kidneys immersed in iced Collins solution were successfully preserved for 30 hours.\(^{49}\) This simple method of storing kidneys on ice (\(~4^\circ C\)) in a preservation solution is referred to as static cold storage (SCS) and it is the clinical standard of care for kidney preservation across the globe.\(^{45,46}\) While several preservation solutions have been developed since, University of Wisconsin (UW) solution and Histidine-Tryptophane–Ketoglutarate solution (HTK) are two of the frontrunners.\(^{50}\) Their compositions\(^{46,50–52}\) are listed in Table 1. UW solution is widely accepted as the gold standard cold preservation solution for liver and kidney preservation.\(^{45,46}\) It mimics intracellular ion concentrations and contains antioxidant and colloid elements. In comparison, HTK solution has lower potassium levels because it was
designed for preserving cardiac grafts; however, it has since been found to be effective for the preservation of abdominal grafts and comparable to UW solution for kidney preservation.\textsuperscript{46,50,53}

Table 1- The composition of two commonly used kidney preservation solutions.

<table>
<thead>
<tr>
<th>Solution Type</th>
<th>UW Solution (Bridge to Life ®)</th>
<th>HTK (Custodiol®)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular</td>
<td>Intracellular, low K\textsuperscript{+}</td>
<td></td>
</tr>
<tr>
<td>K\textsuperscript{+} (mM)</td>
<td>125</td>
<td>10</td>
</tr>
<tr>
<td>Na\textsuperscript{+} (mM)</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td>Buffer</td>
<td>Phosphate</td>
<td>Histidine</td>
</tr>
<tr>
<td>Cell membrane stability</td>
<td>Lactobionate, Raffinose pentahydrate</td>
<td>Tryptophan, α-Ketoglutarate</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.2</td>
</tr>
<tr>
<td>Adenosine (mM)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Antioxidant</td>
<td>Glutathione, Allopurinol</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Colloid</td>
<td>Hydroxyethyl Starch</td>
<td>Mannitol*</td>
</tr>
</tbody>
</table>

*not a colloid but helps with preventing cellular edema like hydroxyethyl starch

One of the consequences of hypothermic preservation is cold IRI, which has negative effects on renal graft survival and function.\textsuperscript{45,54,55} Hypothermia exacerbates the effects of ischemia by further reducing the activity of the Na\textsuperscript{+}/K\textsuperscript{+} ATPase, which contributes to cell swelling.\textsuperscript{42,49} It also causes a decrease in osmotic pressure that leads to interstitial edema and preservation solutions try to prevent this with colloid components like hydroxyethyl starch.\textsuperscript{47,56,57} To reduce the consequences of cold ischemia, the use of
dynamic preservation via perfusion pumps re-emerged and hypothermic machine perfusion (HMP) devices were created. HMP refers to the continuous pulsatile perfusion of the donor organ with cold preservation solution, which improves the circulation of its components. Studies on the benefits of HMP compared to SCS are inconsistent. While some studies show that HMP can improve renal graft outcomes, others report that it is comparable to SCS. However, a growing body of evidence suggests that HMP improves the outcomes of deceased donor kidneys compared to SCS. Unfortunately, there are additional drawbacks to HMP, such as expense and complexity, that limit its use for ex situ organ procurement. While both SCS and HMP are approved methods for donor kidney preservation, these additional factors explain why SCS is used more frequently and is considered the clinical standard of care.

Importantly, the duration of cold preservation, which is referred to as cold ischemia time, can significantly impact renal graft outcomes. Several studies have shown that longer cold ischemia times are strongly associated with DGF, especially in deceased donor kidney transplants. A study conducted by Serrano et al. evaluated the outcomes of 81,945 deceased donor kidney transplants in the US and found that longer cold ischemia times were linked to increased rates of DGF, which led to longer lengths of stay and higher costs. In addition, Debout et al. evaluated the outcomes of 3,829 deceased donor kidney transplants in France and demonstrated a proportional relationship between each additional hour of cold ischemia and the risk of graft failure. Longer cold ischemia times are especially detrimental for DCD kidneys. These kidneys experience warm ischemia from the time when life support is withdrawn until cardiac death, which can take up to 2 hours or longer, followed by cold ischemia during preservation. A study by Gill et al. has
shown that DCD kidneys that experience combinations of warm ischemia times exceeding 48 minutes and cold ischemia times exceeding 48 hours have a higher risk of graft failure.69 Thus, strategies to mitigate the kidney injury that occurs during cold ischemia are of interest. Many approaches have been investigated, including the use of gasotransmitters to mitigate cold IRI and kidney preservation at higher temperatures to circumvent cold IRI altogether.70,71 These approaches are described more thoroughly in subsequent sections.

1.5 The use of exogenous hydrogen sulfide therapy to mitigate renal IRI

In 1996, a landmark paper by Abe and Kimura reported on the endogenous production and signaling capability of hydrogen sulfide (H2S).72 This led to its classification as a gasotransmitter, along with nitric oxide and carbon monoxide. Since then many groups around the globe have studied its chemical and pathophysiological effects.73,74 In recent years, the effects of H2S on renal pathophysiology have been studied in greater detail and exogenous H2S therapy has been shown to mitigate warm and cold renal IRI.70,75 Emerging evidence suggests that exogenous H2S can protect against IRI-induced oxidative stress, apoptosis, mitochondrial dysfunction, inflammation, and vasoconstriction, which translates to improved renal graft outcomes.70 Several studies, including some conducted by our own group, have shown that exogenous H2S can improve renal graft function and survival using various models of renal IRI and transplantation.76–80

1.5.1 H2S donors

Considering the low endogenous concentrations of H2S with the body, the development of physiologically effective H2S donors has been a challenge. Initial studies
used H₂S gas; however, the risk of toxicity and the inability to control H₂S concentration precisely limited its application. The most widely used source of H₂S are inorganic sulfide salts like sodium hydrosulfide (NaHS) and sodium sulfide (Na₂S). Although they are referred to as donors, they are solid analogs of H₂S gas and they instantly release H₂S. Several studies, including some by our group, have shown that NaHS can mitigate renal IRI, but the immediate H₂S release that occurs with the use of NaHS also makes it difficult to control H₂S concentrations precisely.

Uncontrolled and rapid H₂S release can have damaging effects in vivo and it is not ideal for organ preservation studies. This has led to the development of synthetic H₂S donors like GYY4137 and AP39 that can facilitate slower and more controlled H₂S release upon hydrolysis. These compounds have been used successfully in various in vivo models of disease. Considering the importance of mitochondrial dysfunction in IRI, mitochondria-targeted H₂S supplementation is of particular interest. Synthetic H₂S donor AP39 is mitochondria-targeted via its positively-charged triphenylphosphonium motif. It has been shown to support the electron transport chain and cellular bioenergetics at nanomolar concentrations. Recent studies also report its effectiveness in mitigating cold myocardial, pancreatic, and renal IRI. The chemical structures of these H₂S donors are shown in Fig. 1-2. Subsequent sections that summarize the protective effects of H₂S in the context of cold IRI will include evidence resulting from the use of these H₂S donors.
Figure 1-2. The chemical structures of commonly used H₂S donors.
1.5.2 \( \text{H}_2\text{S} \) protects against oxidative stress

The generation of ROS during IRI causes graft injury. Cold preservation solutions contain antioxidant components, such as glutathione, to combat ROS. Mechanistically, an increase in \( \text{H}_2\text{S} \) levels reduces ROS production by contributing to the synthesis of glutathione and by reducing the activity of NADPH oxidase, which is a major source of ROS production in the kidney.\(^{87}\) Treatment with NaHS has been shown to reduce the expression of NADPH oxidase 4 in a mouse model of warm renal IRI. The same study also showed that treatment with NaHS reduced superoxide formation, lipid peroxidation and the ratio of oxidized glutathione to total glutathione.\(^{81}\) In addition to enhancing antioxidant defenses by contributing to glutathione synthesis, \( \text{H}_2\text{S} \) also increases the expression of other antioxidant enzymes, such as catalase.\(^{81}\) Using an \textit{in vitro} model of cold renal IRI, we have previously shown that treatment of tubular renal epithelial cells with AP39-supplemented UW solution reduces ROS levels compared to treatment with UW alone.\(^{77}\)

Recent studies have also shown that cold preservation with AP39-supplemented UW solution reduces ROS levels in \textit{in vitro} models of myocardial and pancreatic IRI;\(^{85,86}\) however, the exact mechanism underlying this trend remains unknown. It has also been suggested that \( \text{H}_2\text{S} \) interacts with other gasotransmitters (nitric oxide and carbon monoxide) and cumulative antioxidant effects occur due to their activation of \( \text{K}^+\text{ATP} \) channels.\(^{88–90}\) This effect could be contributing to the findings of the studies discussed above and more research is needed to determine the exact mechanisms underlying the antioxidant effects of \( \text{H}_2\text{S} \) donors, especially AP39.
1.5.3 H$_2$S maintains mitochondrial homeostasis and inhibits IRI-induced apoptosis

A growing body of literature shows that H$_2$S inhibits apoptosis in various models of IRI. Meng et al. have shown that GYY4137 modulates apoptotic gene expression using *in vitro* and *in vivo* models of myocardial IRI.$^{82}$ They found that treatment with H$_2$S upregulated B-cell lymphoma-2 (BCL-2), an anti-apoptotic gene, and downregulated BAX, a pro-apoptotic gene. GYY4137 also reduced the activity of caspase 3, a key effector enzyme involved in carrying out apoptosis.$^{82}$ Additionally, our collaborators have shown that treatment with AP39 reduces apoptosis in their *in vitro* model of myocardial IRI and they found that BAX was downregulated in the AP39 group.$^{86}$ Our results with renal IRI models align with these findings. Using an *in vitro* model of cold renal IRI, we have previously shown that supplementing UW solution with GYY4137 and AP39 improves tubular epithelial cell viability.$^{77}$ In addition, we found that SCS of rat kidneys in NaHS-supplemented UW solution reduces renal apoptosis using *in vivo* models of syngeneic and allogeneic murine renal transplantation.$^{77,80}$ Although not yet confirmed, the intrinsic apoptotic pathway genes implicated as H$_2$S targets in myocardial IRI may also apply to renal IRI. In addition to modulating the expression of genes of this pathway, H$_2$S donors (including AP39) can reduce cytochrome c, which suppresses sulfide signaling (through persulfidation) and, subsequently, the pro-apoptotic activity of caspase 9.$^{91}$

One interesting aspect of our previous *in vitro* results was that nanomolar concentrations of mitochondria-targeted H$_2$S donor AP39 were 1000-fold more potent in preserving cell viability than similar concentrations of GYY4137.$^{77}$ Since the mitochondria are well established as key sites of IRI, it is important to consider the impact of H$_2$S on
mitochondrial homeostasis. Several studies have reported that AP39 preserves mitochondrial membrane potential, which prevents the formation of the mitochondrial permeability transition pore that initiates apoptosis.\textsuperscript{77,86} Another possible mechanism that may be contributing to the enhanced anti-apoptotic effect of mitochondria-targeted H\textsubscript{2}S is its effect on the oxidative phosphorylation machinery. During ischemia, a lack of blood flow causes lower oxygen delivery to the tissues, which impairs this machinery and results in ATP depletion.\textsuperscript{22} ATP depletion has been shown to cause apoptosis and necrosis in murine proximal tubular cells.\textsuperscript{92} The effects of H\textsubscript{2}S on metabolism are complicated. At high concentrations, H\textsubscript{2}S can inhibit complex IV and this inhibition is thought to be the mechanism underlying the hypometabolic states induced by H\textsubscript{2}S.\textsuperscript{93} However, H\textsubscript{2}S also has the ability to maintain metabolism by acting as an electron donor. Electron donation to the ETC maintains ATP synthesis, which promotes cell survival at a local level and improves graft function at a systemic level. Szczesny et al. have shown that low concentrations of AP39 (30-100nM) stimulate the ETC in endothelial cells subjected to oxidative stress while high concentrations (300nM or greater) have an inhibitory effect.\textsuperscript{83} In summary, exogenous H\textsubscript{2}S donors, especially mitochondria-targeted donor AP39, can reduce tissue injury and increase viability during IRI by controlling the expression of apoptotic genes and by maintaining mitochondrial homeostasis.

1.5.4 H\textsubscript{2}S counteracts IRI-induced vasoconstriction

One of the factors that contributes to renal IRI and DGF after transplantation is vasoconstriction. Cold preservation of kidneys has been shown to cause a time-dependent increase in renal vasoconstriction by upregulating the expression of renal endothelin-1.\textsuperscript{94} Recent evidence suggests that H\textsubscript{2}S can counteract the effects of vasoconstriction by
inducing vasodilation. Xia et al. have reported an increase in RBF and glomerular filtration rate after the infusion of NaHS into the intrarenal arteries of rats.\textsuperscript{95} Using an \textit{in vivo} porcine model of DCD renal transplantation, we have previously shown that HMP with NaHS-supplemented UW solution improves RBF and reduces intrarenal resistance compared to HMP with UW alone.\textsuperscript{96} From a mechanistic standpoint, the H\textsubscript{2}S-mediated increase in RBF is not fully understood. However, a study evaluating the effect of NaHS in a myocardial IRI model suggests that the activation of K\textsuperscript{+}\textsubscript{ATP} channels contributes to this effect, which could be applicable to the kidney as the blockade of these channels contributes to renal IRI.\textsuperscript{97,98} It is interesting to note that cyclosporine, an immunosuppressive agent that is widely used after renal transplantation, also contributes to vasoconstriction. Its nephrotoxic effects include interstitial fibrosis and renal atrophy.\textsuperscript{99} A recent study has shown that infusion of Na\textsubscript{2}S during the reperfusion of porcine kidneys after 2 hours of cold preservation improved urine output and RBF, indicating a reversal of the vasoconstriction and glomerular atrophy caused by cyclosporine.\textsuperscript{100} Overall, these studies show that H\textsubscript{2}S-induced vasodilation can reverse IRI-induced and drug-induced vasoconstriction during in the acute post-transplant period along with increasing RBF and reducing renal resistance.

1.5.5 H\textsubscript{2}S suppresses IRI-induced inflammation

In addition to its antioxidant, anti-apoptotic and vasodilatory effects, recent studies suggest that H\textsubscript{2}S suppresses inflammatory responses in various disease models. Early work by our group showed that prolonged SCS of rat kidneys in NaHS-supplemented UW solution improved post-transplant outcomes (serum creatinine, urine output and survival) vs. UW solution alone.\textsuperscript{80} Importantly, treatment with NaHS reduced the infiltration of leukocytes (macrophages and neutrophils) and the expression of pro-inflammatory
cytokines, such as interferon-gamma. Ahmad et al. have shown that AP39 reduced neutrophil infiltration in a renal IRI model in rats. The exact mechanism underlying the anti-inflammatory effects of H$_2$S in renal IRI has not been elucidated. However, the findings described above are supported by studies that have evaluated the use of H$_2$S donors in other disease models. In a study on the effects of H$_2$S on rat cardiomyocytes infected with Coxsackie virus B3, it was found that GYY4137 inhibits the expression of the following pro-inflammatory cytokines: TNF-α, interleukin-1β (IL-1β) and IL-6. Additionally, both GYY4137 and AP39 have been shown to inhibit the expression of TNF-α and IL-6 in the context of airway hyperreactivity. Furthermore, AP39 attenuated the increase of TNF-α and IL-6 in a mouse model of burn injury. Interestingly, our collaborators have reported that SCS of murine heart grafts in AP39-supplemented UW solution protects heart graft function using an in vivo model of heterotopic heart transplantation. In addition, treatment with AP39 reduced the expression of TNF-α, IL-1β and IL-6 and increased cell viability in their in vitro model of cold myocardial IRI. These studies on AP39 have been published fairly recently and there is still plenty to discover.

While research on the anti-inflammatory effects of H$_2$S in the kidney is in its early stages, the literature suggests that H$_2$S donors like AP39 can suppress key inflammatory mediators of renal IRI.

In summary, H$_2$S confers protection against renal IRI by causing vasodilation, reducing inflammation, preventing apoptosis, and minimizing oxidative stress. Additionally, supplementing cold preservation solutions, such as UW solution, with exogenous H$_2$S donors shows potential as a novel therapeutic strategy to minimize cold IRI-induced damage.
1.6 Alternatives to cold preservation

(Sections 1.6.1 and 1.6.2 have been adapted from Juriasingani et al.)

While improvements to cold preservation methods, such as exogenous H$_2$S therapy can improve renal graft outcomes, the context of cold IRI and the damage associated with it would still be present. Longer cold ischemia times increase the incidence of DGF, especially for marginal kidneys. Thus, research evaluating alternatives to cold preservation that use temperatures closer to 37°C has gained momentum.

1.6.1 Normothermic preservation

The alternative to cold preservation that has been studied the most is normothermic machine perfusion (NMP), which refers to the oxygenated ex vivo perfusion of kidneys at 37°C. Hosgood et al. (UK) and Selzner et al. (Canada) have led critical research efforts to establish this approach. Both groups utilize erythrocyte-based, leukocyte-depleted perfusates that were supplemented with various additives for the duration of NMP.

Hosgood et al. have shown that NMP after SCS or HMP improves ex vivo graft outcomes using a porcine DCD kidney perfusion model. In 2011, they performed the first in man renal transplantation where, using paired kidneys, they showed that 30 min of NMP after SCS improved post-transplant outcomes compared to SCS only. Since then, they have developed a quality scoring system based on studies evaluating NMP using declined human kidneys. In 2018, they validated this system by transplanting human kidneys based on their quality scores. Currently, they are leading an ongoing phase II clinical trial to evaluate the effectiveness of transplanting DCD kidneys after NMP vs. SCS. In North America, Selzner et al. have compared prolonged NMP to prolonged SCS along with several combinations of NMP after SCS. They have shown that prolonged NMP...
(16 hours) improves outcomes compared to prolonged SCS and brief durations of NMP after SCS. They are currently leading a phase I clinical trial to evaluate the feasibility of NMP using their perfusate and perfusion apparatus.

Several exogenous therapies have also been shown to improve renal graft outcomes in the normothermic setting. These studies administer compounds prior to or during ex vivo reperfusion with blood, which can be seen as analogous to NMP since both involve kidney perfusion at 37°C. It has been shown that supplementing autologous blood with siRNA targeting caspase 3 during ex vivo reperfusion reduced apoptosis along with improving RBF and O₂ consumption. Additionally, administering NaHS intravenously 10 mins prior to and after the start of ex vivo reperfusion improved RBF and the function of DCD pig kidneys. Thus, in addition to eliminating the ill effects of cold IRI, the use of NMP could facilitate the circulation of promising therapeutics within kidney grafts to further improve their outcomes. Overall, the recent interest in NMP reflects a major shift in the field of kidney preservation. However, this technique has not yet been approved for clinical use and several barriers (discussed in section 1.6.3) need to be overcome before it can be implemented clinically.

1.6.2 Subnormothermic preservation

Due to the interest in NMP and improving HMP, questions about whether subnormothermic temperatures (20-34°C) may be beneficial for preserving renal grafts remain unanswered. The interest in subnormothermic preservation stems from the idea that cold IRI can be prevented at these temperatures without raising metabolic demand to physiological levels, as is the case with normothermic preservation. Very few studies have evaluated the impact of subnormothermic perfusion on kidney outcomes compared to SCS
and HMP. In the early 2000s, a group based in the Netherlands showed that a period of perfusion at 32-34°C with metabolic support following prolonged SCS improved the outcomes of pig and declined human kidneys.\textsuperscript{119-121} However, this approach was never directly compared to SCS and the research was later abandoned. In 2014, Hoyer et al. compared the effects of SCS, oxygenated HMP and oxygenated subnormothermic perfusion at 20°C on DCD porcine kidney outcomes. Subnormothermic kidney perfusion with a modified version of HTK solution at 20°C led to higher blood flow, urine output and creatinine clearance than SCS and HMP during reperfusion with autologous blood.\textsuperscript{122} Since established preservation solutions appear to work at ~20°C, it is possible that supplementing these solutions with H\textsubscript{2}S and other exogenous therapies may improve renal outcomes during subnormothermic preservation at ~20°C like it does with SCS. As is the case with normothermic preservation, subnormothermic preservation has not been approved for clinical use yet and several barriers (discussed in section 1.6.3) need to be overcome before it can be implemented clinically.

