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Protective benefits of sodium thiosulfate-supplemented UW solution against prolonged cold ischemia-reperfusion injury during renal transplantation.

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Abstract

Kidney transplantation is the preferred treatment for patients with end-stage renal disease. However, ischemia-reperfusion injury (IRI) is an inevitable consequence of renal transplantation, with prolonged IRI periods associated with decreased graft survival and function. We have previously demonstrated that the supplementation of University of Wisconsin (UW) organ preservation solution with hydrogen sulfide (H_2S) donor molecules, such as AP39, leads to improved renal graft function and reduced transplantation-associated IRI. However, the approval of these experimental donor drugs for clinical use may be years away. In this study, we investigate the effects of an FDA-approved H_2S donor molecule, sodium thiosulfate (STS), to determine whether STS could mitigate cold renal IRI. In an *in vitro* model of renal IRI, adding STS to serum free media improved cell viability for cold preservation at 4^0C in a dose-dependent manner. Using a syngeneic renal transplantation model, we investigate the effect of adding STS to University of Wisconsin (UW) preservation solution, the clinical standard for preservation solution in static cold storage. Adding 150µM STS to UW solution improves graft survival, urine output and serum creatinine and blood urea nitrogen levels compared to preservation storage with UW solution alone.. Histopathological examination reveals a reduction in apoptosis and acute tubular necrosis along with decreased macrophage and neutrophil infiltration. Additionally, STS-supplemented preservation solution induced mitochondrial biogenesis and reduced renal apoptosis and inflammation gene expression. These data suggest that STS treatment on cold IRI-associated renal injury could represent a novel clinically applicable strategy to minimize the detrimental outcomes of prolonged cold IRI during renal transplantation.

Summary for Lay Audience

Kidney transplantation is inherently associated with ischemia-reperfusion injury (IRI), a biological event that causes tissue damage to the transplanted kidney organ. Current methods to minimize IRI involves preserving the kidney in University of Wisconsin (UW) solution at cold temperatures. However, this kidney preservation method has persisted in its most simple form, as this method has not changed since its conception 60 years ago. Contrastingly, due to its low supply, more kidneys with increased cold IRI times are being accepted and used for transplantation to keep up with its high demand. Sodium thiosulfate (STS) is a clinical drug used to treat patients with calciphylaxis and cyanide poisoning. In the past decade, numerous studies have elucidated the protective effects of STS on hypertension and IRI on other organs, such as liver, heart and brain. Here we sought to determine if STS plays a beneficial role in minimizing cold IRI associated with kidney transplantation. We concluded that preservation of renal grafts in STS-supplemented UW solution protects against prolonged IRI by suppressing apoptotic and inflammatory pathways, and thereby improving graft function and recipient survival. With STS being an FDA-approved compound, its protective benefits could serve as a novel clinically applicable therapeutic strategy to mitigate the harmful outcomes of prolonged IRI during kidney transplantation.

Keywords

Keywords: Sodium thiosulfate, ischemia-reperfusion injury, kidney transplantation, kidney preservation, graft survival

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1 Introduction

1.1 End stage renal disease

End-stage renal disease (ESRD) is the last stage of chronic kidney disease (CKD) after which the kidney is no longer able to support an individual's day-to-day needs [1]. Patients with ESRD exhibit a glomerular filtration rate (GFR) of less than 15 mL/min, a marker of significant kidney damage [1]. In North America, the most common causes of CKD are diabetes, hypertension, and obstructive uropathy [1]. In 2019, ESRD affected over 41,000 Canadians, a 33% increase since 2010 [2], and is therefore a major burden to the healthcare system.