1.6.3 Challenges with the clinical translation of these approaches

The clinical implementation of normothermic and subnormothermic perfusion is limited by several factors. One key factor is the lack of a standardized perfusion protocol. The literature on this subject reports the efficacy of various temperatures with the use of diverse perfusates, including blood-based, erythrocyte-based and acellular solutions.\textsuperscript{115,122-124} Additionally, diverse additives ranging from colloids and nutrients to antibiotics and anticoagulants have been used to optimize kidney graft outcomes in these studies.\textsuperscript{123} Since a multitude of temperatures and perfusates have been effective, the development of a standardized clinical protocol is difficult and the use of whole blood or red blood cells in
Another limiting factor is the lack of an approved portable perfusion apparatus that can support these approaches during *ex situ* organ procurement. Existing HMP devices are built to circulate acellular solutions and maintain cold conditions (4°C). In their current form, these devices do not support higher temperatures, oxygenation, the circulation of blood, the addition of nutrients and the collection of samples (perfusate and urine). The lack of industry support and the focus on improving HMP strategies has delayed the development of perfusion devices that support normothermic and subnormothermic kidney perfusion. Since kidneys can tolerate prolonged cold ischemia and dialysis can be used in cases of DGF, there is little incentive to shift away from HMP and invest in novel technologies. However, strategies that facilitate the use of marginal grafts are critical for increasing transplant rates and it is clear that SCS and HMP are not ideal methods for preserving these grafts. Cost is an additional limiting factor in this context. The clinical trials and technological advancements needed to facilitate the clinical implementation of normothermic and subnormothermic kidney perfusion require significant monetary resources. From an industry standpoint, investing in these approaches may not seem advantageous until it is evident that they lead to a significant improvement of kidney outcomes compared to HMP. However, the development of clinical normothermic and subnormothermic kidney preservation protocols is essential for facilitating the assessment, preservation and transplantation of marginal kidneys.

### 1.6.4 The use of blood substitutes in kidney preservation

The potential use of red blood cells and whole blood for normothermic and subnormothermic kidney preservation complicates the clinical translation of these
approaches. While banked red blood cells are readily available, their use after longer banking times can lead to inflammation and hemolysis.\textsuperscript{123} Meanwhile, the presence of leukocytes and platelets in whole blood can lead to thrombosis and amplify IRI through endothelial activation and neutrophil infiltration.\textsuperscript{123,125} The use of plasma, following the depletion of leukocytes and platelets, can help maintain osmotic pressure but the risk of thrombosis remains due to the presence of fibrinogen.\textsuperscript{123} Paradoxically, the large doses of heparin that are being used to prevent thrombosis can cause the aggregation of red blood cells.\textsuperscript{123} From a logistical standpoint, regional shortages of blood and the unreliability of blood collection from DCD donors (blood loss due to trauma/gradual cessation of blood flow) limit the availability and use of blood or plasma for kidney preservation. Additionally, the timely screening and cross-matching of blood to ensure its compatibility to the graft could be challenging, especially considering the unpredictability of DCD kidney procurement. Furthermore, existing portable perfusion devices are designed for the use of acellular solutions that are less viscous than blood. Thus, acellular oxygen-carrying perfusates are of interest in order to circumvent the challenges described above and meet the increased metabolic demand that occurs during kidney preservation outside of cold conditions.\textsuperscript{46,123}

Blood substitutes, such as hemoglobin-based oxygen carriers, have been used in the literature to replace the use of blood in kidney preservation protocols. In the early 2000s, Brasile et al. used pyridoxilated bovine hemoglobin in their exsanguinous metabolic support solution, which was used to perfuse porcine and declined human kidneys at 32-34°C.\textsuperscript{119–121} Since then, Hemopure (Hemoglobin-based oxygen carrier- 201, HbO\textsubscript{2} Therapeutics LLC) has emerged as a promising candidate for kidney perfusion. Hemopure
is an acellular solution of polymerized bovine hemoglobin.\textsuperscript{126,127} It is similar to human hemoglobin in terms of its oxygen carrying capacity and it releases oxygen more readily.\textsuperscript{128} Additionally, it is stable for a long period of time at a wide range of temperatures (3 years, 3–30°C).\textsuperscript{126} Using declined human kidneys, Aburawi et al. have reported that 6 hours of NMP with Hemopure vs. packed red blood cells leads to comparable kidney outcomes.\textsuperscript{129} A recent study by our centre has shown that 4 hours of subnormothermic perfusion at 22°C with Hemopure reduced DCD pig kidney injury compared to SCS.\textsuperscript{130} Additionally, subnormothermic perfusion with Hemopure vs. autologous blood led to equivalent oxygen saturation, RBF, graft function and urinary damage markers. Thus, Hemopure is a promising alternative to blood in the context of subnormothermic kidney perfusion.

1.7 Rationale and Objectives

1.7.1 Rationale

Renal transplantation is the treatment of choice for patients with ESRD.\textsuperscript{4} However, the demand for kidney transplants exceeds the supply of donor kidneys.\textsuperscript{7} Renal grafts from deceased donors are being used more frequently to meet the demand.\textsuperscript{12} However, these kidneys exhibit higher rates of DGF and their long-term graft survival is lower than kidneys procured from living donors.\textsuperscript{17,18} Despite these measures, 3,261 Canadians were still on the waiting list at the end of 2019 and 72 patients died during that year while waiting for a kidney transplant.\textsuperscript{7} Additionally, some transplant recipients require more than one transplant within their lifetime due to the limited lifespan of kidney grafts as approximately 10% of all the kidney transplants performed in Canada between 2004 and 2013 were re-
transplants. Furthermore, several thousand deceased donor kidneys are discarded each year in North America due to stringent selection criteria and logistical factors. Taken together, these factors indicate the need for novel strategies than enhance graft longevity and facilitate the use of more marginal kidneys to increase transplant rates.

One of the major barriers limiting the attainment of these goals is IRI, an inherent consequence of the blood flow interruptions that occur during the transplantation process. IRI contributes to DGF and lower graft survival. DCD kidneys experience particularly severe IRI due to the warm ischemic injury that occurs as a consequence of cardiac death. Organ preservation is a key phase of the transplantation process in terms of mitigating IRI. Current preservation methods rely on the use of cold conditions (4°C) and acellular solutions. However, the resulting cold ischemia is linked to poorer post-transplant outcomes. Thus, there is growing interest in alternative preservation approaches that can eliminate the ill effects of cold IRI. Recent research suggests that organ preservation at normothermic and subnormothermic temperatures is preferable, particularly for DCD kidneys, but there is no consensus on an optimal temperature or protocol.

Supplementing UW solution with exogenous H2S donors, particularly AP39, has been shown to improve renal graft outcomes following prolonged periods of static cold storage. These improvements can be attributed to the diverse protective effects of administering low concentrations of H2S. Seeing cold preservation solutions are effective for subnormothermic kidney preservation at ~20°C, the effect of H2S supplementation in this context is of interest. Additionally, since the research on normothermic and subnormothermic preservation routinely employs oxygenated perfusion with blood and other oxygen-carrying perfusates, the effect of adding of H2S to these protocols is also
of interest. The primary objective of my thesis was to evaluate the effect of subnormothermic preservation at 21°C with AP39 on DCD renal outcomes compared to static cold storage, the clinical standard of care. Cutting edge in vitro and ex vivo models were used to sequentially investigate the effect of adding AP39 to UW solution, blood and Hemopure during subnormothermic preservation. Through this approach, I sought to establish a novel kidney preservation method that is feasible for clinical translation by using established pre-clinical porcine models and considering anticipated obstacles, such as the complexities associated with using blood.

1.7.2 Objectives

Objective 1. Evaluate the effectiveness of AP39-supplemented UW solution for static subnormothermic storage at 21°C using an in vitro model of renal IRI and an ex vivo model of DCD porcine renal graft preservation.

Objective 2. Investigate whether ex vivo subnormothermic perfusion at 21°C with AP39-supplemented blood improves DCD porcine renal graft outcomes compared to static cold storage.

Objective 3. Using a novel blood-free model of preservation and reperfusion, investigate whether ex vivo subnormothermic perfusion at 21°C with AP39-supplemented Hemopure improves DCD porcine renal graft outcomes compared to static cold storage.
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2 H$_2$S supplementation: A novel method for successful organ preservation at subnormothermic temperatures

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2.1 Abstract

Renal transplantation is the preferred treatment for end-stage renal disease. Currently, there is a large gap between the supply and demand for transplantable kidneys. The use of sub-optimal grafts obtained via donation after cardiac death (DCD) is on the rise. While static cold storage (SCS) in University of Wisconsin (UW) solution on ice (4°C) is the clinical standard of care for renal graft preservation, cold storage has been associated with negative graft outcomes. The alternative, normothermic machine perfusion, involves mechanical perfusion of the organ at physiological or normothermic temperature (37°C) and this technique is expensive, complicated and globally inaccessible. As such, simpler alternatives are of interest. Preliminary results revealed that UW solution is more protective at 21°C than 37°C and subnormothermic preservation is of interest because it may facilitate the use of existing solutions while preventing cold injury. We have previously shown that SCS in UW solution supplemented with mitochondria-targeted H₂S donor AP39 improves renal graft outcomes. As such, it was hypothesized subnormothermic preservation at 21°C with AP39 will also improve renal outcomes. Using an in vitro model of hypoxia and reoxygenation, we found that treating porcine tubular epithelial cells with UW+5uM AP39 during 18h hypoxia at 21°C significantly increased renal tubular epithelial cell viability after 24h of reoxygenation at 37°C compared to UW alone. Also, AP39-supplemented UW solution was significantly more cytoprotective during hypoxia at 21°C than hypoxia at 37°C, regardless of AP39 concentration. Using an ex vivo DCD organ preservation model, we found that DCD porcine kidneys stored for 24h in UW+200nM AP39 at 21°C showed significantly lower tissue necrosis than DCD porcine kidneys preserved using SCS in UW solution, the clinical standard of care. Overall, our findings suggest that exogenous H₂S
supplementation improves the viability of the gold standard organ preservation solution, UW solution, for subnormothermic preservation at 21°C.

2.2 Introduction

Renal transplantation is the treatment of choice for patients with end-stage renal disease. Several studies have shown that renal transplantation is superior to dialysis in terms of long-term survival outcomes, quality of life and cost-effectiveness [1–3]. The global incidence of end-stage renal disease is on the rise and there is a limited supply of donor kidneys that are suitable for transplantation. This has led to an ever-increasing number of patients on transplant waitlists. Organs from living donors are preferred for optimal transplant outcomes. However, sub-optimal grafts from donation after cardiac death (DCD) donors are being used more frequently to bridge the gap between the supply and demand for transplantable kidneys [4,5]. One of the factors that influences transplant outcomes and contributes to the preference for living donors is ischemia-reperfusion injury (IRI). IRI is an inevitable consequence of the loss and subsequent gain of blood flow during transplantation. Delayed graft function (DGF) is an important consequence of IRI that is strongly linked to acute graft rejection and early graft loss [6]. IRI is an especially important factor to consider with DCD kidneys because they experience prolonged warm ischemia upon withdrawal of life support until cardiac arrest, which leads to increased rates of DGF [4]. Considering the increase in end-stage renal disease and the demand for transplantable kidneys, strategies to mitigate renal IRI are of interest in order to maximize renal graft survival and expand the pool of donors.

When considering strategies to mitigate IRI, it is important to consider the cellular mechanisms underlying the clinical manifestations of IRI. Ischemia is characterized by a
cessation of blood flow that results in hypoxia. Hypoxia decreases electron transport chain (ETC) activity and causes cells to switch to anaerobic respiration. The resulting decrease in ATP production inhibits the activity of membrane-bound ATPases, such as the Na+/K+ ATPase [7,8]. The accumulation of Na+ ions impairs other transporters, which causes the accumulation of intracellular Ca^{2+} [9]. The accumulation of Ca^{2+} in the cytoplasm and, eventually, the mitochondrial matrix has been shown to increase mitochondrial permeability and cause the production of reactive oxygen species (ROS), both of which are exacerbated during reperfusion [10–12]. Reperfusion is characterized by the influx of oxygen upon re-establishment of blood flow, which paradoxically further damages ischemic tissues instead of reversing the ischemic damage. The leaked electrons, due to impaired ETC activity, reduce the newly present oxygen, which leads to an excess of ROS that cannot be cleared [10,13–15]. ROS-mediated damage to ETC complexes leads to more pronounced ATP depletion, which can cause cell death [12]. ROS also damage mitochondrial membrane lipids which, along with Ca^{2+}-induced mitochondrial swelling, causes the opening of mitochondrial permeability transition pores (MPTPs). Due to the increased mitochondrial membrane permeability, mediators of the intrinsic apoptotic pathway are released into the cytoplasm, which initiates apoptosis [10, 11]. Thus, the cellular mechanisms underlying IRI are complicated.

Optimal preservation of the donor organ prior to transplantation is essential for mitigating the effects of IRI, especially for sub-optimal DCD grafts. The current clinical standard of care for renal graft preservation is static cold storage (SCS) in University of Wisconsin (UW) solution on ice at 4°C [16]. However, the resulting cold IRI has been linked to increased risk of DGF, graft failure and patient death along with reduced long-
term graft survival [16–18]. To limit these negative effects, hypothermic machine perfusion (HMP), where the donor organ is continuously perfused with preservation solution, was introduced [16]. While the increased distribution of nutrients and the clearance of toxic metabolites with HMP are perceived benefits, studies comparing HMP to SCS are inconsistent [19–22]. There are additional drawbacks to HMP, such as expense and complexity, that make this approach less feasible than SCS [21,23]. Since donor organs are routinely preserved in cold conditions until the time of transplantation, the repercussions of cold IRI are an important concern in most renal transplantation cases, especially those involving DCD grafts due to the prior warm ischemic injury as cardiac death occurs. As a result, novel organ preservation strategies are of interest to circumvent the damage caused by cold IRI.

An emerging strategy with therapeutic potential against IRI is exogenous hydrogen sulfide (H$_2$S) therapy. H$_2$S is an endogenously produced gasotransmitter with diverse cytoprotective effects. Recent studies, including some conducted by our own group, have shown that H$_2$S therapy can mitigate oxidative stress, inflammation, apoptosis and mitochondrial dysfunction caused by IRI [24–27]. Using rat models of syngeneic and allogeneic renal transplantation, we have previously shown that prolonged SCS in NaHS-supplemented UW solution can improve post-transplant graft survival and function [25,26]. However, the immediate and robust release of H$_2$S caused by inorganic sulfide salts like NaHS upon suspension makes it difficult to control H$_2$S concentrations precisely [28]. This has led to the development of synthetic, slow-releasing H$_2$S donors. AP39 is a synthetic H$_2$S donor that is also mitochondria-targeted via its triphenylphosphonium motif [29]. Using a rat model of syngeneic renal transplantation, we have previously shown that
prolonged SCS in AP39-supplemented UW solution improves post-transplant graft outcomes [27]. Additionally, using an in vitro model of renal IRI, it was found that AP39 is 1000-fold more cytoprotective than non-targeting H₂S donors [27]. Thus, cold storage of renal grafts in H₂S-supplemented preservation solutions can improve outcomes, however, the premise of cold IRI remains.

To prevent the negative effects of cold IRI altogether, organ preservation at temperatures closer to physiological temperature (37°C) is of interest. The existing technique is normothermic machine perfusion (NMP) where the organ is perfused with oxygenated autologous blood, erythrocyte-based solutions or acellular solutions at 37°C [30]. Several studies have shown that NMP improves renal graft outcomes compared to SCS [31–35]. Additionally, subnormothermic machine perfusion (SMP) of DCD porcine grafts at 20°C has been shown to improve graft outcomes compared to HMP and SCS [36]. However, the challenges of NMP and SMP include technical complexity, economic viability, and logistical problems as no standard perfusion apparatus or protocol exists. Additionally, these approaches are not very suitable for ex situ organ procurement [23]. Due to these challenges, simpler alternatives are of interest. One possible approach is a subnormothermic equivalent of SCS. Therefore, this study aims to investigate the effectiveness of H₂S-supplemented UW solution for static subnormothermic preservation using an in vitro model of renal IRI and an ex vivo porcine model of DCD renal graft preservation.
2.3 Materials and Methods

In vitro model of renal IRI

An in vitro model of renal ischemia-reperfusion injury (Fig. 2-1) was used to investigate the protective effects of AP39-supplemented UW solution during hypoxic storage at 10°C, 21°C and 37°C.

Figure 2-1 In vitro model of renal IRI. A summary of the in vitro renal IRI model used to analyze the protective effects of AP39-supplemented UW solution during cold, subnormothermic and normothermic organ preservation.
Cell culture

LLC-PK1 cells (ATCC, USA), proximal tubular epithelial cells from porcine kidneys, were used in the in vitro experiments because proximal tubule cells are susceptible to ischemic injury [37]. Porcine kidney cells were also used for consistency with the pig organ preservation model used for the second aim of this study. The cells were cultured in 75cm² flasks using 10mL of Media 199 containing 10% fetal bovine serum (FBS) inactivated by heat at 60°C for 30 minutes and 1% penicillin/streptomycin (P/S). The cells were incubated at normal growth conditions of 37°C, 5% CO₂, and 21% O₂. At ~80-90% confluence, 0.25% trypsin-EDTA was used to passage or split the cells for experiments. All cells used were below the 20th passage. All supplies, except the cells, were purchased from Thermo Fisher Scientific, USA.

Cell preparation for experiments

LLC-PK1 cells were seeded in 6-well plates (4x10⁵ cells/well). Once the cells were ~80-90% confluent in Media 199 (10% FBS, 1% P/S), the media was aspirated and phosphate-buffered saline (PBS) was used to wash the cells. Cells were then assigned to experimental treatments.

Experimental conditions and treatments

A 1mM stock solution of AP39 was made by suspending AP39 (provided by Dr. Matt Whiteman) in 100% (v/v) dimethyl sulfoxide (DMSO) (AMRESCO Inc, USA). The 1mM stock solution of AP39 was then diluted in Belzer UW® Cold Storage Solution (Bridge to Life, USA) to make the following six treatment groups: UW only and UW supplemented with 5nM, 50nM, 500nM, 5µM or 10µM AP39. UW solution was used instead of media because UW solution is clinically used for organ preservation. All
treatments were normalized to contain 0.5% (v/v) DMSO to control for any cell death caused by DMSO. The treatments were applied in duplicate to the LLC-PK1 cells in the 6-well plates by adding 1mL of treatment per well. Negative control cells were treated with UW solution with 0.5% DMSO. The plates were then incubated at 10°C, 21°C or 37°C for 18h in hypoxic growth conditions (4% CO₂, 0.5% O₂, 95% N₂) to simulate ischemia during cold, subnormothermic and normothermic organ preservation conditions. These conditions were created using a HypOxystation® H85 hypoxia chamber (HYPOXYGEN, USA). Following hypoxia, the treatment solutions were aspirated and 2mL of Media 199 (10% FBS, 1%P/S) was added to each well. The plates were reoxygenation via incubation in normoxic growth conditions (37°C, 5% CO₂, 21% O₂) for 24h to simulate reperfusion and associated injury. Positive control cells were grown in 6-well plates in normoxic growth conditions (37°C, 5% CO₂, 21% O₂) in Media 199 (10% FBS, 1%P/S) for the 18h hypoxia period and the 24h reoxygenation period.

**Analysis of cellular viability, apoptosis and necrosis**

The cells samples were analyzed for viability, apoptosis and necrosis after reoxygenation. The reoxygenation media was collected in labeled flow tubes. Each well was washed with 2mL of 1X PBS to remove any traces of FBS. Subsequently, 500µL of 0.25% trypsin-EDTA was added to each well and the plates were incubated at 37°C for 3-6 minutes to accelerate the detachment of the monolayer of cells. Following incubation, 500µL of Media 199 (10% FBS, 1%P/S) was added to each well to inactivate the trypsin and the media containing the detached cells was collected and added to the labeled flow tubes. The flow tubes were pelleted via centrifugation (600 x g for 6 minutes) and then resuspended in 1X PBS followed by identical centrifugation. The cells were then
resuspended in 250µL of 1X Annexin-V Binding Buffer (BioLegend, USA). Following resuspension, 2µL of FITC-conjugated Annexin-V (FITC-Annexin-V) (BioLegend, USA) and 4µL of 7-Aminoactinomycin D (7-AAD) (BioLegend, USA) were added to each sample to stain for apoptosis and necrosis respectively. In order to optimize detection, all samples were incubated in the dark for 15 minutes. The stained samples were then analyzed for cell viability, apoptosis, and necrosis using the CytoFLEX S (Beckman Coulter, USA). One sample of unstained live cells and two samples of heat-killed cells (90°C, 12 minutes), stained with either FITC-Annexin-V or 7-AAD, were used for compensation. FlowJo® V10 (FlowJo LLC, USA) was used to appropriately gate the data for statistical analysis.