There are currently two forms of treatment for renal failure in ESRD patients: dialysis and renal transplantation. Dialysis is a treatment for kidney failure by removing waste, toxins, and excess fluids to prevent them from building up in one's body while maintaining a safe level of chemicals, such as sodium, potassium, and bicarbonate, in the blood [3]. This process can be done by direct filtering of the blood through a dialysis machine (hemodialysis) or a surgical implanted catheter in the abdomen filled with dialysate to filter blood through the peritoneum via passive diffusion (peritoneal dialysis) [4]. However, long term risks of dialysis include increased infections, anemia, low blood pressure, and cardiovascular complications. The 10-year survival rates of patients with ESRD in Canada undergoing dialysis are less than 15%, which is similar to other chronic illnesses such as cancer [2]. Another treatment for ESRD is kidney transplantation, a surgical procedure to procure a healthy kidney from a living or decreased donor and subsequent engraftment of the kidney into a recipient. Compared to dialysis, kidney transplantation is the treatment of choice for patients with ESRD as it offers improved survival and quality of life. Patients who receive renal transplantation show a 79.8% 5-year survival rate for deceased donors and 92.4% for living donors, compared to a 42.1% survival for ESRD patients treated with dialysis alone [2]. Renal transplantation is also the more cost-effective long-term treatment for ESRD, since the total annual cost per patient

on dialysis is approximately $100,000 [4]. In addition, ESRD patients who received renal transplantation displayed better quality of life compared to patients undergoing chronic dialysis treatment due to the decreased cardiovascular complications, fewer dietary restrictions, and the elimination of multiple visits per week to dialysis clinics for hemodialysis [5]. Overall, it is evident that renal transplantation is a preferred choice for the treatment of ESRD.

1.2 Obstacles for renal transplantation

1.2.1 Availability of Donor Organs

Although renal transplantation is the optimal choice for treating patients with ESRD, the lack of available donor organs is a primary challenge that limits the implementation of kidney transplantation to such patients. In the past decade, the demand for kidney transplantation has increased due to prevalence of chronic diseases such as hypertension and diabetes. As such during this period, the kidney transplantation waiting lists have grown by 62% in the United States and 20% in Canada, with over 105,000 patients on the kidney transplantation wait list in North America [6,7]. On the other hand, the total number of living kidney donors remains stagnant, with an average of 400 cases per year in Canada [2]. This increase in demand over supply of living donor kidneys increases mortality rates of ESRD patients as they are forced to undergo longer dialysis periods while the transplantation waiting lists grows.

1.2.2 Ischemia reperfusion injury

Another major challenge in renal transplantation is that organ procurement is inherently associated with ischemia-reperfusion injury (IRI). Renal IRI is an inevitable consequence of transplantation and is defined as tissue injury due to temporary cessation of blood flow upon procurement (ischemia) and subsequent restoration of blood flow to the ischemic graft upon transplantation (reperfusion) [8]. The first major molecular event of IRI occurs when cells are deprived of adequate oxygen due to cessation of blood flow, resulting in energy depletion since the cells are unable to synthesize ATP [9]. The depletion of ATP elicits a rise in inorganic phosphate and inhibits Na^+/K^+ pumps, resulting in influx of Ca^{2+} in the cytoplasm, and increased mitochondrial inner membrane permeability [10].

In addition, prolonged ischemic time can damage multiple protein complexes in the electron transport chain (ETC), ultimately leading it to be more prone to electron leakage [11]. The second major molecular event in IRI occurs when blood flow is restored to the ischemic tissue and is often characterized by increased ROS formation due to mitochondrial complex I dysfunction and decreased ATP production and cell death [12]. ROS can impair ETC complexes, which further inhibits ATP production and increases electron leakage [13]. Cellular ATP depletion leads to an increase in expression of pro-apoptotic proteins such as BID and BAX, which causes mitochondrial swelling and induces the efflux of cytochrome c and apoptosis-inducing factor [14]. Correspondingly, these factors activate caspase 3 apoptotic signaling cascades, which initiates cellular apoptosis. The pathophysiology of IRI in the kidney also involves the triggering of inflammation cascades that damages renal tissue. Activation of Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway can recruit pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 6 (IL6), all of which plays a major role in renal damage induced by IRI [15-18]. Additionally, these inflammatory mediators and ROS elicit leukocytes and neutrophil infiltration into the ischemic tissue, which leads to swelling of the endothelial cell and promotes programmed necrosis [19-21]. Kaminski et al. showed that activation of neutrophils can release ROS and other mediators that accelerates IRI, ultimately leading to acute and chronic kidney failure [22]. Recent studies found a new type of programmed cell death that is closely related to the pathophysiological process of IRI: ferroptosis [23]. Ferroptosis is characterized by large amount of iron accumulation, intracellular lipid ROS, and lipid peroxidation during the iron-dependent cell death process [24]. A study in 2014 found that ferroptosis was functionally relevant in IRI and acute tubular necrosis and showed that a ferroptosis inhibitor protected against this injury [25]. Another study highlights ferroptosis as the main death pathway of renal tubular cells in an acute kidney injury mouse model [26]. An additional mode of regulated programmed cell death is necroptosis, whose pathway is characterized by TNFα, interferon, Fas ligands, and oxidative stress [27]. Recent studies found that renal IRI led to increased levels of necroptosis in human kidney cells, as evident by the upregulation of necroptotic markers including oxidative stress, inflammatory cytokines, MLKL, RIPK1, and RIPK3 [28]. Interestingly, an *in vivo* study found that