Data analysis

All statistical analyses were conducted using GraphPad Prism Version 7. For results obtained from one set of hypoxia and reoxygenation experiments (10°C, 21°C or 37°C hypoxia), one-way analysis of variance (ANOVA) and Tukey’s post-hoc test were performed to determine the statistical differences between multiple treatment groups. To compare the results of all treatment groups between two different sets of hypoxia and reoxygenation experiments (21°C hypoxia vs. 37°C hypoxia or 10°C hypoxia vs. 21°C hypoxia), multiple t-tests with the Holm-Sidak method for multiple comparisons were performed. Statistical significance was accepted at p<0.05.

Subnormothermic temperature and H₂S donor selection

Prior to the conditions used with the in vitro model in this study, preliminary experiments were performed to test the protective effects of UW solution at various temperatures and to test the cytoprotective effects of adding two different H₂S donors to UW solution. The novel subnormothermic temperature (21°C) was selected based on
preliminary data which showed that UW solution was more cytoprotective during 12h of subnormothermic hypoxia at 21°C than normothermic hypoxia at 37°C (Fig. 2A). Preliminary data also showed that UW solution supplemented with mitochondria-targeted H₂S donor AP39 was more cytoprotective during 18h of hypoxia at 21°C than UW solution supplemented with non-specific H₂S donor GYY4137 (Fig. 2-2). Hence, AP39 was the only donor used for both aims of this study.

Figure 2-2 Preliminary results. (A) Renal cell viability (%) following treatment with UW solution during 12h of subnormothermic (21°C) or normothermic (37°C) hypoxia and 24h of reoxygenation at 27°C in Media 199 (n=3). (B) Renal cell viability (%) following treatment with UW, UW+GYY4137 or UW+AP39 during 18h of subnormothermic hypoxia (21°C) and 24h of reoxygenation at 37°C in Media 199 (n=3).
**Ex vivo model of DCD porcine kidney preservation**

Tissue necrosis was visualized to evaluate the impact of prolonged *ex vivo* porcine kidney storage in AP39-supplemented UW solution at 21°C after causing warm ischemic injury that mimics DCD injury (Fig. 2-3).

**Figure 2-3 Ex vivo model of DCD porcine kidney preservation.** A summary of the experimental procedure used to visualize the level of tissue necrosis of DCD porcine kidneys following prolonged *ex vivo* static storage for 24h in UW+200nM AP39 21°C vs. UW solution at 4°C or at 21°C.

**Surgery & induction of warm ischemia to mimic DCD**

Kidneys were obtained from pigs weighing 30-65 kg that were being euthanized as a part of other studies at Western University. The pigs were tranquilized and prepared for surgery according to standard operating procedure. A midline incision was used to expose the kidneys. The right and left kidneys with their ureters were dissected free from the retro
peritoneum while leaving the blood supply to the ureter intact and then the ureter was divided. The renal vascular pedicles were left as long as possible by freeing the renal artery and vein up to the aorta and vena cava. The pedicles were clamped for 60 minutes to induce warm ischemia in the donor organs following intravenous infusion of 3000 units of heparin. This was done to mimic kidney injury that occurs during clinical DCD organ procurement. Subsequently, nephrectomies of the left and right kidneys were performed. All the surgeries were performed by transplant fellows at University Hospital, London, Ontario, Canada. Other research groups harvested other organs of the donor animal and the donor animal was euthanized. The surgical procedures received ethics approval from the Western University Council for Animal Care.

**Ex vivo organ preservation treatments**

The kidneys were randomly assigned to one of three preservation treatment groups for the prolonged organ storage period of 24h (Fig. 2-3). The first group of kidneys were flushed with and stored in UW solution on ice (4°C), which reflects SCS, the current clinical standard of care for organ preservation. The second group of kidneys were flushed with and stored in UW solution at 21°C. The third groups of kidneys were flushed with UW solution supplemented with 200nM AP39 and stored in the same solution at 21°C. The groups labels are UW-4°C, UW-21°C and UW+AP39-21°C respectively. The dose of AP39 added to UW solution was determined based on previous prolonged cold storage experiments with rat kidneys where adding 200nM AP39 to UW solution during cold storage resulted in lesser tissue injury and improved post-transplant graft outcome [27].
Fluorescence staining to evaluate cellular necrosis

Following the 24h storage period, the kidneys were perfused with 50mL of 5µM ethidium homodimer-1 (EthD-1) (Invitrogen™, USA) diluted in 1X PBS at a rate of 5mL/min. EthD-1 is a fluorescent marker that binds to double-stranded DNA when the plasma membrane of cells is compromised, which indicates necrosis. Subsequently, the kidneys were washed by perfusion with 25mL of PBS at a rate of 1mL/min to flush out the excess EthD-1 stain.

Imaging and data analysis

Following EthD-1 staining, kidney sections were stored in formalin for a minimum of 72 hours, stained with the nuclear marker 4,6-diamidino-2-phenylindole (DAPI) and analyzed via fluorescent microscopy. An Olympus (Toronto, Canada) IX83 Inverted Microscope was used to capture five images per section for EthD-1 analysis and to capture the equivalent DAPI images. All images were captured at 20X magnification. The total proportion (%) of EthD-1+ area was quantified using ImageJ software v 1.50 (National Institutes of Health, USA). Background fluorescence was subtracted and the RGB color threshold was adjusted uniformly for all images to accurately quantify the level of EthD-1+ staining. GraphPad Prism 7 was used to present the EthD-1+ % area values and one-way ANOVA and Tukey’s post-hoc test were performed to determine statistically significant differences between treatment groups. Statistical significance was accepted at p<0.05.
2.4 Results

Supplementation of UW solution with mitochondria-targeted H2S donor AP39 increases renal tubular epithelial cell viability and decreases apoptosis during *in vitro* subnormothermic renal IRI

To investigate the effect of supplementing UW solution with AP39 on renal tubular epithelial cell viability during subnormothermic IRI, LLC-PK1 cells were treated with UW solution supplemented with various concentrations of AP39 and subjected to 18h of hypoxia at 21°C to mimic subnormothermic ischemia during organ storage. This was followed by 24h of reoxygenation in media at 37°C to mimic reperfusion. Flow cytometry analysis after staining for necrosis and apoptosis revealed a dose-dependent increase in the percentage of unstained viable cells as the concentration of AP39 added to UW solution was increased (Fig. 2-4A, 2-4B). While all the experimental samples exhibited significantly lower renal tubular epithelial cell viability (p<0.05) than control cells grown in normoxic conditions, cells treated with UW solution supplemented with 5µM and 10µM AP39 exhibited significantly higher viability (p<0.05) than cells treated with only UW solution (Fig. 2-4B). The reverse trend was observed with apoptosis, where cells treated with UW solution supplemented with 5µM and 10µM AP39 exhibited significantly lower apoptosis (p<0.05) than cells treated with UW solution alone. Interestingly, the level of apoptosis exhibited by cells treated with UW solution supplemented with 5µM AP39 was comparable to that of control cells grown in normoxic conditions (Fig. 2-4C). Additionally, the dose-dependent increase in viability and decrease in apoptosis appears to reach optimum levels when UW solution is supplemented 5µM AP39 and the addition of a higher dose, 10µM AP39, begins to reverse these trends (Fig. 2-4B, 2-4C).
Figure 2-4 AP39-supplemented UW solution improves renal tubular epithelial cell survival in a dose-dependent manner during subnormothermic IRI. LLC-PK1 cells were treated with UW solution supplemented with different concentrations of H$_2$S donor AP39. The treated cells were placed in hypoxia for 18h at 21°C and later reoxygenated in Media 199 for 24h at 37°C. Following reoxygenation, cells were stained with FITC-Annexin-V and 7-AAD for flow cytometry analysis to quantify apoptosis and necrosis. Control cells were grown in normoxic growth conditions in Media 199. All data were
analyzed using FlowJo V10. (A) Representative images of flow cytometry results for all treatment groups. (B) Mean cell viability (%) (n=5) as determined by the ratio of cells negative for FITC-Annexin-V and 7-AAD staining. (C) Mean apoptosis (%) (n=5) as determined by the ratio of cells positively stained for FITC-Annexin-V, but not 7-AAD. Data represent mean ± SEM. Means were analyzed using one-way ANOVA and Tukey’s post-hoc test. *, p<0.05 vs. control. †, p<0.05 vs. UW Solution. ns, not significant (p>0.05).

**AP39-supplemented UW solution is more cytoprotective during subnormothermic IRI than during warm IRI**

LLC-PK1 cells were treated with UW solution supplemented with various concentrations of AP39 and subjected to 18h of hypoxia at 21°C or 37°C to mimic ischemic injury during subnormothermic or normothermic organ preservation. This was followed by 24h of reoxygenation in media at 37°C to mimic reperfusion injury. Flow cytometry analysis revealed a dose-dependent increase in renal tubular epithelial cell viability following warm IRI. While all the experimental samples exhibited significantly lower viability (p<0.05) compared to control cells grown in normoxic conditions, cells treated with UW solution supplemented with 10µM AP39 exhibited significantly higher viability (p<0.05) than cells treated with UW solution alone (Fig. 2-5A). Additionally, all the experimental samples displayed significantly higher viability (p<0.05) after subnormothermic IRI than after warm IRI (Fig. 2-5B).
Figure 2-5 AP39-supplemented UW solution is more cytoprotective during subnormothermic IRI than warm IRI. (A) Mean cell viability (%) after 18h of hypoxia at 37°C (n=5) and 24h of reoxygenation at 37°C as determined by the ratio of cells negative for FITC-Annexin-V and 7-AAD staining. (B) Mean cell viability (%) after 18h of hypoxia at 21°C (n=5) or 37°C (n=5) and 24h of reoxygenation at 37°C. Data represent mean ± SEM. Means in (A) were analyzed using one-way ANOVA and Tukey’s post-hoc test. *, p<0.05 vs. control. †, p<0.05 vs. UW Solution. Means in (B) were analyzed using multiple t-tests and the Holm-Sidak method for multiple comparisons. *, p<0.05.
AP39-supplemented UW solution is more cytoprotective during cold IRI than during subnormothermic IRI

LLC-PK1 cells were treated with UW solution supplemented with various concentrations of AP39 and subjected to 18h of hypoxia at 10°C or 21°C to mimic ischemia during cold or subnormothermic organ preservation. This was followed by 24h of reoxygenation in media at 37°C to mimic reperfusion. Flow cytometry analysis revealed a dose-dependent increase in renal tubular epithelial cell viability following 18h of hypoxia at 10°C followed by 24h of reoxygenation at 37°C. While all the experimental samples exhibited significantly lower viability (p<0.05) compared to control cells grown in normoxic conditions, cells treated with UW solution supplemented with 5µM AP39 exhibited significantly higher viability (p<0.05) than cells treated with UW solution alone (Fig. 2-6A). Additionally, cells treated with UW solution alone or UW solution supplemented with 5nM, 50nM, 500nM and 5µM AP39 displayed significantly higher viability (p<0.05) after cold IRI than after subnormothermic IRI. However, the difference in viability exhibited by both groups decreases as the dose of AP39 increases (Fig. 2-6B).
Figure 2-6 AP39-supplemented UW solution is more cytoprotective during cold IRI than subnormothermic IRI. (A) Mean cell viability (%) after 18h of hypoxia at 10°C (n=5) and 24h of reoxygenation at 37°C as determined by the ratio of cells negative for FITC-Annexin-V and 7-AAD staining. (B) Mean cell viability (%) after 18h of hypoxia at 10°C (n=5) or 21°C (n=5) and 24h of reoxygenation at 37°C. Data represent mean ± SEM. Means in (A) were analyzed using one-way ANOVA and Tukey’s post-hoc test. *, p<0.05 vs. control. †, p<0.05 vs. UW Solution. Means in (B) were analyzed using multiple t-tests and the Holm-Sidak method for multiple comparisons. *, p<0.05.
Prolonged static subnormothermic storage of DCD kidneys in AP39-supplemented UW solution at 21°C reduces tissue necrosis compared to static cold storage in UW solution at 4°C

Porcine kidneys were nephrectomized after 1hr of renal pedicle clamping to mimic clinical DCD organ donation. The kidneys were then flushed with and stored in either UW solution at 4°C, UW solution at 21°C or UW solution supplemented with 200nM AP39 at 21°C for 24h. Following storage, the kidneys were flushed with 5µM EthD-1 stain to visualize tissue necrosis. Formalin-fixed sections were stained with DAPI and analyzed for EthD-1 and DAPI fluorescence using a confocal microscope (Fig. 2-7A). Fluorescence in 5 random fields of view per kidney was quantified using ImageJ to determine the mean EthD-1+ area and DCD porcine kidneys stored in AP39-supplemented UW solution at 21°C exhibited the lowest mean EthD-1+ area. The mean EthD-1+ area of DCD kidneys preserved in UW solution at 21°C was significantly higher than that of DCD kidneys stored in AP39-supplemented UW solution at 21°C (p<0.001) and DCD kidneys stored in UW solution at 4°C (p<0.05). Importantly, DCD kidneys stored in AP39-supplemented UW solution at 21°C exhibited significantly lower (p<0.05) mean EthD-1+ area than DCD kidneys preserved using SCS in UW solution at 4°C, which reflects the standard of care (Fig. 2-7B).
Figure 2-7 Prolonged static subnормothermic storage of DCD kidneys in AP39-supplemented UW solution at 21°C reduces tissue necrosis compared to prolonged static cold storage in UW solution at 4°C. Porcine kidneys were nephrectomized after 1hr of renal pedicle clamping to mimic DCD organ donation. The kidneys were flushed
with and stored in either UW at 4°C (n=3), UW at 21°C (n=3) or UW+200nM AP39 at 21°C (n=6) for 24h. The kidneys were then flushed with 5μM EthD-1, stored in formalin, sectioned and stained with DAPI. (A) Representative DAPI, EthD-1 (20X magnification) and ImageJ quantification images for each treatment group. (B) Mean EthD-1+ area (%) determined by analyzing five random fields of view for each kidney sample. Dots represent mean EthD-1+ area (%) for individual kidneys and lines represent the mean EthD-1+ area (%) ± SEM for each treatment group. Means were compared using one-way ANOVA and Tukey’s post-hoc test. *, p<0.05. **, p<0.01.
2.5 Discussion

IRI is an inevitable consequence of the transplantation process and it is a major concern because renal IRI has been associated with DGF, acute graft rejection and early graft loss following renal transplantation [6]. Organ preservation prior to transplantation is one of the key steps of interest in terms of mitigating renal IRI. Currently, the clinical standard of care for renal graft preservation is SCS at 4°C in a preservation solution such as UW solution [16]. The hypothermic conditions reduce cellular metabolism to promote survival until transplantation. However, the resulting cold IRI has been linked to increased DGF, increased risk of graft failure and decreased long-term graft survival [16–18]. While exogenous H$_2$S therapy has been shown to mitigate the effects of cold IRI, normothermic and subnormothermic preservation have become an important avenue of interest to prevent cold IRI. While NMP and SMP have been shown to improve renal graft outcomes compared to SCS, the cost and complexity of these techniques along with the lack of an optimized standard protocol are challenges that need to be overcome [23, 32–37]. As a result, a normothermic or subnormothermic equivalent of SCS, where the organ is kept at 37°C or at room temperature in a preservation solution along with other therapeutic compounds, is of interest. In this study, we demonstrate, for the first time, that mitochondria-targeted H$_2$S donor AP39 makes the gold standard preservation solution, UW solution, viable for subnormothermic organ preservation at 21°C.

Firstly, the effectiveness of UW solution, with and without AP39, at various organ preservation temperatures was investigated using an in vitro model of renal IRI. Our preliminary results showed that UW solution is more cytoprotective during subnormothermic IRI than normothermic IRI. Consistent with the preliminary results, the
cytoprotective effects of AP39-supplemented UW solution were also temperature-dependent as it was the most cytoprotective during cold IRI and the least cytoprotective following normothermic IRI. These results were expected because UW solution was designed for cold storage and they are consistent with our previous findings about the effects of AP39 in cold IRI [27]. However, as the dose of AP39 added to UW solution increased, the gap between the renal tubular epithelial cell viability levels following cold IRI and subnormothermic IRI decreased. Therefore, we postulated that preservation at 21°C with AP39-supplemented UW solution has the potential to match cold storage in UW solution alone, depending on the concentration of AP39 used. Overall, our in vitro results show that mitochondria-targeted H₂S therapy makes the existing gold standard preservation solution, UW solution, viable for subnormothermic organ preservation of renal grafts at 21°C.

Our findings of the temperature-dependent protective effects of UW solution are also consistent with a study by Iwai et al., however, an important factor to consider is the type of preservation solution being used [38]. UW solution is an intracellular-type preservation solution, which means that it has a low-sodium/high-potassium composition that mimics intracellular fluid [38]. In this study, we chose to evaluate H₂S-supplemented UW solution for subnormothermic and normothermic preservation due to our previous work, which shows that cold storage with H₂S-supplemented UW solution improved renal graft outcomes [25–27]. However, in addition to comparing the outcomes of renal grafts preserved in UW solution at 4°C, 23°C and 37°C, Iwai et al. also compared UW solution to an extracellular-type solution, which has a high-sodium/low-potassium composition that mimics extracellular fluid, called ET-Kyoto (ETK) solution [38]. They found that ETK
solution was more suitable for normothermic preservation than UW solution, however, both solutions were comparable for subnormothermic preservation [38]. They suggested that the difference in the performance of ETK and UW solution during normothermic preservation is due to the difference in the phase temperature of the main carbohydrates, which are added to prevent cellular edema [38]. ETK solution contains trehalose, which has a phase transition temperature of 24°C, while UW solution contains raffinose, which has a phase transition temperature of 17°C [38–40]. This may explain why UW solution is unsuitable for normothermic preservation and somewhat suitable for subnormothermic preservation. The addition of AP39 to UW solution in this study likely improves its viability for subnormothermic preservation due to the diverse cytoprotective effects of H2S. Further research that compares intracellular-type solutions to extracellular-type solutions is needed to establish the ideal static subnormothermic preservation protocol and the optimal exogenous H2S therapy. However, we chose to use UW solution in this study because it is the most mainstream preservation solution used worldwide and any findings using UW solution would be widely applicable to clinical practice. In addition to low sodium, high potassium and raffinose, UW solution also contains antioxidant components, such as allopurinol and glutathione, along with lactobionate and hydroxyethyl starch to prevent cellular edema [41]. There is no existing evidence to suggest that AP39 chemically interacts with any of the components of UW solution, however, further research is needed to confirm this.

With both the cold IRI and subnormothermic IRI models in the in vitro arm of this study, 5 μM AP39 was the most effective concentration that led to the highest renal tubular epithelial cell viability. Cells treated with UW+ 5 μM AP39 during
subnormothermic IRI not only exhibited significantly higher viability than cells treated with UW solution alone, but they also exhibited significantly lower apoptosis. This suggests that the anti-apoptotic effects of H\textsubscript{2}S play an important role in making UW solution viable for subnormothermic preservation. Apoptosis has been established as a major mechanism underlying renal IRI-induced tissue damage and both the intrinsic and extrinsic apoptotic pathways play a role [42]. Renal IRI has been shown to upregulate the expression of pro-apoptotic genes such as FADD, Bad, Bak and p53 [43]. Bax and Bak oligomers serve as the outer mitochondrial membrane component of the MPTP. The opening of MPTPs is a key step in the intrinsic apoptotic pathway that allows pro-apoptotic factors, such as cytochrome c, to be released into the cytoplasm. Cytochrome c facilitates the oligomerization of caspase-9 and Apaf-1, which activates caspase-9 and allows it to cleave other caspases to carry out widespread apoptotic events [42]. H\textsubscript{2}S has been shown to counteract multiple pro-apoptotic mechanisms. Via persulfidation, AP39 can induce cytochrome c-dependent protein sulfide signaling, which leads to inactivation of caspase 9 via persulfidation [44]. H\textsubscript{2}S also decreases the expression of pro-apoptotic genes, such as Bid, which can facilitate crosstalk between the intrinsic and extrinsic apoptotic pathways [26,42]. Additionally, H\textsubscript{2}S can decrease ROS production by scavenging ROS directly or by upregulating cellular antioxidant defenses [45]. Using \textit{in vitro} and \textit{in vivo} models of renal IRI and transplantation, we have previously shown that treatment with AP39 mitigates tissue apoptosis, reduces ROS production and prevents mitochondrial depolarization during cold IRI [27]. Thus, H\textsubscript{2}S facilitates a variety of direct and indirect anti-apoptotic mechanisms, especially in the mitochondria, which may be contributing to the superior effects of mitochondria-targeted H\textsubscript{2}S donors, such as AP39, compared to non-
targeted donors [27]. Future studies are warranted to elucidate the mechanisms underlying the cytoprotective effects of AP39-supplemented UW solution during subnormothermic preservation that were observed in this study.