apoptosis co-occurred with necroptosis in mice following renal IRI, as shown by increased levels of cleaved caspase 3 and positive TUNEL cells [29]. The pathophysiology of renal IRI is very complex but some pathological pathways such as excessive ROS production, ATP depletion, induction of cellular apoptotic pathways, activation of neutrophils, and other inflammatory mediators are major molecular events associated with IRI. These complex processes are graphically depicted in Figure 1.

1.3 Current clinically used methods of organ preservation in renal transplantation

During renal transplantation, the ischemic period is divided into warm ischemic time (WIT) and cold ischemic time (CIT) [30]. The former is defined as the loss of blood flow to the organ which occurs when the renal arteries and veins are ligated. This interval of warm ischemia is then followed by a period of cold ischemia of various time periods wherein the organ is conserved in a hypothermic solution at 4◦C to slow cellular activity and reduce production of toxic metabolites before being transplanted into the recipient [31-32]. By slowing down the harmful effects of IRI during CIT, physicians can accomplish tissue typing and cross-matching as well as organ transportation and preparation of the recipient [33]. Immunosuppressive regimens have advanced substantially in recent years along with our understanding of allograft rejection, yet organ preservation persists in its most simple form; static cold storage (SCS) is the most prevalent method for renal allograft preservation and this method has not changed since its conception over 60 years ago [34]. Although SCS is the current standard of care for organ preservation, these methods have been maximized and prolonged periods (>24h) of cold IRI are still associated with increased rates of acute tubular necrosis, decreased graft survival, and delayed graft function [35-38]. In recent years, we have seen a shift away from the traditional paradigm of SCS to a more dynamic organ storage, such as the use of machine perfusion (MP). Traditionally, SCS organs are bathed in preservation solution inside sterile plastic bags covered with ice prior to transportation to the recipient. MP, on the other hand, is a pump that continuously perfuses the kidney with preservation solution [39]. Immediately after procurement, the organ is attached to the machine via the renal artery, and a device generates a continuous flow via a peristaltic pump [39]. Studies that compared MP to SCS