Based on the in vitro results, we decided to investigate the effects of storing renal grafts in AP39-supplemented UW solution at 21°C. Tissue necrosis was visualized after DCD porcine kidneys were preserved for 24h using SCS at 4°C in UW solution, which reflects the clinical standard of care, or subnormothermic storage at 21°C in UW solution with or without 200nM AP39. The dose of AP39 added to the UW solution was determined based on the in vivo results of our previous study [27]. Prolonged subnormothermic storage at 21°C in AP39-supplemented UW solution led to significantly lower tissue necrosis than subnormothermic storage in UW solution and SCS in UW solution alone at 4°C, which is the clinical standard of care. These results are consistent with our in vitro results, which showed that the addition of AP39 makes UW solution more viable for subnormothermic organ preservation at 21°C. These results also suggest that this approach has the potential to match or surpass SCS in UW solution alone, depending on the concentration of AP39 used. The effectiveness of 200nM AP39 in this model is consistent with our previous findings [27].

As discussed earlier, a large body of evidence shows the role of apoptosis in tubular cell death caused by renal IRI and several studies have shown the anti-apoptotic effects of H2S in renal IRI [26,27,42,43]. However, emerging evidence suggests that RIPK3/RIP3-induced necroptosis is also involved in IRI-induced tubular cell death [46,47]. Using a mouse model of allotransplantation, Lau et al. showed that RIPK3⁻/⁻ donor kidneys exhibit tissue necrosis, which was determined via EthD-1 fluorescence, along with
improved post-transplant outcomes [46]. Interestingly, a recent study by Lin et al. shows that exogenous H$_2$S can reduce the expression of RIP3 and reduce necroptotic injury of hyperglycemic human umbilical vein endothelial cells [48]. While the effect of exogenous H$_2$S on RIP3-induced necroptosis has not be studied in the context of renal IRI, it is possible that such an effect is contributing to the decreased level of tissue necrosis observed after prolonged DCD kidney preservation in AP39-supplemented UW solution at 21°C. Future studies evaluating RIP3 expression after preservation of renal grafts with and without H$_2$S are needed to confirm this effect.

While our prolonged \textit{ex vivo} DCD kidney storage model allows visualization of tissue injury, the lack of post-transplant graft function analysis and small sample sizes are limitations. However, there are several key advantages of using this model. Firstly, the warm ischemic injury inflicted in this model not only mimics clinical DCD organ donation but is likely more severe due to the complete cessation of blood flow via clamping rather than the gradual ischemic injury that occurs during cardiac death. Thus, seeing as DCD kidneys stored in AP39-supplemented UW solution at 21°C show low tissue necrosis in this model, this storage treatment would likely be as or more effective in reducing the tissue necrosis of discarded human DCD kidneys in future studies. Secondly, the prolonged 24h storage time is at the higher end of conventional organ preservation times in clinical renal transplantation, which range from 6-36h [49,50]. Both of these aspects reflect clinically relevant conditions and the use of porcine kidneys is a third advantage, due to their similarity to human kidneys in terms of size and structure [51,52]. Overall, our results show that static storage of DCD kidneys in AP39-supplemented UW solution at 21°C is better than the clinical standard of care in terms of reducing renal tissue injury. However, further
research is needed to evaluate the function of DCD kidneys preserved in AP39-supplemented UW solution at 21°C.

In conclusion, we demonstrate, for the first time, that exogenous H$_2$S therapy with mitochondria-targeted H$_2$S donor AP39 improves the viability of the gold standard kidney preservation solution, UW solution, for subnormothermic preservation at 21°C. Subnormothermic preservation at 21°C is likely effective because it prevents the damage caused by cold storage while allowing the use of conventional preservation solutions that were designed for cold storage, such as UW solution. Additionally, supplementing UW solution with H$_2$S likely mitigates the negative effects of the higher metabolic demand at 21°C compared to 4°C with cold storage. Thus, subnormothermic preservation at 21°C with exogenous H$_2$S therapy represents a novel avenue in organ preservation. However, further research, using *ex vivo* reperfusion and *in vivo* transplantation models, is needed to evaluate the function of renal grafts preserved using static subnormothermic storage at 21°C in H$_2$S-supplemented UW solution and this study will provide the foundation for that work. In terms of post-transplant function, this approach also needs to be compared to the clinical standard of care, SCS in UW solution, along with SMP at 21°C and NMP with H$_2$S-supplemented blood or other solutions. It is possible that subnormothermic preservation at 21°C using H$_2$S, including static storage and machine perfusion, may match or surpass SCS, because it prevents cold IRI-induced damage, and NMP, because it promotes recovery in a phase of lower metabolic demand at 21°C rather than at 37°C. Ultimately, if successful, these studies could substantiate the translation of static subnormothermic preservation at 21°C with H$_2$S into clinical practice. The clinical implementation of this approach would eliminate the negative consequences of cold
storage and it could expand the pool of acceptable donor organs by promoting the recovery of sub-optimal donor organs.

2.6 References


Chapter 3

3 Subnormothermic perfusion with H$_2$S donor AP39 improves DCD porcine renal graft outcomes in an *ex vivo* model of kidney preservation and reperfusion

This chapter has been accepted for publication.


MDPI
3.1 Abstract

Cold preservation is the standard of care for renal grafts. However, research on alternatives like perfusion at higher temperatures and supplementing preservation solutions with hydrogen sulfide (H$_2$S) has gained momentum. In this study, we investigate whether adding H$_2$S donor AP39 to porcine blood during subnormothermic perfusion at 21°C improves renal graft outcomes. Porcine kidneys were nephrectomized after 30 minutes of clamping the renal pedicles and treated to 4h of static cold storage on ice (SCS) or *ex vivo* subnormothermic perfusion at 21°C with autologous blood alone (SNT) or with AP39 (SNTAP). All kidneys were reperfused *ex vivo* with autologous blood at 37°C for 4h. Urine output, histopathology and RNAseq were used to evaluate renal graft function, injury and gene expression profiles respectively. The SNTAP group exhibited significantly higher urine output than other groups during preservation and reperfusion, along with significantly lower apoptotic injury compared to the SCS group. The SNTAP group also exhibited differential pro-survival gene expression patterns compared to the SCS (downregulation of pro-apoptotic genes) and SNT (downregulation of hypoxia response genes) groups. Subnormothermic perfusion at 21°C with H$_2$S-supplemented blood improves renal graft outcomes. Further research is needed to facilitate the clinical translation of this approach.

3.2 Introduction

Over the past 25 years, hydrogen sulfide (H$_2$S) has become known as the third gasotransmitter, following nitric oxide and carbon monoxide, and the body of literature about its roles in all cells and tissues continues to expand [1–3]. One interesting therapeutic application of H$_2$S is its use in kidney graft preservation to improve renal transplant outcomes. Renal transplantation is the preferred treatment for end stage renal disease because it enhances quality of life and survival compared to hemodialysis [4]. The demand for transplantable kidneys, however, outweits the supply. As a result, kidneys obtained via donation after cardiac death (DCD) are being used more often in transplant centres across the globe [5]. DCD kidneys typically exhibit higher rates of delayed graft function (DGF) and poor long-term survival [6,7]. This is due to the greater ischemic injury experienced by DCD grafts compared to living donor organs. Kidneys from living donors experience some cold ischemic injury from the time of harvest to reimplantation. However, DCD kidneys experience prolonged periods of warm ischemia, as cardiac death occurs, and cold ischemia, during organ preservation. This leads to more severe ischemia-reperfusion injury (IRI) upon reimplantation, which contributes to DGF and other poor outcomes exhibited by DCD kidneys [8].

Organ preservation is a critical phase in terms of mitigating IRI. The clinical standard of care for renal graft preservation is static cold storage (SCS), where the organ is placed on ice (~4°C) in a bag with preservation solution. The alternative is hypothermic machine perfusion (HMP), where the organ is perfused with cold preservation solution. While this approach improves the distribution of nutrients and improves DCD graft outcomes compared to SCS [9,10], it is used less frequently due to its higher cost and
logistical challenges [11,12]. Importantly, both approaches are limited by the shutdown of cellular metabolism inherent to cold preservation, which causes renal injury while improving survival in cold conditions. Longer cold ischemia times are linked to poorer DCD graft outcomes, including higher rates of DGF [13]. As a result, therapeutic strategies that mitigate cold IRI of interest. One therapeutic approach is supplementing preservation solutions with H$_2$S due to its vasodilatory, anti-apoptotic and other cytoprotective effects during IRI [14–16]. We have previously shown that supplementing preservation solutions with H$_2$S, especially mitochondria-targeted H$_2$S donor AP39, during SCS improves murine renal graft outcomes [15,16]. However, the context of cold preservation remains. Thus, alternative approaches that facilitate kidney preservation at higher temperatures (20-37°C) are also of interest [17].

Normothermic machine perfusion, which involves ex vivo perfusion of the kidney at 37°C with a blood-based or acellular solutions, has been shown to improve renal graft outcomes compared to SCS and HMP in several porcine DCD renal transplantation studies [18,19]. In a 2011 pilot surgical case, it was reported that a short duration of this approach after SCS improved clinical post-transplant outcomes compared to SCS alone [20]. Additionally, several ongoing clinical trials are looking to validate and establish a clinical protocol for normothermic preservation. However, in moving away from cold preservation, research into subnormothermic preservation at temperatures (15-35°C) has also gained momentum. In 2014, Hoyer et al. reported that, compared to SCS and HMP, subnormothermic machine perfusion of DCD porcine kidneys at 20°C improved blood flow, output and creatinine clearance during reperfusion with autologous blood [21]. Our group has previously shown that adding AP39 to University of Wisconsin (UW) solution,
a preservation solution that is routinely used for SCS, made it viable for subnormothermic preservation at 21°C [22]. We have also shown that subnormothermic perfusion at 21°C improved DCD porcine renal graft outcomes compared to SCS, subnormothermic perfusion at 15°C, and normothermic perfusion at 37°C [23]. In this study, we investigate whether subnormothermic perfusion at 21°C with H₂S donor AP39 improves DCD porcine renal graft outcomes compared to SCS and subnormothermic perfusion without AP39, using ex vivo pulsatile perfusion for preservation and reperfusion.
3.3 Materials and Methods

Animal care and surgery

Ten yorkshire pigs (60-70kg), purchased from a regional farm, were tranquilized and routinely prepped for surgery. A midline incision was used to expose the kidneys (n=20). The renal pedicles were clamped in situ for 30 minutes to induce warm ischemia, to mimic DCD injury, following intravenous infusion of 10000 units of heparin. By completely stopping renal blood flow, we replicate an extreme clinical DCD scenario where there is no oxygen being supplied to the kidneys. This approach has been used in many other studies within this field [18,21]. The ureters and arteries were cannulated to facilitate ex vivo perfusion and urine collection. Subsequently, both kidneys were nephrectomized and the donor animal was euthanized. Autologous blood used for perfusion was collected via cannulation of the inferior vena cava before and after clamping the renal pedicles.[23] Blood collected prior to clamping is referred to as non-stressed blood because there is no interruption of blood flow. However, blood collected after clamping is referred to as stressed blood due to the buildup of metabolites and signaling molecules due resulting from the lack of blood flow through the kidneys. Surgeries were performed by transplant fellows at University Hospital, London, Canada. All procedures were approved by University of Western Ontario’s Animal Use Committee.

Treatments and ex vivo perfusion setup

The kidneys were assigned to one of three preservation treatments (Fig. 3-1A). The first group of kidneys were flushed with and stored in UW solution on ice for 4h (SCS), which reflects the clinical standard of care. The second group of kidneys were flushed with UW solution and treated to 4h of subnormothermic perfusion at 21°C with non-stressed
blood (SNT). The third group of kidneys were flushed with UW solution supplemented with 200 nM AP39 and treated to 4h of subnormothermic perfusion at 21°C with non-stressed blood supplemented with 200 nM AP39 (SNTAP). After the 4h preservation period, all kidneys were perfused with stressed autologous blood for 4h at 37°C to simulate reperfusion after renal transplantation. A total of ten pig kidney perfusion experiments were performed. In the first seven experiments, left and right kidneys were randomly assigned to either the SCS group (n=7) or the SNTAP group (n=7), with both kidneys connected to the same cassette during reperfusion with stressed blood. Pairs of kidneys sharing the blood reservoir was unavoidable due to the limitation of having a single pulsatile pump. In the following three experiments, both the left and right kidneys were assigned to the SNT group (n=6) as this was an additional control group to represent the effects of subnormothermic perfusion without AP39. The dose of AP39 was chosen based on a previous in vivo murine transplantation study by our group [10]. The kidneys were perfused using an ex vivo pulsatile perfusion apparatus (Fig. 3-1B), which was identical to the setup used in a previous study by our centre [23]. Through adjusting the flow, the mean perfusion pressure was maintained at 60 mmHg after an initial 5-minute period of gradual increase. The blood used in the perfusion circuits was oxygenated using an external oxygen gas supply and no supplemental nutrients, vasodilators or diuretics were added at any point. This was done to evaluate the effect of subnormothermic perfusion with and without AP39 compared to SCS in a simplistic manner without any confounding variables. Urine output was recorded hourly and PlasmaLyte solution (Baxter, IL) was used to compensate for fluid volume loss. Kidney samples were collected after reperfusion with stressed blood,
kidneys were bivalved sagittally. One half was stored at -80°C for RNA sequencing (RNAseq) analysis and the other half was stored in formalin for histopathological analyses.

**AP39**

AP39, synthesized in-house by Prof. Whiteman [24] (Exeter, UK), was dissolved in dimethyl sulfoxide to achieve a 1 mM stock concentration. Two hundred microliters of the stock was added to 1 L bags of preservation solution used for flushing, and blood used for perfusion, to achieve a treatment concentration of 200 nM AP39 [15].

**Histopathological imaging and quantification**

The formalin-fixed kidney sections, including cortex and medulla, were stained with Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) along with Hematoxylin and Eosin (H&E) to determine the level of apoptosis and acute tubular necrosis (ATN) respectively. TUNEL and H&E imaging was done using the Nikon Instruments Eclipse 90i digital microscope at 10x magnification (Nikon Instruments, New York). To quantify apoptotic injury, 10 random images of TUNEL staining were captured per sample. The images were run through Image J v.1.50 (National Institute of Health, USA) to determine %TUNEL+ Area, the ratio of the brown tubular area (TUNEL+) and the total tubular area. To quantify ATN, H&E slides were scored for ATN by a blinded renal pathologist as per the following scheme: 1= <11%, 2= 11-24%, 3= 25-45%, 4= 46-75%, 5= >75%.

**Statistical Analysis**

GraphPad Prism 8 was used to create graphs and conduct statistical analyses. Student’s t-test or one-way ANOVA followed by Tukey’s post-hoc test was used for
comparisons of two or three experimental groups respectively. Statistical significance was accepted at p<0.05.

**RNASeq**

Total RNA was extracted from frozen renal cortical tissues using RNEasy kits (Qiagen, Germany) following vendor’s protocol. Total RNA samples were quantified using the NanoDrop (Thermo Fisher Scientific, USA), and quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., USA) and the RNA 6000 Nano kit (Caliper Life Sciences, USA). They were then processed using the Vazyme VAHTS Total RNA-seq (H/M/R) Library Prep Kit for Illumina (Vazyme, China) which includes rRNA reduction. Three samples (n=3) from each experimental group and one control sample from a baseline porcine kidney with no DCD injury (CTR, n=1) were sequenced at the London Regional Genomics Centre (Robarts Research Institute, Canada) using the Illumina NextSeq 500 (Illumina Inc., USA).

Briefly, samples were rRNA depleted then fragmented, cDNA was synthesized, indexed, cleaned-up and amplified via PCR. Libraries were then equimolarly pooled into one library and size distribution was assessed on an Agilent High Sensitivity DNA Bioanalyzer chip and quantified using the Qubit 2.0 Fluorimeter (Thermo Fisher Scientific, USA). The library was sequenced on an Illumina NextSeq 500 as a single end run, 1x76 bp, using a High Output v2 kit (75 cycles). Fastq data files were downloaded from BaseSpace and analyzed using Partek Flow (Partek, USA). After importation, data was aligned to the Sus scrofa genome using STAR 2.6.1d and annotated using Ensembl v 97.
The resulting raw gene counts were analyzed using a workflow summarized in Fig. 3-1C. A final gene counts file was prepared after removing genes that could not be annotated using Ensembl Biomart and genes that did not have matching human gene IDs approved by HUGO Gene Nomenclature Committee, which is necessary for functional enrichment analysis. Using RStudio v. 3.6.1 (Boston, USA; session info in Supplementary Material), sample distribution mapping and principal component analysis were performed to visualize the difference in expression profiles of samples in each group. The DESeq2 package was used to identify differentially expressed genes for the following comparisons: SNTAP vs. SCS and SNTAP vs. SNT (alpha=0.05). Based on the protocol detailed by Reimand et al [24], Gene Ontology (GO) annotations were found for the differentially expressed genes using g:Profiler. These annotations were analyzed using Cytoscape v.3.7.2 to develop network enrichment maps, which facilitated the identification of certain genes and pathways of interest for both comparisons.
Figure 3-1 Summary of Methods. (A) Overview of experimental design. (B) Schematic of *ex vivo* perfusion setup used for porcine kidney perfusion and reperfusion. (C) RNAseq analysis workflow.
3.4 Results

Subnormothermic perfusion with AP39-supplemented blood improves urine output and reduces tissue injury compared to static cold storage and subnormothermic perfusion without AP39

To investigate whether subnormothermic perfusion with AP39-supplemented blood can improve ex vivo DCD renal graft function, porcine kidneys were assigned to 4h of cold storage or subnormothermic perfusion with non-stressed blood at 21°C, with or without AP39. During preservation, DCD kidneys perfused with AP39-supplemented blood (SNTAP group) exhibited significantly higher (p<0.05) urine output than the kidneys perfused with blood on its own (SNT group) (Fig. 3-2A). No urine output was recorded for the static cold storage (SCS group) as those kidneys were placed on ice. The preservation period was followed by 4h of reperfusion with stressed blood at 37°C. During reperfusion, the SNTAP group also exhibited significantly higher urine output than the SCS group (p<0.05) and the SNT group (p<0.01). No significant difference in urine output was observed between the SCS and SNT group (Fig. 3-2B). Following reperfusion, the kidneys were sagittally bivalved and formalin-fixed sections were stained with TUNEL and H&E to determine apoptotic injury and ATN respectively (Fig. 3-3A). The SNTAP group exhibited the lowest mean %TUNEL+ area and mean ATN score (Fig 3-3B & 3-3C). While no statistically significant differences were found amongst the mean ATN scores (Fig. 3-3C), the SNTAP group exhibited significantly lower mean %TUNEL+ area than the SCS group (p<0.05), indicating significantly lower apoptotic injury (Fig. 3B).
Figure 3-2 Subnormothermic perfusion with AP39 improves urine output during preservation and reperfusion. Porcine kidneys were nephrectomized after 30 minutes of clamping to mimic DCD injury and subjected to various preservation treatments for 4h. This was followed by 4h of reperfusion with stressed autologous blood. Urine output was collected during both perfusion periods for all groups except for the static cold storage group, where kidneys were on ice during the 4h of preservation. (A) Total urine output (mL) collected during 4h of preservation perfusion. (B) Total urine output (mL) collected during 4h of reperfusion with stressed blood. Lines represent mean ± SEM. Values in (A) were compared using student’s unpaired t-test and values in (B) were compared using one-way ANOVA followed by Tukey’s post-hoc test. *, p<0.05. **, p<0.01. Circles represent values for static cold storage (SCS, n = 7). Squares represent values for subnormothermic perfusion (SNT, n = 6). Triangles represent values for subnormothermic perfusion with 200 nM AP39 (SNTAP, n = 7).
Figure 3-3 Subnornothermic perfusion with AP39 reduces tissue apoptosis and acute tubular necrosis. (A) Representative TUNEL and H&E images of formalin-fixed DCD kidney sections after 4h of preservation treatment and 4h of reperfusion. Images were taken at 10x magnification (scale bar = 100 µm). (B) Mean %TUNEL+ area as determined by ImageJ using a ratio of TUNEL+ area (brown) to total tubular area. Each individual data point represents the mean %TUNEL+ area of 10 random fields of view of one porcine kidney sample. (C) ATN scores based on H&E staining. Each individual data point represents the score assigned to one porcine kidney sample. Lines in (B) and (C) represent mean ± SEM. Values in (B) and (C) were compared using one-way ANOVA followed by Tukey’s post-hoc test. *, p<0.05. Circles represent values for static cold storage (SCS, n = 7). Squares represent values for subnornothermic perfusion (SNT, n = 6). Triangles represent values for subnornothermic perfusion with 200 nM AP39 (SNTAP, n = 7).

Adding AP39 to blood during subnornothermic perfusion leads to differential pro-survival gene expression patterns compared to static cold storage and subnornothermic perfusion without AP39.

To investigate the effect of AP39 on gene expression in this study, RNAseq was performed on frozen porcine kidney sections collected after reperfusion. Using RStudio, principal component analysis was performed to visualize the kidney samples in all groups (Fig. 3-4). Using the DESeq2 package in RStudio, it was determined that 214 genes were differentially expressed in the SNTAP group vs. the SCS group (alpha=0.05, Figure S1 in Supplementary Material that is available online). Through network enrichment analysis,
clusters of interest were identified based on GO annotations for the 214 genes, including response to heat, response to stress, regulation of transcription and negative regulation of cell death (Fig. 3-5A). Relative to the SCS group, the SNTAP group exhibited downregulation of pro-apoptotic (BCL10) and heat shock response (HSPD1, HSPA1A) genes, along with regulators of those pathways (BAG3, DDIT3). Additionally, proliferation (MAPK7) and oxidative stress response (NRROS) genes were upregulated in the SNTAP group (Fig. 3-5B). Next, it was determined that 614 genes were differentially expressed in the SNTAP group vs. the SNT group (alpha=0.05). Clusters of interest were identified based on GO annotations, including response to hypoxia, regulation of transcription and response to endogenous stimulus (Fig 3-6A.) Relative to the SNT group, several genes associated with the HIF-1α-mediated hypoxia response pathway (EGR1, PCK1, PDK3, RGCC) were downregulated in the SNTAP group. The expression of genes mediating the TGF-β pathway (SMAD3, NRROS) and HIF-1α degradation (AJUBA) was upregulated also observed in the SNTAP group (Fig. 3-6B). Additionally, the downregulation of pro-inflammatory (IL6, HMGB2) and pro-cell death (HOXD8, HOXD10) genes was observed in the SNTAP group, along with upregulation of proliferation (MAPK7) and oxidative stress response (NRROS) genes (Fig. 6C).
Figure 3-4 Principal component analysis of r-log normalized gene counts for all porcine kidney samples. Frozen renal cortical sections were used for RNAseq analysis. RStudio was used to perform the principal component analysis with the full set of r-log normalized gene counts all samples and to visualize the clustering of samples by group. CTR, control baseline kidney with no DCD injury or preservation treatment (n=1). SCS, static cold storage (n=3). SNT, subnormothermic perfusion (n=3). SNTAP, subnormothermic perfusion with 200 nM AP39 (n=3). Numbers following group names denote individual samples.
A

Negative regulation of apoptotic process
response to stress
HSF1-dependent transactivation
response to heat
regulation of protein stability
Regulation of HSP70-mediated heat shock response
regulation of RNA biosynthetic process
negative regulation of transcription from RNA polymerase II promoter in response to stress
regulation of transcription from RNA polymerase II promoter in response to stress
negative regulation of transcription from RNA polymerase II promoter in response to stress

B

log2FoldChange
(for SNTAP, relative to SCS)

Negative regulation of apoptosis
Transcriptional regulation
Response to stress

-4
-2
0
2
4
6

AIF1
BCL10
PDK4
SOD1
HSPA1A
SIN3A
MAPK7
BAG3
DDIT3
CREB3L3
DNAJB1
CYP1A1
CRYAB
NRROS
NOTCH4
Figure 3-5 Gene expression analysis after preservation using subnormothermic perfusion with AP39 vs. static cold storage. Frozen renal cortical sections (n=3 for each group) were used for RNAseq analysis. Differentially expressed genes were identified using RStudio (DESeq2, alpha = 0.05). GO annotations were found for those genes using g:Profiler and network enrichment analysis was performed using Cytoscape. (A) Network enrichment map showing certain nodes of interest (FDR Q value <1.0, Jaccard Overlap combined coefficient > 0.375 with combined constant = 0.5). (B) Gene expression values (log2FoldChange ± SEM, determined using RStudio) of certain genes of interest chosen from three nodes of interest from the enrichment map in (A) – response to stress, negative regulation of apoptotic process and regulation of DNA-templated transcription in response to stress.
A

positive regulation of MAP kinase activity
positive regulation of protein serine/threonine kinase activity
positive regulation of macromolecule metabolic process
response to decreased oxygen levels
response to hypoxia
response to oxygen levels
response to endogenous stimulus
positive regulation of transcription by RNA polymerase II
positive regulation of transcription, DNA-templated

B

log2FoldChange
(for SNTAP, relative to SNT)

-3
-2
-1
0
1
2

Response to hypoxia/decreased oxygen levels

C

log2FoldChange
(for SNTAP, relative to SNT)

-5
-4
-3
-2
-1
0
1
2

Transcriptional regulation

Response to endogenous stimulus
Figure 3-6 Gene expression analysis after preservation using subnormothermic perfusion with or without AP39. Frozen renal cortical sections (n=3 for each group) were used for RNAseq analysis. Differentially expressed genes were identified using RStudio (DESeq2, alpha = 0.05). GO annotations were found for those genes using g:Profiler and network enrichment analysis was performed using Cytoscape. (A) Network enrichment map showing certain nodes of interest (FDR Q value <1.0, Jaccard Overlap combined coefficient > 0.375 with combined constant = 0.5). (B) Gene expression values (log2FoldChange ± SEM, determined using RStudio) of all the genes in three connected nodes from the enrichment map in (A) – response to oxygen levels, hypoxia, decreased oxygen levels. C) Gene expression values (log2FoldChange ± SEM, determined using RStudio) of certain genes of interest from two nodes of interest from the enrichment map in (A) – regulation of DNA-templated transcription and response to endogenous stimulus.
3.5 Discussion

This study shows that subnormothermic perfusion of DCD pig kidneys with AP39-supplemented blood (SNTAP) improves the urine output, reduces tissue injury and leads to differential pro-survival gene expression compared to static cold storage (SCS) and subnormothermic perfusion with blood alone (SNT) in our ex vivo model of preservation (four hours) and reperfusion (four hours).

The SNTAP group displayed a significantly higher urine output than the SNT group during preservation and both the SNT and SCS groups during reperfusion. This is likely due to an increase in vasodilation leading to greater renal blood flow (RBF) and subsequent diuresis. Vasoconstriction is a critical consequence of warm ischemic injury during DCD organ procurement and cold ischemic injury during SCS or HMP [26]. H2S has been shown to enhance vasodilation and RBF in several studies. Xia et al. showed that the infusion of NaHS in the intrarenal arteries of rats enhanced RBF and the glomerular filtration rate [27]. Additionally, using a porcine model of renal transplantation, Hosgood and Nicholson showed that the infusion of H2S ten minutes prior to and after reperfusion improves RBF and renal function [28]. Previous studies have also shown that H2S inhibits platelet aggregation in vitro and in vivo [29,30], which could contribute to increased blood flow. Although AP39 has not been directly linked to RBF or platelet aggregation to date, our RNAseq results (discussed below) suggest a potential vasodilatory mechanism that may contribute to the significantly higher urine output of the SNTAP group.

The SNTAP group also exhibited significantly lower tissue apoptosis than the SCS group, based on TUNEL staining. This finding was supported by the downregulation of pro-apoptotic genes in the SNTAP group relative to the SCS group. Several studies have
reported that AP39 reduces renal apoptosis [15, 31, 32], and it has been shown to downregulate Bax [32], a pro-apoptotic gene in the intrinsic apoptotic pathway. This study shows, for the first time, that AP39 may be exerting anti-apoptotic effects through downregulating BCL10 [33,34], a pro-apoptotic regulatory gene, and DDIT3 [35], a transcriptional regulator of endoplasmic reticulum stress-induced apoptosis. Notably, the expression of heat shock proteins (HSPD1 and HSPA1A) and BAG3, an HSP cochaperone [36], was downregulated in the SNTAP group. Seeing as these factors normally promote cell survival during heat-induced stress [37,38], it is counterintuitive that they are downregulated in the SNTAP group (21°C) compared to the SCS group (4°C). However, the resulting anti-survival effects are likely counteracted by the downregulation of apoptotic genes and upregulation of MAPK7, a key mediator of cell proliferation and cell survival [39].

Interestingly, several genes associated with the HIF1α-mediated hypoxia response, which contributes to acute kidney injury and renal IRI [40], were downregulated in the SNTAP group relative to the SNT group. The downregulation of PCK1 [41] and PDK3, metabolic targets activated by HIF1α [42], may help with managing the metabolic demand at 21°C that is not being met with metabolites, thereby mitigating tissue injury. The downregulation of RGCC, a negative regulator of HIF1α-mediated angiogenesis [43], and EGR1, a negative coregulator of erythropoietin-receptor expression [44], likely promotes vascularization. This may contribute to the improved urine output of the SNTAP group through improving the RBF. The upregulation of AJUBA, a mediator of HIF1α degradation [45], likely helps to balance the positive and negative effects of HIF1α and other genes associated with it. Additionally, the SNTAP group exhibited downregulation
of the pro-apoptotic genes (HOXD8 and HOXD10) [46,47], which could further explain the lower tissue apoptosis in the SNTAP group.

The SNTAP group also exhibited upregulation of MAPK7 and NRROS compared to both the SCS and SNT groups. AP39 has been shown to reduce reactive oxygen species (ROS) levels \textit{in vitro} [15,32]. The downregulation of NRROS, which negatively regulates ROS production [48] and activates TGF-\(\beta\)1 [49,50], could underlie the antioxidant and protective responses observed by previous studies on AP39. Additionally, the downregulation of IL6 was observed in the SNTAP group relative to the SNT group, which matches the findings of a previous study looking at the effect of AP39 in myocardial IRI [32]. IL6 is a key proinflammatory cytokine in the progression of acute kidney injury [51], and its downregulation by AP39 may contribute to the improved outcomes of the SNTAP group compared to the SNT group.

There are two limitations to the perfusion model used in this study. Firstly, the perfusion times used are relatively short. However, the four-hour duration was deemed appropriate, because this was the first time that our novel approach towards organ preservation was attempted on large mammalian kidneys, and hence, we wanted to prove its feasibility. Further testing using longer periods of preservation and reperfusion will be necessary for clinical translation. Secondly, the limitation of having only one organ perfusion pump due to financial constraints required us to place the two kidneys on the same circuit during the reperfusion phase; however, the urine output from each kidney was collected separately. Perfusing both kidneys on separate pumps would have prevented their metabolites from pooling into a shared blood reservoir. Despite this limitation, the SNTAP group exhibited significantly higher urine output during reperfusion and a significantly
lower TUNEL score than the SCS group. This suggests that if we had reperfused the kidneys on two separate pumps, our positive findings would have been accentuated to a greater extent, which further supports the use of our novel approach.

While the number of samples we could perform RNAseq testing on was limited by the cost, the consistency in gene expression across the samples in most groups facilitated a reliable analysis within the constraints of this limitation. Additionally, the findings of our study were strengthened by the approach of clamping the renal pedicle to mimic a DCD injury. Inducing the complete cessation of the RBF via clamping mimics an extreme clinical DCD scenario where no oxygen is supplied to the kidneys. Thus, the positive outcomes observed in this study are more likely to translate to and be accentuated in real clinical DCD scenarios where there is a more gradual cessation of blood flow as cardiac death occurs. Evidently, further in vitro research is needed to confirm the mechanisms underlying AP39’s protective effects. Future studies using in vivo models of renal transplantation and clinically approved H$_2$S donors are also needed to support the findings of this study.

In conclusion, this study demonstrates that subnormothermic perfusion with AP39-supplemented blood can improve ex vivo DCD porcine renal graft function. We postulate that the subnormothermic temperature allows for maintenance of the vasculature, unlike static cold storage, with a metabolic demand that is lower than normal due to the deviation from the physiological temperature (37°C). The presence of H$_2$S helps mitigate some of the negative consequences of being unable to meet the metabolic demand at 21°C in a nutrient-deficient environment, such as inflammation, ROS and apoptosis. Overall, this study adds to the growing body of literature that supports the use of subnormothermic
temperatures and H$_2$S in organ preservation to improve DCD renal graft outcomes. These strategies could facilitate the use of more marginal grafts, which could increase the pool of transplantable organs.

3.6 Supplementary Material

**Supplementary Figure S1**


**Figure S1: Heatmap of the differential gene expression analysis after preservation using subnormothermic perfusion with AP39 vs. static cold storage.** Porcine kidneys were nephrectomized after 30 min of clamping to mimic DCD injury and subjected to subnormothermic perfusion with AP39 (SNTAP) or static cold storage (SCS) for 4 h. This was followed by 4 h of reperfusion with stressed autologous blood. Frozen renal cortical sections (n= 3 for each group) were used for the RNAseq analysis. Differentially expressed genes were identified using RStudio (DESeq2, alpha = 0.05), and a heap map was generated. CTR, control baseline kidney with no DCD injury or preservation treatment (n=1).

**R Studio Session Information:**

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Running under: Windows 10 x64 (build 17763)
Matrix products: default
locale:
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[3] LC_MONETARY=English_Canada.1252 LC_NUMERIC=C
[5] LC_TIME=English_Canada.1252
attached base packages:
[1] grid parallel stats4 stats graphics grDevices utils datasets
[9] methods base
other attached packages:
[1] DEGreport_1.22.0  readxl_1.3.1  readr_1.3.1
[4] vsn_3.54.0  hexbin_1.28.0  ggbpiplot_0.55
[7] scales_1.0.0  plyr_1.8.4  ggplot2_3.2.1
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[100] digest_0.6.22  xtable_1.8-4  tidyr_1.0.0
[103] munsell_0.5.0
3.7 References


44. Su, T.; Liu, P.; Ti, X.; Wu, S.; Xue, X.; Wang, Z.; Dioum, E.; Zhang, Q. HIF1α, EGR1 and SP1 co-regulate the erythropoietin receptor expression under hypoxia:


Chapter 4

4 Evaluating the effects of subnormothermic perfusion with AP39 in a novel blood-free model of ex vivo kidney preservation and reperfusion

This chapter has been accepted for publication.


MDPI
4.1 Abstract

The use of blood for normothermic and subnormothermic kidney preservation hinders the translation of these approaches and promising therapeutics. This study evaluates whether adding hydrogen sulfide donor AP39 to Hemopure, a blood substitute, during subnormothermic perfusion improves kidney outcomes. After 30 minutes of renal pedicle clamping, porcine kidneys were treated to 4h of static cold storage (SCS-4°C) or subnormothermic perfusion at 21°C with Hemopure (H-21°C), Hemopure + 200nM AP39 (H200nM-21°C) or Hemopure + 1µM AP39 (H1µM-21°C). Then, kidneys were reperfused with Hemopure at 37°C for 4h with metabolic support. Perfusate composition, tissue oxygenation, urinalysis and histopathology were analyzed. During preservation, the H200nM-21°C group exhibited significantly higher urine output than the other groups and significantly higher tissue oxygenation than the H1µM-21°C group at 1h and 2h. During reperfusion, the H200nM-21°C group exhibited significantly higher urine output and lower urine protein than the other groups. Additionally, the H200nM-21°C group exhibited higher perfusate pO2 levels than the other groups and significantly lower apoptotic injury than the H-21°C and the H1µM-21°C groups. Thus, subnormothermic perfusion at 21°C with Hemopure + 200nM AP39 improves renal outcomes. Additionally, our novel blood-free model of ex vivo kidney preservation and reperfusion could be useful for studying other therapeutics.
4.2 Introduction

Renal transplantation is preferred treatment for end-stage renal disease because it improves long-term survival and quality of life compared to hemodialysis.\textsuperscript{1–3} However, there is a critical shortage of donor kidneys across the globe due to the increasing need for kidney transplants. In Canada, the prevalence of end-stage renal disease has risen by 33\% between 2010 and 2019.\textsuperscript{4} Despite the 1,648 adult kidney transplants that were performed in 2019, 3,261 individuals remained on the waiting list.\textsuperscript{5} To meet the rising demand, kidneys from marginal donors are being used more frequently. While donation after neurological death does not impact organ health, donation after cardiac death (DCD) leads to warm ischemic injury as blood flow decreases and the heart stops. This is of concern because DCD kidneys are being transplanted more frequently and often lead to poorer patient outcomes compared to kidneys from other donors.\textsuperscript{6} Additionally, clinical cold preservation methods (4°C), such as static cold storage (SCS), exacerbate DCD kidney outcomes due to the combination of cold and warm ischemic injury.\textsuperscript{7–9}

Several strategies have been explored to improve cold preservation methods, such as the addition of hydrogen sulfide (H\textsubscript{2}S), a gasotransmitter with cytoprotective properties, to preservation solutions.\textsuperscript{10–12} Our group and others have previously shown that adding nanomolar concentrations of H\textsubscript{2}S donor AP39 to cold preservation solution improves renal and cardiac graft outcomes.\textsuperscript{11,13} Additionally, there is rising interest in alternatives to cold preservation that employ normothermic (36-37°C) and subnormothermic (15-35°C) temperatures.\textsuperscript{14} Several porcine and discarded human kidney studies have shown that normothermic and subnormothermic machine perfusion improve DCD renal graft outcomes compared to SCS on ice.\textsuperscript{15–20} However, a major challenge in this context is the need for oxygenation and nutrients to meet the metabolic demand of the kidney at these
higher temperatures. Previous studies have primarily used erythrocyte-based solutions or autologous whole blood for this purpose\textsuperscript{16,18,21}, but the use of blood is a challenge for the clinical translation of these approaches. Pervasive shortages of banked blood and the challenges associated with obtaining blood from DCD donors limit the availability of blood for \textit{ex vivo} kidney preservation.

The use of blood substitutes, especially hemoglobin-based oxygen carriers (HBOC), can solve the problem of oxygenation and circumvent the need for blood in the context of normothermic and subnormothermic preservation. One promising candidate is Hemopure (also known as HBOC-201), which is made of highly purified bovine hemoglobin.\textsuperscript{22,23} Clinically, Hemopure is used to treat anemia in patients who cannot receive blood transfusions.\textsuperscript{24,25} However, several recent studies have shown that \textit{ex vivo} kidney preservation with Hemopure can improve renal graft outcomes. In 2019, Aburawi et al. reported that normothermic perfusion (37°C) of discarded human kidneys with Hemopure vs. packed red blood cells led to comparable outcomes.\textsuperscript{26} Subsequently a recent study by our center has shown that subnormothermic perfusion (22°C) of DCD porcine kidneys with Hemopure vs. whole blood exhibited similar outcomes, which further confirms the feasibility of Hemopure for blood-free renal graft preservation.\textsuperscript{19}

While recent evidence supports the use of Hemopure for kidney preservation, its potential as a platform to deliver therapeutics that could further enhance renal graft outcomes remains unexplored. We have previously shown that adding H\textsubscript{2}S donor AP39 to University of Wisconsin (UW) solution, a preservation solution that is commonly used for SCS, made it suitable for subnormothermic preservation.\textsuperscript{27} Additionally, we have shown that subnormothermic perfusion (21°C) of DCD porcine kidneys with AP39-supplemented...
whole blood improves renal graft outcomes and reduces tissue injury using an \textit{ex vivo} model of blood-based preservation and reperfusion.\textsuperscript{20} In this study, we investigate whether subnормothermic perfusion at 21°C with AP39-supplemented Hemopure improves DCD porcine renal graft outcomes compared to SCS and subnормothermic perfusion with Hemopure alone. To evaluate this aim, we use a novel blood-free model of \textit{ex vivo} preservation and reperfusion. In this model, after 4 hours of blood-free preservation, all kidneys are reperfused (37°C) for 4 hours with Hemopure, supplemented with a diuretic and metabolic support, to mimic the post-transplant milieu.

4.3 Materials and Methods

\textbf{Animal care and surgery}

Yorkshire pigs (60-70kg), purchased from a regional farm, were tranquilized and routinely prepped for surgery. A midline incision was used to expose the kidneys. Following intravenous infusion of 10000 U of heparin, the renal pedicles were clamped \textit{in situ} for 30 minutes to induce warm ischemia and mimic DCD injury. The complete cessation of renal blood flow replicates an extreme clinical DCD scenario where no oxygen is being supplied to the kidneys. This approach has been used in many other studies within the field and previous studies by our center. During the clamping period, the ureters and arteries were cannulated to facilitate \textit{ex vivo} perfusion and urine collection. Subsequently, both kidneys were nephrectomized and the donor animal was euthanized. Surgeries were performed by transplant fellows at University Hospital, London, Canada. All procedures were approved by University of Western Ontario’s Animal Use Committee (Animal Use Protocol 2018-090).
**Ex vivo perfusion setup**

The *ex vivo* perfusion setup used in this study is identical to the setup used in previous studies\textsuperscript{18–20} by our center (Fig. 4-1). Mean perfusion pressure was maintained at 60 mmHg through adjusting the flow of the perfusate. Fresh perfusate (1L) was prepared for preservation and reperfusion by mixing 250 mL of Hemopure (generously provided by HbO2 Therapeutics, USA) with 750 mL of PlasmaLyte solution (Baxter International Inc., USA). The perfusate was supplemented with the following: heparin (5000 U), sodium bicarbonate (8.4%, 10mL) and Ancef (1g). Pairs of kidneys were perfused together due to having a single pulsatile pump. However, each kidney was considered as one replicate because its urine output was collected individually.