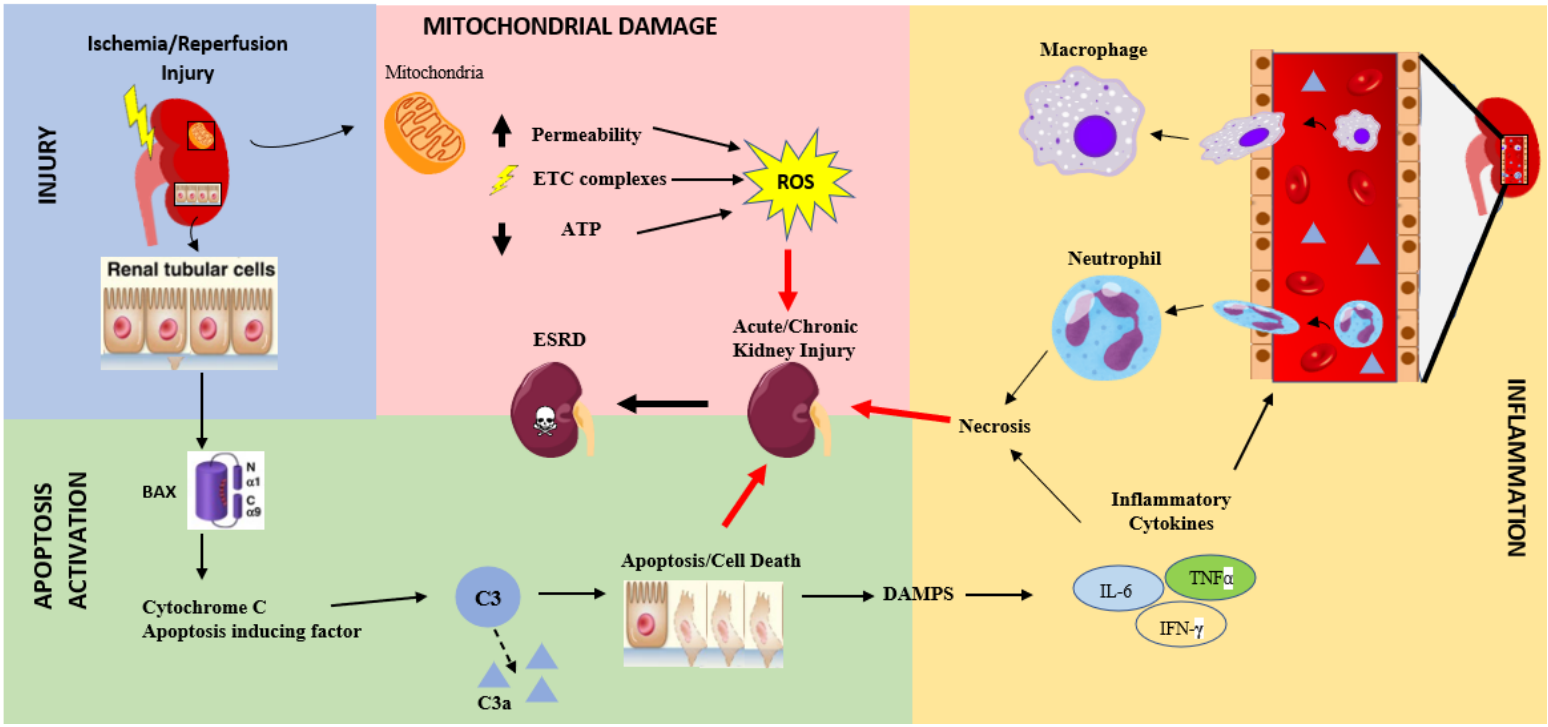


Figure 1: Pathophysiology of kidney ischemia reperfusion injury during transplantation. Renal IRI is an inevitable obstacle of transplantation and is defined as tissue injury due to temporary cessation of blood flow upon procurement (ischemia) and subsequent restoration of blood flow to the ischemic tissue upon transplantation (reperfusion). This process increases mitochondrial permeability and decreases ATP levels, resulting in an increase in ROS production. In addition, the metabolic deficit triggers pro-apoptotic markers BAX, ultimately leading to apoptosis via the complement pathway. ROS and DAMPs can also trigger inflammatory cytokines and consequently cause innate immune cells to adhere to inflamed renal endothelium and extravasate to the interstitial space, where they release more damaging ROS. These renal injuries resulting from IRI results in renal cell death and graft dysfunction in the post-transplant period.

have shown that MP kidneys had lower incidence of delayed graft function, while providing additional parameters, including flow, temperature, and pressure, that allow a more elaborate evaluation of ischemic damage and organ viability to predict post-transplant outcomes [40-43]. Often, transplant centers differ in the timing and nature of MP utilized in terms of its availability and transportability. Therefore, MP is frequently combined with periods of SCS, making a debate on what the ideal perfusion period and timing of perfusion are [44]. An additional potential use of MP for surgeons is the assessment of graft quality and viability prior to transplantation through the analysis of perfusion flow and quantifiable biomarkers within the perfusate [45-46].