![Figure 4-1 The *ex vivo* pulsatile perfusion setup used for blood-free preservation and reperfusion. A pair of kidneys, placed in the perfusion cassette, are receiving externally pumped perfusate.](image)

**Figure 4-1** The *ex vivo* pulsatile perfusion setup used for blood-free preservation and reperfusion. A pair of kidneys, placed in the perfusion cassette, are receiving externally pumped perfusate.
oxygenated perfusate through their arteries. Their ureters are connected to urine collection bags to measure and collect each kidney’s urine output individually. A water-based heat exchanger is connected to the perfusate oxygenator to control the temperature of the perfusate. The image specifically reflects the setup for reperfusion due to presence of dextrose and insulin drips.

**Blood-free preservation treatments**

Pairs of kidneys were assigned to one of four treatment groups (Fig. 4-2). The first group of kidneys were flushed with and stored in Histidine-Tryptophan-Ketoglutarate (HTK) solution (Custodiol®, USA) on ice for 4h (SCS-4°C), which reflects the clinical standard of care. The second group of kidneys were flushed with HTK solution and treated to 4h of subnormothermic perfusion at 21°C with Hemopure (H-21°C). The third group of kidneys were flushed with HTK solution + 200 nM AP39 and treated to 4h of subnormothermic perfusion at 21°C with Hemopure + 200 nM AP39 (H200nM-21°C). The fourth group of kidneys were flushed with HTK solution + 1 µM AP39 and treated to 4h of subnormothermic perfusion at 21°C with Hemopure + 1 µM AP39 (H1µM-21°C). For the three preservation treatments involving subnormothermic perfusion, urine output was recorded hourly and the volume loss was replaced with the addition of PlasmaLyte. Additionally, tissue oxygenation was measured hourly using the InSpectra StO2 Spot Check Tissue Perfusion Monitor (Hutchinson Technology, USA).

**Blood-free reperfusion protocol**

Following 4h of preservation, kidneys were reperfused for 4h using our novel blood-free reperfusion model (Fig. 4-2). Due to budget constraints, the same perfusion cassettes were used throughout preservation and reperfusion. To prevent the mixing of the
perfusates from both halves of the experiment, the perfusion circuit was drained and flushed with 2 L of saline between preservation and reperfusion. After the saline flush, 1 L of fresh Hemopure/PlasmaLyte solution was added for reperfusion and the temperature was set to 37°C. At the start of reperfusion, 4 g of mannitol was added to the perfusate to mimic the post-operative administration of a diuretic to renal transplant recipients. Additionally, we implemented 5% dextrose and insulin drips to provide metabolic support to the kidneys, maintaining a perfusate glucose concentration of ~150 mg/dL.

During reperfusion, urine samples were collected, and urine output was recorded hourly. The volume lost was replaced with the addition of PlasmaLyte. Tissue oxygenation was measured hourly as described earlier. Additionally, perfusate parameters (pH, pO₂, and lactate) were measured using the iSTAT Handheld Blood Analyzer (Abbott Laboratories, USA) to allow for relative comparison between groups. Sodium bicarbonate was injected as needed to adjust perfusate pH. After 4h of reperfusion, kidney sections (cortex and medulla) were cut and stored in formalin for histopathological analyses.
AP39, synthesized in-house by Prof. Whiteman\textsuperscript{28}, was dissolved in dimethyl sulfoxide to achieve a 1mM stock concentration. To achieve a treatment concentration of 200 nM AP39, 200 µL of the stock was added to 1 L of preservation solution and perfusate. Similarly, for a concentration of 1 µM AP39, 1 mL of the stock was added to 1 L of preservation solution and perfusate. The doses were chosen based on previous studies by our group.

Urinalysis

Most of the urine samples collected were heavily pigmented, due to the presence of hemoglobin from the Hemopure, which prevented the use of conventional urinalysis methods. A 1:3 dilution of urine in Hemoglobin (Biotech Support Group, USA) allowed us to obtain clearer urine samples after 10 minutes of vigorous shaking and centrifugation.
at 12,000g. Urine protein and creatinine levels were analyzed using the IDEXX Urine Analyzer (IDEXX Laboratories, USA), but creatinine values remained undetectable.

**Histopathology imaging and scoring**

Formalin-fixed kidney sections, including cortex and medulla, were embedded in paraffin and mounted onto microscope slides. The sections were stained with Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and Hematoxylin and Eosin (H&E) to determine the level of apoptosis and acute tubular necrosis respectively. TUNEL and H&E imaging was done using the Nikon Instruments Eclipse 90i digital microscope at 10x magnification (Nikon Instruments, New York). Both sets of slides were scored by a blinded renal pathologist as per the following scheme: 1= <11%, 2= 11-24%, 3= 25-45%, 4= 46-75%, 5= >75%.

**Statistical analyses**

GraphPad Prism 9 was used to create graphs and conduct statistical analyses. One-way or two-way ANOVA followed by Tukey’s post-hoc test was used for comparisons of three or more experimental groups. Statistical significance was accepted at p<0.05.
4.4 Results

Subnormothermic perfusion with Hemopure + 200nM AP39 at 21°C improves gross kidney morphology and perfusate pO₂ levels during blood-free reperfusion

Kidneys were imaged prior to preservation (after flushing) and at the end of reperfusion to evaluate differences in gross morphology (Fig. 4-3). The images taken prior to preservation show that the kidneys in each group had similar gross morphology and were thoroughly flushed following the induction of warm ischemia, which is reflected by the pale colour. At the end of reperfusion, the H200nM-21°C kidneys looked the reddest, which is an indicator of consistent perfusion and overall organ health. On the other hand, the H1µM-21°C kidneys looked much darker than the other groups at the end of reperfusion, which is an indicator of thrombosis and tissue injury (Fig. 4-3). Additionally, perfusate samples were analyzed hourly during reperfusion to evaluate relative changes in pO₂, pH and lactate levels (Fig. 4-4, Table 2). The H200nM-21°C group exhibited higher perfusate pO₂ levels than the SCS-4°C, H-21°C and H1µM-21°C groups throughout the reperfusion period. Additionally, the perfusate pO₂ levels stayed relatively consistent over time for each group (Fig. 4-4A). The H1µM-21°C group exhibited higher perfusate pH than the other three groups throughout the reperfusion period. Apart from the elevated pH of this group and the high variability at the 1h timepoint, perfusate pH was maintained within the range of 7.1 to 7.4. Interestingly, perfusate pH decreased over time for each group (Fig. 4-4B). Furthermore, all four groups exhibited comparable increases in lactate levels throughout the reperfusion period, which contributes to the decrease in perfusate pH described earlier (Fig. 4-4B). None of the differences in perfusate parameter levels were statistically significant.
Figure 4-3 Gross morphology of the kidneys prior to preservation and at the end of reperfusion. Images were taken prior to perfusion to document the consistent flushing of the kidneys following the induction of warm ischemia. Images were taken at the end of reperfusion to document the gross morphology of kidneys by the end of the experiment. One pair of representative images were chosen for each preservation treatment group. Treatment groups: SCS-4°C, static cold storage on ice at 4°C. H-21°C, perfusion with Hemopure at 21°C. H200nM-21°C, perfusion with Hemopure + 200 nM Ap39 at 21°C. H1µM-21°C, perfusion with Hemopure + 1µM Ap39 at 21°C.
Figure 4-4 Perfusate parameters during blood-free reperfusion. (A) $pO_2$ (mmHg), (B) pH and (C) Lactate (mmol/L) during blood-free reperfusion. Since pairs of kidneys were reperfused with shared perfusate, singular readings were obtained at each timepoint for each pair of kidneys. The iSTAT Analyzer was unable to detect tissue oxygenation for one pair of kidneys (n=1) in the H1µM-21°C group. Individual points on each graph represent the mean value at a specific timepoint for pairs of kidneys within a single treatment group. SEM values are not presented on the graphs to allow for clear visualization of trends without overlapping error bars. Mean ± SEM are listed in Table 2. After a Geisser-Greenhouse correction, values were compared using repeated measures two-way ANOVA followed by Tukey’s post-hoc test and no significant differences were found. Treatment groups: SCS-4°C, static cold storage on ice at 4°C (n=3). H-21°C, perfusion with
Hemopure at 21°C (n=3). H200nM-21°C, perfusion with Hemopure + 200nM AP39 at 21°C (n=3). H1µM-21°C, perfusion with Hemopure + 1µM AP39 at 21°C (n=2).

Table 2- Mean perfusate parameters ± SEM during blood-free preservation and reperfusion.

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Reperfusion Timepoint</th>
<th>pO₂ (mmHg)</th>
<th>pH</th>
<th>Lactate (mmol/L)</th>
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<tr>
<td></td>
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<td></td>
<td>1h</td>
<td>2h</td>
<td>3h</td>
<td>4h</td>
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<tr>
<td>SCS-4°C (n = 3)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>H-21°C (n = 3)</td>
<td>227.00 ± 6.43</td>
<td>231.00 ± 15.04</td>
<td>231.00 ± 9.644</td>
<td>221.33 ± 9.493</td>
</tr>
<tr>
<td>H200nM-21°C (n = 3)</td>
<td>215.66 ± 3.71</td>
<td>221.33 ± 9.59</td>
<td>224.33 ± 6.17</td>
<td>221.33 ± 3.28</td>
</tr>
<tr>
<td>H1µM-21°C (n = 2)</td>
<td>242.66 ± 14.19</td>
<td>242.66 ± 18.97</td>
<td>238.66 ± 5.78</td>
<td>241.00 ± 15.50</td>
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<tr>
<td></td>
<td>213.00 ± 13.00</td>
<td>214.00 ± 13.00</td>
<td>215.50 ± 5.50</td>
<td>212.00 ± 8.00</td>
</tr>
<tr>
<td></td>
<td>7.40 ± 0.11</td>
<td>7.27 ± 0.09</td>
<td>7.17 ± 0.08</td>
<td>7.05 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>7.67 ± 0.13</td>
<td>7.35 ± 0.13</td>
<td>7.49 ± 0.12</td>
<td>7.38 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>7.17 ± 0.04</td>
<td>7.29 ± 0.23</td>
<td>7.24 ± 0.11</td>
<td>7.13 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>7.84 ± 0.10</td>
<td>7.71 ± 0.09</td>
<td>7.56 ± 0.08</td>
<td>7.44 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>4.39 ± 0.59</td>
<td>6.51 ± 0.84</td>
<td>8.22 ± 0.69</td>
<td>9.54 ± 0.68</td>
</tr>
<tr>
<td></td>
<td>3.34 ± 0.35</td>
<td>4.84 ± 0.81</td>
<td>6.49 ± 0.99</td>
<td>8.35 ± 1.36</td>
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<tr>
<td></td>
<td>3.78 ± 0.12</td>
<td>5.73 ± 0.12</td>
<td>7.023 ± 0.68</td>
<td>9.64 ± 1.02</td>
</tr>
<tr>
<td></td>
<td>2.90 ± 0.90</td>
<td>5.79 ± 0.79</td>
<td>8.29 ± 0.29</td>
<td>10.69 ± 0.48</td>
</tr>
</tbody>
</table>

Note: SCS-4°C, static cold storage on ice at 4°C. H-21°C, perfusion with Hemopure at 21°C. H200nM-21°C, perfusion with Hemopure + 200 nM Ap39 at 21°C. H1µM-21°C, perfusion with Hemopure + 1µM Ap39 at 21°C.
Subnormothermic perfusion with Hemopure + 200nM AP39 at 21°C improves tissue oxygenation during blood-free preservation

To evaluate the impact of the treatments on organ perfusion, tissue oxygenation was measured hourly during preservation and reperfusion (Fig. 4-5, Table 3). During preservation, the H200nM-21°C group exhibited higher tissue oxygenation at all timepoints relative to the H-21°C and H1µM-21°C groups. Statistically, the H200nM-21°C group exhibited significantly higher tissue oxygenation than the H1µM-21°C group at the 1h and 2h timepoints during preservation (Fig. 4-5A). During reperfusion, the H-21°C group exhibited the highest tissue oxygenation, followed by the H200nM-21°C group. The SCS-4°C and the H1µM-21°C groups exhibited similar tissue oxygenation trends and the levels for both groups were lower than the other two groups at the 2h, 3h and 4h timepoints (Fig. 4-5B). Interestingly, the tissue oxygenation levels for each group stayed relatively consistent throughout the preservation period (Fig. 4-5A), while the levels gradually increased for each group throughout the reperfusion period (Fig. 4-5B).
Figure 4-5 Mean tissue oxygenation during blood-free preservation and reperfusion.

(A) Tissue oxygenation (%) during 4h of blood-free preservation with Hemopure (H-21°C, n=5), Hemopure + 200nM AP39 (H200nM-21°C, n=5) or Hemopure + 1µM AP39 (H1µM-21°C, n=4). No values were recorded for the static cold storage (SCS-4°C, n=6) group as the kidneys were on ice. (B) Tissue oxygenation (%) during 4h of blood-free reperfusion with Hemopure and metabolic support at 37°C. The InSpectra StO2 Spot Check Tissue Perfusion Monitor was unable to detect tissue oxygenation for one pair of kidneys (n=2) in the H1µM-21°C group. Individual points on each graph represent the
mean tissue oxygenation (%) level at a specific timepoint for individual kidneys within a single treatment group. SEM values are not presented on the graphs to allow for clear visualization of trends without overlapping error bars. Mean ± SEM are listed in Table 3.

After a Geisser-Greenhouse correction, values were compared using repeated measures two-way ANOVA followed by Tukey’s post-hoc test. *, p<0.05 for H200nM-21°C compared to H1µM-21°C.

Table 3- Mean tissue oxygenation (%) ± SEM (%) during blood-free preservation and reperfusion.

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Mean Tissue Oxygenation (%) ± SEM (%)</th>
<th>Preservation Timepoint</th>
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<td></td>
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<td>1h</td>
<td>2h</td>
<td>3h</td>
<td>4h</td>
</tr>
<tr>
<td>SCS-4°C (n=6)</td>
<td>No data collected while kidneys were on ice</td>
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<tr>
<td>H-21°C (n = 5)</td>
<td>65.37 ± 1.65</td>
<td>63.86 ± 1.94</td>
<td>63.11 ± 2.04</td>
<td>63.06 ± 2.02</td>
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<tr>
<td>H200nM-21°C (n = 5)</td>
<td><strong>68.04 ± 1.02</strong></td>
<td><strong>69.29 ± 1.76</strong></td>
<td>67.62 ± 3.36</td>
<td>68.43 ± 4.10</td>
<td></td>
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<tr>
<td>H1µM-21°C (n = 4)</td>
<td>61.93 ± 1.42</td>
<td><strong>60.04 ± 1.78</strong></td>
<td>60.75± 2.02</td>
<td>61.29 ± 1.21</td>
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<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Reperfusion Timepoint</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1h</td>
<td>2h</td>
<td>3h</td>
<td>4h</td>
</tr>
<tr>
<td>SCS-4°C (n = 6)</td>
<td>67.12 ± 0.88</td>
<td>68.12 ± 0.47</td>
<td>73.29 ± 0.40</td>
<td>77.57 ± 0.24</td>
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<tr>
<td>H-21°C (n = 5)</td>
<td>68.31 ± 6.20</td>
<td>77.29 ± 6.15</td>
<td>81.80 ± 5.55</td>
<td>84.71 ± 5.52</td>
</tr>
<tr>
<td>H200nM-21°C (n = 5)</td>
<td>66.35 ± 1.91</td>
<td>72.90 ± 2.34</td>
<td>77.73 ± 2.62</td>
<td>79.00 ± 2.44</td>
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<tr>
<td>H1µM-21°C (n = 4)</td>
<td>62.61 ± 0.82</td>
<td>68.46 ± 0.89</td>
<td>72.46 ± 0.91</td>
<td>76.57 ± 2.91</td>
</tr>
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**Note**- Bolded values in each column were significantly different relative to each other (p<0.05) as per repeated measures Two-way ANOVA and Tukey’s post-hoc test after a Geisser-Greenhouse correction. SCS-4°C, static cold storage on ice at 4°C. H-21°C, perfusion with Hemopure at 21°C. H200nM-21°C, perfusion with Hemopure + 200 nM Ap39 at 21°C. H1µM-21°C, perfusion with Hemopure + 1µM Ap39 at 21°C.
Subnormothermic perfusion with Hemopure + 200nM AP39 at 21°C improves kidney function during blood-free preservation and reperfusion

To evaluate kidney function, urine output was measured during both halves of the experiment and urinalysis was performed (Fig. 6). During preservation, the H200nM-21°C group exhibited significantly higher urine output than the H-21°C and H1µM-21°C groups (Fig. 4-6A). This trend continued during reperfusion as the H200nM-21°C group exhibited significantly higher urine output than the SCS-4°C, H-21°C and H1µM-21°C groups (Fig. 4-6B). Since no urine was collected for the SCS-4°C group during preservation as the kidneys were on ice, urine samples collected at the 1h and 4h timepoints during reperfusion were analyzed to compare all four groups. Samples collected from the H200nM-21°C group at the 1h timepoint exhibited lower urine protein levels than all the other groups; however, only the difference between the H-21°C and the H200nM-21°C group was statistically significant (Fig. 4-6C). This trend continued as the samples collected from the H200nM-21°C group at the 4h timepoint also exhibited lower urine protein levels than all the other groups. However, no statistically significant differences were found between urine protein levels at the 4h timepoint (Fig. 4-6D).
Figure 4-6 Urine parameters during blood-free preservation and reperfusion. (A) Total urine output (mL) during 4h of blood-free preservation with Hemopure (H-21°C, n=5), Hemopure + 200nM AP39 (H200nM-21°C, n=5) or Hemopure + 1µM AP39 (H1µM-21°C, n=6). No values were recorded for the static cold storage (SCS-4°C, n=6) group as the kidneys were on ice. (B) Total urine output (mL) during 4h of blood-free reperfusion with Hemopure and metabolic support at 37°C. (C) Urine protein (g/L) levels in urine samples collected 1h after the start of reperfusion. (D) Urine protein (g/L) levels
in urine samples collected in the final hour (4h) of reperfusion. An outlier was excluded from the H1µM-21°C group in (D). Individual points on each graph reflect values for individual kidneys and lines represent the mean ± SEM. Values were compared using one-way ANOVA followed by Tukey’s post-hoc test. *, p<0.05. **, p<0.01. ***, p<0.001. ****, p<0.0001.

**Subnormothermic perfusion with Hemopure + 200nM AP39 at 21°C reduces apoptotic kidney injury following blood-free preservation and reperfusion**

To evaluate apoptotic tissue injury, kidney sections were stained with TUNEL (Fig. 4-7A) and scored by a blinded renal pathologist (Fig. 4-7B). The H200nM-21°C group received lower TUNEL scores than all the other groups, which indicates that this group exhibited the lowest apoptotic injury. Statistically, the TUNEL scores of the H200nM-21°C group were significantly lower than the scores of the H-21°C and H1µM-21°C groups. Although the TUNEL scores of the H200nM-21°C group were also lower than the scores of the SCS-4°C group, the difference was not statistically significant (Fig. 4-7B).

To evaluate acute tubular necrosis (ATN), kidney sections were stained with H&E (Fig. 4-8A) and scored by a blinded renal pathologist (Fig. 4-8B). The SCS-4°C, H-21°C and H200nM-21°C groups received similar ATN scores. However, the ATN scores of the H1µM-21°C group were significantly higher than the scores of the H-21°C and H200nM-21°C groups, which indicates that this group exhibited more severe ATN. Although the ATN scores of the H1µM-21°C group were also higher than the scores of the SCS-4°C group, the difference was not statistically significant (Fig. 4-8B).
Figure 4-7 Apoptotic tissue injury following blood-free preservation and reperfusion.

(A) Representative TUNEL images of formalin-fixed kidney sections after 4h of blood-free preservation treatment and 4h of blood-free reperfusion. Images were taken at 10x magnification (scale bar = 100 µm). Black arrows represent apoptotic cells. (B) TUNEL scores assigned by a blinded renal pathologist (1= <11%, 2= 11-24%, 3= 25-45%, 4= 46-75%, 5= >75%). Each individual data point represents the score assigned to one porcine kidney sample. Lines represent mean ± SEM. Values were compared using one-way ANOVA followed by Tukey’s post-hoc test. *, p<0.05. **, p<0.01. Treatment groups: SCS-4°C, static cold storage on ice at 4°C (n=6). H-21°C, perfusion with Hemopure at 21°C (n=5). H200nM-21°C, perfusion with Hemopure + 200nM AP39 at 21°C (n=5). H1µM-21°C, perfusion with Hemopure + 1µM AP39 at 21°C (n=6).
Figure 4-8 Acute tubular necrosis following blood-free preservation and reperfusion.

(A) Representative H&E images of formalin-fixed kidney sections after 4h of blood-free preservation treatment and 4h of blood-free reperfusion. Images were taken at 10x magnification (scale bar = 100 µm). Black arrows indicate areas of acute tubular injury, including tubular cell sloughing, epithelial flattening and tubular dilation. (B) Acute tubular necrosis (ATN) scores assigned by a blinded renal pathologist (1= <11%, 2= 11-24%, 3= 25-45%, 4= 46-75%, 5= >75%). Each individual data point represents the score assigned to one porcine kidney sample. Lines represent mean ± SEM. Values were compared using one-way ANOVA followed by Tukey’s post-hoc test. *, p<0.05. Treatment groups: SCS-4°C, static cold storage on ice at 4°C (n=6). H-21°C, perfusion with Hemopure at 21°C (n=5). H200nM-21°C, perfusion with Hemopure + 200nM AP39 at 21°C (n=5). H1µM-21°C, perfusion with Hemopure + 1µM AP39 at 21°C (n=6).
4.5 Discussion

This study establishes a novel blood-free model of *ex vivo* kidney preservation and reperfusion using Hemopure, a hemoglobin-based oxygen carrier that serves as a blood substitute. Using this model, we show that subnормothermic perfusion of DCD pig kidneys at 21°C with AP39-supplemented Hemopure improves graft function and reduces tissue injury compared to static cold storage and subnормothermic perfusion with Hemopure alone.

The primary finding of this study is that the H200nM-21°C group displayed significantly improved or comparable outcomes relative to the SCS-4°C group, which reflects the clinical standard of care for kidney preservation. Importantly, the H200nM-21°C group exhibited significantly higher urine output than the SCS-4°C group during reperfusion. The immediacy of post-operative urine output is a critical renal transplant outcome as it determines whether dialysis is needed to address delayed graft function. In 2018, Hosgood et al. successfully transplanted declined human kidneys after assessing several parameters, including urine output, during 1h of *ex vivo* normothermic perfusion.29 The five kidneys that were transplanted based on their criteria had higher urine outputs than those that were not transplanted and only one kidney exhibited delayed graft function. Although additional research is needed to validate their method, their findings suggest that *ex vivo* urine output can be used to determine if a kidney is suitable for transplant. While we have yet to evaluate our novel preservation approach using declined human kidneys, the difference in urine output observed in this study has promising implications.

In addition to showing that our novel approach matches the clinical standard of care, this study strengthens the evidence supporting the use of 200 nM AP39 in kidney preservation. We have previously shown that prolonged SCS in UW + 200 nM AP39
improves recipient outcomes in an *in vivo* model of murine renal transplantation.\textsuperscript{11} Additionally, this dose has shown efficacy in our recent studies on the use of AP39 in subnormothermic kidney preservation.\textsuperscript{20,27} From a mechanistic standpoint, our *in vitro* research has shown that 200 nM AP39 preserves mitochondrial membrane potential along with reducing apoptosis and the production of reactive oxygen species.\textsuperscript{11} Furthermore, the RNA sequencing analysis of DCD pig kidneys preserved with AP39-supplemented blood in our previous study implicated the downregulation of pro-apoptotic and hypoxia response genes as potential mechanisms underlying the protective effects of AP39.\textsuperscript{20} The inclusion of the H1µM-21°C group in this study is our first attempt at using a higher dose of AP39 in a mammalian model. The 1 µM dose was chosen based on its efficacy in a frostbite model (unpublished). Seeing as the H1µM-21°C group exhibited significantly lower urine output and significantly higher tissue injury than the H200nM-21°C group, this study further supports the use of a low dose of (200 nM) AP39. Interestingly, the differences in the outcomes of the H-21°C and SCS-4°C groups do not match expectations and showed comparable outcomes. A previous study conducted at our center showed that subnormothermic perfusion with Hemopure significantly improved urine output and reduced tissue injury compared to SCS.\textsuperscript{19}

One of the focal points of this study is our novel blood-free model of preservation and reperfusion. While Hemopure has been used for normothermic and subnormothermic kidney preservation before,\textsuperscript{19,20,26} we are the first to use it consecutively for subnormothermic preservation at 21°C and reperfusion at 37°C. With the efficacy of AP39-supplemented Hemopure during preservation, we have circumvented a major roadblock to translation – the acquisition of human blood for DCD kidney preservation. Additionally,
we used Hemopure for reperfusion to establish a completely blood-free platform for evaluating targeted therapies. While no standard perfusate composition or perfusion protocol exists, the supplements added to the Hemopure/PlasmaLyte mixture in this study align with those used in previous studies reviewed by Elliot et al.\textsuperscript{30}

On the other hand, our novel perfusion model has several limitations. Although we used Hemopure to establish a blood-free model of preservation and reperfusion, the lack of white blood cells in our perfusate largely excludes the inflammatory component of reperfusion with blood. Additionally, the pigmented nature of Hemopure, due to the hemoglobin, prevented the use of colorimetric and fluorescent assays to detect inflammatory markers in the perfusate. Furthermore, pairs of kidneys were connected to the same circuit and perfused using a shared Hemopure reservoir due to the limitation of having only one organ perfusion pump. Although we collected urine output separately for each kidney, the perfusate parameter readings were less robust, as only one reading was obtained due to the perfusate being shared by pairs of kidneys. Moreover, we were unable to detect perfusate parameters (pO\textsubscript{2}, pH and Lactate) with our iSTAT analysis platform since it was designed for use with blood (not Hemopure) at normothermic temperatures rather than subnormothermic temperatures. Our attempt to use the IDEXX analysis platform to detect perfusate levels during preservation also failed. Moreover, our study lacks a number of controls such as cold or subnormothermic perfusion group without Hemopure. This is because the high cost (CAD 5000/pig experiment) limits the number of groups we could evaluate. Thus, we designed our study around the premise of comparing the outcomes of our novel approach to that of SCS, which is the clinical standard of care, and perfusion with Hemopure alone, which is the control for the effects of temperature and
oxygenated perfusion. Lastly, while perfusion was kept at constant pressure by adjusting the flow, we are unable to report flow data.

As mentioned above, this study advances our previous findings that showed the efficacy of preserving DCD pig kidneys using subnormothermic perfusion at 21°C with AP39-supplemented blood. There is some overlap in the strengths and limitations of both studies due to the overlap in methodology. Per our previous study, we induced warm ischemic injury by clamping the renal pedicle. This approach mimics an extreme clinical DCD scenario where no oxygen is supplied to the kidneys due to a complete cessation of blood flow. Thus, the positive outcomes observed would likely be heightened in real clinical DCD scenarios, where there is a gradual reduction in blood flow as the donor’s heart stops pumping blood. Additionally, the 4h duration of the preservation and reperfusion periods is relatively short. However, this was deemed appropriate for establishing a novel model and for facilitating comparisons to our previous study.

While no mechanistic advances were made, our methodology has improved with the addition of new approaches to evaluate urine protein levels and tissue oxygenation in real time. It is important to note that whereas this study reports a novel approach for subnormothermic kidney preservation with H₂S-supplemented blood substitute, future ex vivo perfusion studies using declined human kidneys and longer perfusion times are needed to support our findings. Additionally, more in vitro research is required to establish the exact mechanisms underlying the protective effects of AP39. Furthermore, research with clinically approved H₂S donors and in vivo models of renal transplantation is also needed to facilitate the clinical translation of our novel approach.
In conclusion, this study demonstrates that subnormothermic perfusion at 21 °C with AP39-supplemented Hemopure improves *ex vivo* DCD porcine renal graft outcomes. Our findings contribute to the expanding body of literature that supports the use of H₂S and subnormothermic preservation to improve kidney outcomes following transplantation. Additionally, we have established a novel blood-free model of *ex vivo* kidney preservation and reperfusion that will be useful for evaluating other therapeutics, such as other gasotransmitters and gene therapies.
4.6 References


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Chapter 5

5 Discussion and Conclusion
5.1 Discussion

Renal transplantation is the preferred treatment for patients with end-stage kidney disease.\textsuperscript{1} However, the ischemia-reperfusion injury (IRI) that occurs during organ procurement and reimplantation limits renal graft longevity. Optimal organ preservation is critical for mitigating IRI. Currently, cold/hypothermic temperatures (~4°C) are used for preservation; however, this approach can exacerbate graft outcomes.\textsuperscript{2} Grafts that are affected by severe IRI, such as those obtained from donors after cardiac death (DCD), are being used more frequently to meet the rising demand for kidney transplants and these organs are particularly susceptible to cold ischemic injury.\textsuperscript{3,4} In recent years, the body of literature evaluating therapeutics and novel approaches that could improve renal graft preservation has grown exponentially. Our group has previously shown that supplementing cold preservation solutions with various hydrogen sulfide (H\textsubscript{2}S) donors, including AP39, improves renal cell viability and \textit{in vivo} kidney transplant outcomes.\textsuperscript{5,6} Additionally, substantial research has shown that normothermic (36-37°C) and subnormothermic preservation (20-34°C) improves DCD kidney outcomes compared to cold preservation.\textsuperscript{7-9} In 2014, Hoyer et al. showed that oxygenated subnormothermic perfusion of DCD porcine kidneys at 20°C improved outcomes compared to static cold storage on ice and oxygenated hypothermic perfusion.\textsuperscript{10} The overarching aim of this thesis is to evaluate the merit of subnormothermic preservation with H\textsubscript{2}S donor AP39. We systematically used \textit{in vitro} and \textit{ex vivo} models of kidney preservation to demonstrate the efficacy of subnormothermic preservation at 21°C with AP39 compared to static cold storage, the clinical standard of care, and subnormothermic preservation without AP39. In this chapter, we summarize and discuss our main findings and contextualize their
importance in the fields of kidney preservation and \( \text{H}_2\text{S} \) therapeutic research. Additionally, potential mechanisms of action for AP39 during renal IRI, the strengths and limitations of this thesis, and future directions for basic sciences and translational research are also discussed.

5.1.1 Adding AP39 to preservation solutions, blood and blood substitutes improves their suitability for subnormothermic kidney preservation

A key finding of this thesis is that adding AP39 to preservation solutions, blood and blood substitutes improves their suitability for subnormothermic kidney preservation. This is remarkable considering the diverse compositions of these solutions. First, AP39 was added to University of Wisconsin (UW) solution in our *in vitro* model (Chapter 2). UW solution is an acellular solution that mimics intracellular ionic concentrations and contains other compounds such as adenosine. Clinically, it is used for the cold preservation of livers, kidneys, and pancreas.\(^{11}\) Adding AP39 to UW solution improved renal cell viability following hypoxia at 10°C, 21°C and 37°C in a dose-dependent manner. Additionally, DCD pig kidneys stored in UW+200 nM AP39 at 21°C exhibited lower necrosis than kidneys stored on ice in UW solution alone. Thus, AP39 made UW solution, which was designed to mitigate edema at 4°C, viable for subnormothermic preservation at 21°C. Next, adding 200 nM AP39 to whole pig blood during subnormothermic perfusion at 21°C improved DCD pig kidney outcomes (Chapter 3). Compared to an acellular solution like UW solution, whole blood contains red blood cells and leukocytes. It is possible that \( \text{H}_2\text{S} \) may impact these cells in addition to the kidney,\(^ {12}\) which might be contributing to the improved outcomes of the treatment group (discussed in section 5.3). Furthermore, adding 200 nM AP39 to Hemopure during subnormothermic perfusion at 21°C also improved
DCD pig kidney outcomes (Chapter 4). Hemopure is a hemoglobin-based oxygen carrier that is used as a blood substitute. Like UW solution, Hemopure is an acellular solution as it is composed of chemically stabilized, cross-linked bovine hemoglobin. Its oxygen-carrying capacity rivals that of human blood and it eliminates the complexities associated with acquiring human blood (global shortage) and using it for ex vivo kidney perfusion (screening for pathogens, thrombosis, etc.). For these reasons, Hemopure is an ideal perfusate for the clinical translation of oxygenated subnormothermic preservation. Overall, the consistent improvement in renal outcomes observed after adding 200 nM AP39 to UW solution, whole pig blood and Hemopure during subnormothermic preservation strengthens the validity of our novel approach and our AP39 dose.

An important aspect to consider with all the experimental models used is the lack of nutrients during the subnormothermic preservation period. Several studies on normothermic kidney preservation have supplemented the perfusate with nutrients such as glucose, amino acids, insulin, etc. in the form of drips. However, we chose not to do so for two important reasons. Supplementing UW solution, pig blood and Hemopure with only AP39 allowed us to evaluate the direct impact of the treatment on renal cell viability and kidney outcomes during subnormothermic preservation without any confounding variables. Additionally, the improvement of renal outcomes with AP39 treatment in the absence of nutrients is advantageous for clinical translation. Current kidney preservation methods, static cold storage on ice and hypothermic perfusion pumps, rely on the use of acellular solutions and they do not have the capacity to provide nutrients continuously. If we consider the path of least resistance towards clinical translation, the performance of AP39-supplemented Hemopure at 21°C is optimal. In addition to being an acellular
perfusate, AP39-supplemented Hemopure improves outcomes without the addition of nutrients. Thus, implementing this approach clinically would only require modifications to existing hypothermic perfusion pumps to allow temperature changes and oxygenation. While these changes would require some innovation, this avenue is much more feasible than trying to create a new apparatus that would allow temperature changes and support nutrient drips without compromising on portability, which is critical for DCD organ procurement.

One of the focal points of this thesis is the novel blood-free model of preservation and reperfusion that was developed using Hemopure (Chapter 4). While Hemopure has been used for normothermic and subnormothermic kidney preservation before, we are the first to use it consecutively for subnormothermic preservation at 21°C and reperfusion at 37°C. As discussed above, the efficacy of AP39-supplemented Hemopure is promising because it allows us to circumvent the challenges associated with the use of blood for ex vivo kidney preservation. During reperfusion, Hemopure was supplemented with a diuretic (mannitol) along with dextrose and insulin drips to mimic the clinical post-transplant milieu. Our use of supplements aligns with previous studies reviewed by Elliot et al. and the consistent urine outputs observed within each group during reperfusion further supports our novel protocol. Notably, perfusate lactate levels increased during reperfusion due to the absence of hepatic metabolism to convert lactate to glucose and additional measures would be needed to correct this finding in future studies. Nonetheless, our innovative blood-free platform can be readily used for evaluating novel therapeutics and conducting drug repositioning studies.
Overall, the findings presented in this thesis show that adding AP39 to preservation solutions, blood and blood substitutes improves their suitability for subnormothermic kidney preservation. The consistent efficacy observed at the preservation temperature (21°C) with the addition of 200 nM AP39 to these diverse solutions strengthens the validity of our novel approach and our treatment dose. Additionally, AP39-supplemented Hemopure is particularly suitable for the clinical translation of oxygenated subnormothermic perfusion as it could be implemented with feasible changes to existing kidney perfusion pumps since it is less viscous than blood. Furthermore, due to the innovation needed to advance our research, we established a novel blood-free model of preservation and reperfusion that will be useful for the evaluation of other therapeutics in the context of kidney preservation.

5.1.2 Subnormothermic preservation with AP39 improves DCD renal graft outcomes compared to static cold storage, the clinical standard of care

The main finding of this thesis is that subnormothermic preservation with AP39 leads to improved or comparable outcomes relative to static cold storage, the clinical standard of care. In this section, the results from each experimental model are discussed sequentially to substantiate this conclusion. First, an in vitro model of renal IRI was used to evaluate the efficacy of AP39-supplemented UW solution during cold (10°C), subnormothermic (21°C), and normothermic (37°C) preservation (Chapter 2). Supplementing UW solution with AP39 increased renal cell viability in a concentration-dependent manner at each temperature. Since UW solution was designed for cold preservation, the highest renal cell viability was observed following cold preservation and
the lowest renal cell viability was observed following normothermic preservation. However, when outcomes following cold and subnormothermic preservation were compared, it was found that supplementing UW solution with AP39 reduced the gap in renal cell viability between the two conditions in a concentration-dependent manner. This led to the *ex vivo* model where DCD pig kidneys were treated to 24h of static cold storage on ice (4°C) in UW solution or static subnormothermic storage (21°C) in UW solution with or without AP39. Importantly, static subnormothermic storage in UW + 200 nM AP39 significantly reduced tissue necrosis compared to static cold storage. However, 4h of this approach followed by 4h of *ex vivo* reperfusion using autologous pig blood (unpublished, model from Chapter 3) led to a drastic reduction in oxygenation and darkening of the kidneys was observed (indicative of thrombosis). By this point, our focus had shifted towards evaluating AP39 in perfusion models (Chapters 3 and 4) since oxygenated perfusion is the norm in the literature on alternatives to cold preservation. However, the findings mentioned above may have been a result of the intracellular-type ion composition of UW solution rather than the approach of using AP39-supplemented preservation solution for static subnormothermic storage. Several studies have reported that extracellular-type solutions are more effective for static subnormothermic storage (22-23°C) of kidney grafts than UW solution.\textsuperscript{18,19} Thus, future research investigating the effects of supplementing extracellular-type solutions with H\textsubscript{2}S could be useful for establishing a static subnormothermic storage protocol for kidney grafts.

While static subnormothermic storage would be easier to translate to clinical practice than subnormothermic perfusion, it would not address the increase in metabolic demand that occurs within the kidney at 21°C compared to 4°C. This rise in metabolic
demand is a primary factor underlying the use of oxygenated perfusion by researchers who are investigating novel kidney preservation protocols to replace cold storage. Most of the research conducted in this regard is focused on normothermic perfusion (37°C), which has been shown to improve renal graft outcomes when used after cold preservation or in place of it. The emergence of subnormothermic preservation is more convoluted. In the early 2000s, a group based in the Netherlands showed that a period of perfusion at 32-34°C with metabolic support following prolonged static cold storage improved the outcomes of pig and declined human kidneys. This approach was never directly compared to cold storage and the research was later abandoned. In 2014, two independent studies reported that perfusing pig kidneys and declined human livers with acellular solutions at 20-21°C improved graft outcomes. Around this time, temperatures below normothermic (36-37°C) levels were referred to as ‘subnormothermic’; however, the lower boundary of this range was unknown. A few years later, our centre compared the effect of perfusing DCD pig kidneys with autologous blood at 15°C, 22°C and 37°C to static cold storage. It was found that subnormothermic perfusion at 22°C improved urine output and reduced tissue injury compared to static cold storage and normothermic preservation. While this research was published in 2019, the preliminary findings of that study along with our in vitro findings (Chapter 2) shaped the ex vivo models used to evaluate AP39 (Chapters 3 and 4). Additionally, the thrombosis observed during kidney perfusion at 15°C suggests that this temperature may be too low for blood-based oxygenated perfusion. Based on the evidence summarized above, temperatures in the range of 20-34°C are referred to as ‘subnormothermic’ in this thesis. However, it is possible that acellular solutions may be effective for oxygenated kidney perfusion at temperatures below 20°C since acellular
solutions have been used for oxygenated cold preservation at 4°C. Although the ‘subnormothermic’ designation is somewhat arbitrary since certain temperatures have not been tested, it is clear that preservation at 20-23°C improves renal outcomes compared to cold storage.

Considering the complex milieu described above, it is remarkable that subnormothermic preservation with 200nM AP39 at 21°C improved renal graft outcomes relative to static cold storage in both of our *ex vivo* kidney perfusion models (Chapters 3 and 4). The most important outcome to consider is urine output. Perfusing DCD pig kidneys with AP39-supplemented blood and AP39-supplemented Hemopure at 21°C significantly improved urine output compared to static cold storage, the clinical standard of care. The immediacy of post-operative urine output is an important kidney transplant outcome as it determines whether dialysis is needed to address delayed graft function. In 2018, Hosgood et al. successfully transplanted declined human kidneys after assessing several parameters, including urine output, during 1h of *ex vivo* normothermic perfusion. The five kidneys that were transplanted based on the study’s criteria had higher urine outputs than those that were not transplanted (based on clinical findings, suboptimal biopsies and poor HMP parameters) and only one kidney exhibited delayed graft function. Although additional research is needed to validate their method, their findings suggest that *ex vivo* urine output can be used to determine if a kidney is suitable for transplant. While our novel preservation approach has not been evaluated using declined human kidneys, the difference in urine output observed in this study has promising implications.

Beyond urine output, kidneys perfused with AP39-supplemented blood at 21°C exhibited lower levels of apoptosis, similar levels of acute tubular necrosis and pro-survival
gene expression patterns compared to the kidneys treated with static cold storage (Chapter 3). Furthermore, kidneys perfused with Hemopure+200 nM AP39 at 21°C exhibited lower levels of apoptosis and similar levels of acute tubular necrosis compared to kidneys treated with static cold storage (Chapter 4). These findings match the results of our previous study comparing DCD pig kidney outcomes after static cold storage or subnormothermic preservation with Hemopure or blood.\textsuperscript{16} The Hemopure+200 nM AP39 group also displayed higher tissue oxygenation and perfusate pO\textsubscript{2} levels along with lower urine protein levels compared to the static cold storage group (statistically comparable). Importantly, the poorer outcomes of the Hemopure+1µM group compared to both of these groups further substantiates the use of 200 nM AP39 in future studies.

Overall, the findings presented in this thesis show that subnormothermic kidney preservation with AP39 leads to improved or comparable outcomes relative to static cold storage, the clinical standard of care. Although more research is needed to substantiate our novel approach, the consistent efficacy of our novel approach in all of the porcine models used demonstrates its strong clinical potential. Subnormothermic preservation at 21°C is likely effective because it avoids the damage caused by cold storage while providing a phase of lower metabolic demand than normothermic preservation. Additionally, supplementing perfusion solutions with AP39 mitigates some of the negative effects of the higher metabolic demand that occurs at 21°C compared to 4°C (discussed in section 5.3). Thus, subnormothermic preservation at 21°C with exogenous H\textsubscript{2}S therapy is a promising alternative to cold storage for optimal kidney preservation.
5.1.3 Subnormothermic preservation with AP39 reduces renal apoptosis and induces pro-survival gene expression

Our results consistently show that subnormothermic preservation with AP39 reduces apoptosis compared to subnormothermic preservation without AP39 and static cold storage. In our in vitro model of renal IRI (Chapter 2), treatment with AP39-supplemented UW solution reduced renal cell apoptosis during subnormothermic storage compared to UW solution. In both the ex vivo models (Chapters 3 and 4), subnormothermic perfusion with AP39-supplemented blood and AP39-supplemented Hemopure reduced apoptosis compared to subnormothermic perfusion without AP39 and static cold storage, the clinical standard of care. These results align with previous studies that have investigated the effect of AP39 on renal injury. Ahmad et. al, have previously shown that pretreatment of rats with AP39 reduced apoptosis caused by acute warm renal IRI (37°C) resulting from 30 min of renal vascular clamping followed by 6h of reperfusion. Additionally, our group has previously shown that cold preservation (12°C) of renal epithelial cells with AP39-supplemented UW solution increased cell viability and reduced apoptosis compared to preservation with UW solution alone. While previous studies have reported that AP39 exerts an anti-apoptotic effect during cold and normothermic renal IRI, we are the first to report that this also occurs during subnormothermic kidney preservation at 21°C.

The mechanism(s) underlying the reduction in apoptosis following AP39 administration have not been fully elucidated. However, several studies have suggested potential targets. Using an in vitro model of cold myocardial IRI, our collaborators have reported that AP39 downregulated BAX, a pro-apoptotic factor, at the protein and mRNA level. Vitvitsky et. al have shown that the reduction of cytochrome c, an initiator of
apoptosis, by H$_2$S donors (including AP39) was critical for potentiating sulfide signaling through persulfdidation, which diminished the pro-apoptotic activity of caspase 9.$^{28}$ Our RNAseq results (Chapter 3) showed that subnormothermic perfusion of DCD pig kidneys with AP39-supplemented blood downregulated transcriptional regulators of the intrinsic apoptotic pathway, BCL10,$^{29,30}$ and endoplasmic reticulum stress-induced apoptosis pathway, DDIT3,$^{31}$ compared to static cold storage. While more research is needed to confirm the exact mechanism(s) underlying the anti-apoptotic effects of AP39, the implication of BAX, cytochrome c and BCL10 suggests that the intrinsic apoptotic pathway might be a key target.

Although the direct impact of AP39 on apoptosis is of primary interest, it is important to consider indirect targets that may be contributing to the observed reduction in apoptosis. Due to its mitochondria-targeted nature, AP39 modulates mitochondrial processes that may contribute to reducing apoptosis. Our group has previously shown that cold preservation (12°C) of renal epithelial cells with AP39-supplemented UW solution preserved mitochondrial membrane potential and reduced ROS production compared to preservation with UW solution alone.$^6$ The same trends were observed by Nishime et al. who evaluated mitochondrial membrane potential and ROS production in islet cells isolated from porcine pancreas that were preserved in cold AP39-supplemented UW solution.$^{32}$ Mitochondrial membrane potential is critical for regulating the opening of the mitochondrial permeability transition pore (MPTP), which leads to the release of pro-apoptotic factors, such as cytochrome c, that potentiate apoptosis by engaging caspases.$^{33}$ It is unknown whether AP39 modulates MPTP opening in renal IRI. However, Karwi et al. have shown that AP39 inhibited MPTP opening in rat hearts following myocardial IRI.$^{34}$
It is likely that this occurs independent of Cyclophilin-D, a modulator of the MPTP, as AP39 has been shown to increase mitochondrial Ca\(^{2+}\) retention, which discourages MPTP opening, and reduce cardiac infarct size in mice lacking Cyclophilin-D.\(^{35}\) The effects of AP39 on the targets and processes discussed above are summarized in Fig. 5-1. Evidently, the effects of AP39 on mitochondrial bioenergetics need to be studied further to fully elucidate the mechanisms underlying its protective effects, especially in renal IRI.

**Figure 5-1** A summary of the various mitochondrial targets and cellular processes that AP39 modulates to reduce apoptosis. During IRI, ROS generation and accumulation of Ca\(^{2+}\) in the mitochondria leads to the opening of the MPTP and the release of cytochrome c, which leads to the initiation of apoptosis. AP39 has been shown to exert diverse effects that have anti-apoptotic consequences including reducing ROS generation, increasing Ca\(^{2+}\) retention, inhibiting MPTP opening, downregulating BAX and reducing cytochrome c, which limits apoptosis through potentiating sulfide signaling.
In addition to reducing apoptosis, our RNAseq results (Chapter 3) showed that subnormothermic perfusion of DCD pig kidneys with AP39-supplemented blood (SNTAP) induced other pro-survival gene expression patterns. Compared to subnormothermic perfusion with blood alone (SNT), several genes associated with the HIF1α-mediated hypoxia response, which contributes to acute kidney injury and renal IRI, were downregulated in the SNTAP group. The downregulated genes include metabolic targets of HIF1α, PCK-1 and PDK3, and negative regulators of HIF1α-mediated angiogenesis, EGR1 and RGCC that may help with managing metabolic demand at 21°C and promoting vascularization respectively. While no previous studies have linked AP39 to the HIF1α-mediated hypoxia response, other H2S donors have been shown to improve outcomes in hypoxic conditions through modulating the activation of the HIF1α pathway. If confirmed by future research, these mechanisms could explain why AP39 is so effective in various models of hypoxic/ischemic injury.

Importantly, IL6 was downregulated in the SNTAP group compared to the SNT group. This finding is supported by the reduction/downregulation of IL6 observed after AP39 treatment in the context of cold myocardial IRI, burn injury and airway hyperreactivity. Since IL6 is known to play a critical role in various forms of IRI, including renal IRI and acute kidney injury, investigating the effect of AP39 on IL6 and its downstream targets more directly could be useful to establish the anti-inflammatory effects of AP39. Our RNAseq results also showed the upregulation of MAPK7, a proliferation gene, and NRROS, a negative regulator of ROS production, in the SNTAP group relative to the other groups. If confirmed by future mechanistic analyses, these genes and the pathways associated with them could explain the enhanced cell viability and
reduced ROS production reported by several previous studies on AP39\(^6,27,32\) and our \textit{in vitro} results (Chapter 2).

Since we are the first to investigate the use of AP39 in subnornothermic kidney preservation, substantial future research is needed to establish the exact mechanisms underlying the protective effects of AP39 observed in this context. Three distinct avenues for future research emerge from our findings, namely the effect of AP39 on the intrinsic apoptotic pathway, the HIF1α-mediated hypoxia response and IL6-mediated inflammation. Several methods can be used to evaluate the mechanistic effects of AP39 in the context of subnornothermic preservation. The primary approach would be to genetically knockdown some of the targets identified above, such as BAX or IL6, in a human kidney cell line and evaluate the effect of AP39 treatment on cell viability following hypoxia at 21°C and reoxygenation at 37°C using an \textit{in vitro} model similar to the one used in Chapter 2. Once a target contributing to AP39’s protective effects is identified, cellular pathways involving that target would need to be investigated further and the mechanistic findings would need to be confirmed in tissues from an \textit{in vivo} model of kidney transplantation.

Overall, the findings presented in this thesis show that subnornothermic preservation with AP39 reduces apoptosis and induces pro-survival gene expression. Only a handful of previous studies on the biological applications of AP39 are relevant to the discussion of our results and parallels have been drawn to substantiate our results wherever possible. It is important to note that most of these studies discussed in this section report effective doses in the range of 100 - 400 nM AP39\(^6,27,28,32,44\) and our dose, 200 nM AP39, falls within that range. In addition to comparing our results to the results of previous studies, the novel implications of our RNAseq data have also been highlighted above.
5.2 Strengths and Limitations

The conclusions presented in this thesis are strengthened by several facets of the experimental approaches that we used. The premise of this work revolves on developing an alternative to cold preservation to mitigate the renal graft injury it causes, particularly for DCD kidneys. Therefore, the induction of warm ischemic injury to mimic DCD conditions was a critical factor in developing relevant models. In all three porcine models used (Chapters 2, 3 and 4), the renal pedicle was clamped for 30 or 60 minutes to induce a complete cessation of blood flow. This induces more significant injury than the gradual cessation of blood flow that occurs in a clinical setting as the donor experiences cardiac death. Thus, the results of this study would likely be enhanced in the clinical setting since our approach led to urine output despite the induction of more severe kidney injury. Additionally, the use of 21°C as the ideal temperature for subnormothermic preservation is validated through comparisons with cold (4-10°C) and normothermic (37°C) temperatures in our in vitro model (Chapter 2) and our ex vivo porcine kidney perfusion model. Furthermore, the use of 200 nM AP39 is supported by the improved outcomes observed for this treatment group in all three porcine models relative to the static cold storage group (Chapters 2, 3 and 4) and a treatment group that received a 5x higher dose of AP39 (Chapter 4). Over time, our novel approach has been optimized sequentially by working through the stages of in vitro research before moving on to using several models of static and dynamic ex vivo kidney preservation. Through this process, several major challenges have been overcome, such as the shortage of blood, to transform a novel idea into a viable kidney preservation approach that is effective in pre-clinical models and is pending patent approval.
In order to fully contextualize the findings of this thesis, however, it is important to examine the limitations in addition to considering the strengths. The *ex vivo* porcine kidney perfusion models used to evaluate the efficacy of supplementing blood and Hemopure with AP39 (Chapters 3 and 4) were appropriate considering the novelty of our approach. However, the preservation and reperfusion periods (4h each) were relatively short and the availability of a single pulsatile pump for treating pairs of organs limited the individual analysis of each porcine kidney. Although these limitations somewhat weakened our findings, they facilitated the execution of the vast number of porcine experiments required for this thesis with limited manpower as one person was able to monitor a single pump for the duration of preservation and reperfusion (8h). Despite the clinical relevance of our *ex vivo* porcine kidney perfusion models, evaluating our novel approach using declined human kidneys would have been more indicative of clinical outcomes. Unfortunately, this avenue was limited due to the drop in organ procurement and transplantation during the COVID-19 pandemic. Furthermore, the lack of an *in vivo* transplantation model made it difficult to determine the effects of our novel approach on recipient survival and post-transplant outcomes. While an attempt was made to establish a porcine kidney transplantation model, its success was limited by veterinary challenges and a lack of manpower. Additionally, while my *in vitro* and RNAseq data (Chapters 2 and 3) show the anti-apoptotic and other pro-survival effects of AP39 during subnormothermic preservation, more research is needed to confirm the cellular mechanisms at play. Furthermore, in order to fully understand how AP39 works, the challenges associated with the detecting and quantifying the levels and effects of H₂S (due to its transient nature) need to be overcome, especially in the context of physiological systems.
5.3 Future Directions

Based on the limitations identified above, three clear paths for future research emerge. First, evaluating the effect of *ex vivo* subnormothermic perfusion with AP39 on declined human kidneys, with donor blood and/or Hemopure, would provide invaluable insight on the clinical outcomes of our novel approach. Next, the use of an *in vivo* porcine transplantation model as previously described in the literature would help to establish the safety of our novel approach by evaluating its effect on recipient survival and post-transplant outcomes. In addition, the cellular mechanisms underlying the protective effects of AP39 need to be studied further. Potential targets known to be modulated by AP39 (apoptosis, inflammatory and/or hypoxia response genes) can be knocked down in human kidney cell lines. These cell lines can then be subjected to hypoxia/reoxygenation to mimic renal ischemia-reperfusion injury, with and without AP39 treatment, to determine whether AP39 mitigates the adverse effects of the absence of specific target genes that impact renal cell viability. Additionally, RNAseq or microarray analysis could be conducted on declined human kidneys treated with subnormothermic perfusion with AP39-supplemented blood or Hemopure to further confirm the mechanisms elucidated through *in vitro* research.

While the three avenues listed above are essential for advancing the findings of this thesis, several additional directions for future research may be of interest. The novel *ex vivo* model of blood-free preservation and reperfusion established in this thesis may be useful for drug repositioning studies to evaluate other therapeutics that may improve kidney graft preservation. Future research could also address the downside of using AP39, which is that it has not been approved for clinical use yet. Evaluating a clinically approved
H$_2$S donor, such as sodium thiosulfate, using the models described above may be more advantageous to facilitate the clinical translation of subnormothermic preservation with H$_2$S. Research conducted by our lab (unpublished) shows that supplementing cold preservation solutions with sodium thiosulfate improves renal graft outcomes in a rat model of renal transplantation. These results have also translated to a pilot renal transplantation clinical trial being conducted at our centre. Another factor limiting the translation of subnormothermic preservation with H$_2$S is the lack of portable perfusion devices that allow deviation from cold preservation and support perfusates such as blood or Hemopure. One of our collaborators is working with an industry partner to modify an existing portable perfusion device so that it can support oxygenated subnormothermic perfusion with Hemopure. If such a setup was approved for clinical use after it has been tested using porcine and human kidney grafts, a major barrier to the clinical translation of our approach would be eliminated. Although these changes require a significant financial investment and technical expertise, the possibility of using discarded and marginal kidney grafts to save more lives warrants the undertaking of these efforts. Overall, the research conducted by our group and others is rapidly advancing the clinical translation of subnormothermic kidney preservation and the use of H$_2$S donors in this context.

5.4 Conclusion

The overarching aim of this thesis is to evaluate the merit of subnormothermic kidney preservation at 21°C with H$_2$S donor AP39. Our results showed that adding AP39 to preservation solutions, blood and blood substitutes improved their suitability for subnormothermic kidney preservation. Importantly, we found that subnormothermic
preservation with AP39 improved DCD renal graft outcomes compared to static cold storage, the clinical standard of care. Furthermore, subnormothermic preservation with AP39 reduced renal apoptosis and induced pro-survival gene expression. Through the sequential use of cutting edge kidney preservation models, we transformed a novel yet obscure idea into a viable and effective kidney preservation approach that is pending patent approval (US patent application serial no. 17/127,965). Additional research and innovation are needed to facilitate the clinical implementation of subnormothermic kidney preservation at 21°C with H₂S. Nonetheless, the experimental models and findings presented in this thesis will inform future studies seeking to evaluate novel kidney preservation approaches and advance the clinical use of H₂S therapeutics.

Notably, renal blood flow was stopped completely rather than gradually in our porcine models, which induced more severe warm ischemic injury than the clinical DCD scenario. Thus, the positive impact of subnormothermic preservation with AP39 would likely be enhanced upon clinical translation. While additional research with declined human kidneys and clinically approved H₂S donors is essential for advancing this research, subnormothermic preservation with H₂S shows the potential to enhance the outcomes of marginal kidney grafts from deceased donors with significant warm ischemic injury. Thus, the most important implication of this research is its potential to expand the pool of transplantable kidneys.
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6 Appendices

6.1 Copyright Release for Chapter 1

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- Novel therapeutic strategies for renal graft preservation and their potential impact on the future of clinical transplantation
  - Publication: Current Opinion in Organ Transplantation
  - Publisher: Wolters Kluwer Health, Inc.
  - Date: Aug 1, 2019

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6.2 Copyright Release for Chapter 2

H2S supplementation: A novel method for successful organ preservation at subnormothermic temperatures

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6.4 Animal Ethics Approval

**AUP Number: 2018-090**  
**PI Name: Sener, Alp**  
**AUP Title: Evaluating Subnormothermic Organ Preservation With Hydrogen Sulfide As A Method To Improve Injured Renal Graft Viability.**  
**Official Notification of ACC Approval:** Animal Use Protocol 2018-090 has been approved.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:
   a) Western's Senate MAPPs 7.12, 7.10, and 7.15 [http://www.uwo.ca/univsec/policies_procedures/research.html](http://www.uwo.ca/univsec/policies_procedures/research.html)
   b) University Council on Animal Care Policies and related Animal Care Committee procedures
   c) [http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm](http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm)

2) As per UCAC's Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC. [http://uwo.ca/research/services/animalethics/animal_use_protocols.html](http://uwo.ca/research/services/animalethics/animal_use_protocols.html)

3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
   a) be made familiar with and have direct access to this AUP;
b) complete all required CCAC mandatory training (training@uwo.ca); and
c) be overseen by me to ensure appropriate care and use of animals.

4) As per MAPP 7.15,
a) Practice will align with approved AUP elements;
b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
c) UCAC policies and related ACC procedures will be followed, including but not limited to:
   i) Research Animal Procurement
   ii) Animal Care and Use Records
   iii) Sick Animal Response
   iv) Continuing Care Visits

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets, http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care
Curriculum Vitae – Smriti Juriasingani

EDUCATION

2018 – 2021  Doctor of Philosophy, University of Western Ontario Microbiology & Immunology, Supervisor: Dr. Alp Sener

2017 – 2018  Master of Science, University of Western Ontario Microbiology and Immunology

2013 – 2017  Bachelor of Medical Sciences, University of Western Ontario Honors Specialization in Microbiology & Immunology

HONORS AND AWARDS

2020 – 2021  Ontario Graduate Scholarship

2020  Vanguard First Disclosure Award

2019 – 2020  Ontario Graduate Scholarship

2018  Dr. F W Luney Travel Award

2014 – 2017  Dean’s Honor List

2013 – 2017  Continuing Admission Scholarship

PEER-REVIEWED PUBLICATIONS

Primary Publications

Published


**Accepted/In press**


**Other Publications**


ORAL PRESENTATIONS


POSTER PRESENTATIONS


RESEARCH EXPERIENCE

2016 – 2021 Ph.D. Candidate (previously M.Sc. Candidate & Honors student), University of Western Ontario

2015 – 2016 Research Assistant, University of Western Ontario

TEACHING EXPERIENCE

2019 – 2021 Teaching Assistant, University of Western Ontario

MICROIMM 3620

2021 - 2021 Exam Marker, University of Western Ontario

MICROIMM 3300

2019 – 2020 Exam Proctor, University of Western Ontario

MICROIMM 2500, MICROIMM 4200
SUPERVISORY EXPERIENCE

4th Year Honors Specialization Students:

2019 – 2020  Elena Kum, Lauren Mills
2018 – 2019  Austin Kemp, Katharine Pacoli
2017 – 2018  Max Zhang, Vicky Vo, Kyle Willms

LEADERSHIP/SERVICE EXPERIENCE

2021 – 2021  Microbiology & Immunology (MnI) Representative
Schulich Ethics, Diversity and Inclusion Committee*

2019 – 2021  Graduate Student Representative
MnI Undergraduate Education Committee*

2019 – 2020  University Hospital Graduate Student Representative
MnI Graduate Student Committee*

2019 – 2021  Crisis Support Line Volunteer
ANOVA, London, Ontario

2019 – 2021  VP Advocacy/Journalism (previously Journalist)
Organ Advocacy Initiative*

2018 – 2021  Blog Writer, Communications Team
Bachelor of Medical Sciences Association*

2015 – 2021  Student Coordinator
Volunteers in Progress/Western Peer Leaders*

2015 – 2017  Peer Mentor
Leadership and Academic Mentorship Program*

* denotes organizations associated with the University of Western Ontario