Currently, the most commonly used organ preservation solutions for kidney transplantation are University of Wisconsin (UW) solution, histidine-tryptophan-ketoglutarate (HTK), Celsior, and hyperosmolar citrate (Marshall's solution, HOC) [33]. Out of the listed solutions, UW solution is currently considered to be the gold standard among organ preservation solutions. UW solution was developed by Dr. Belzer and James Southard in the 1980s and was shown to safely allow preservation of human grafts for an extended period [47]. UW solution is made up of a combination of metabolically inert substrates, including raffinose, adenosine, glutathione, allopurinol, hydroxyethyl starch, and lactobionate [48]. In addition, UW solution contains adenosine, which acts as the substrate for the synthesis of ATP during reperfusion and the antioxidant glutathione to act as oxygen radical scavengers and neutralize ROS generated during the reoxygenation part of reperfusion. [35,48]. However, some of the disadvantages to the solution are its high viscosity and high potassium concentration, making it less efficient in quickly flushing a donor graft. HTK was used as an alternative solution based on its low viscosity, low potassium level, and lower cost compared with those of UW solution [49]. Studies comparing the use of UW and HTK for organ preservation have shown similar results in terms of graft and the patient survival, especially if CIT was kept at a minimum [49-52]. However, in studies with prolonged CIT, HTK was shown to be inferior compared to UW due to increased risk of graft loss and thrombosis [51-54]. To keep up with the global rising incidence of ESRD and ever-increasing number of patients on transplant waitlists, many transplant programs accept grafts with extended cold storage times, thus increasing CIT

which ultimately continues to represent a major challenge in mitigating renal IRI and improving clinical outcomes of transplantation.

1.4 Hydrogen Sulfide

One recent promising therapeutic strategy to mitigate IRI during renal transplantation is the use of gasotransmitters. Gasotransmitters are small, endogenously produced molecules and have been shown to have various cytoprotective effects. The family of molecules includes carbon monoxide (CO), nitric oxide (NO), and hydrogen sulfide (H_2S). H_2S is a gasotransmitter that is endogenously produced from the amino acid L-cysteine and 3-mercaptopruvate via 3 enzymes: cystathionine β-synthase, cystathionine γ-lyase, and 3-mercaptopruvate sulfurtransferase [55]. For several decades, H_2S was notoriously known for its toxic effects and death among agricultural and industrial workers at high concentrations. The mechanism underlying the toxic effect of H_2S involves inhibition of cytochrome c oxidase (complex IV), the terminal complex of the mitochondrial electron transport chain, resulting in lack of oxygen use in cells [56]. In the past 25 years, however, this obnoxious-smelling membrane-permeable gas has risen above its negative public image and is now known to play several important functions in physiological processes at low concentrations. In addition to its many cellular signaling properties such as reducing inflammation and stimulating cell proliferation, H_2S can also play a detoxifying role during oxidative stress by increasing the development of the antioxidant glutathione [57]. Furthermore, a 2012 study by Aminzadeh *et al*, found that progression of chronic kidney disease is associated with a reduction of H_2S and its corresponding production enzymes [58]. Given the cytoprotective features of H_2S, its supplementation with preservation solution could contribute to combatting the inflammatory and oxidative stress environment that IRI induces. H_2S can be delivered via gaseous administration or through the use of donor molecules. Our lab has been the first to show that supplementing standard organ preservation solution (UW solution) with H_2S donor molecules (AP39 and GYY147) protects rats and porcine donor kidneys from prolonged warm and cold ischemic injury and improves graft function following transplantation [59-61]. In terms of alleviating the effects of IRI, AP39 demonstrates greater potential because it is targeted to the mitochondria, which has been well established

as a key mediator in IRI, via its triphenylphosphonium motif. Further evidence of this was confirmed using an *in vitro* model of renal IRI, where it was shown that AP39 is 1000-fold more cytoprotective from cell death than non-targeting H_2S donor molecules, such as GYY4137 [62]. We have also shown that prolonged SCS in UW solution supplemented with AP39 leads to improved graft function and reduced tissue injury following allogeneic renal transplantation in rats [63]. These latest findings have pushed the potential clinical use of H_2S donor molecules, especially those targeting the mitochondria, in organ preservation solutions. Unfortunately, these experimental H_2S donor molecules are decades away from clinical use due to the time-consuming process of clinical testing. Therefore, to translate our bench work to clinical practice, we have begun looking into sodium thiosulfate (STS), an already clinically utilized drug known to release H_2S [64].

1.5 Sodium Thiosulfate

Sodium thiosulfate (STS, $Na_2S_2O_3$) is a synthetic, white crystalline solid or powder with no odor and dissolves easily in water. This inorganic compound is typically available in the form of the pentahydrate ($Na_2S_2O_3 \cdot 5H2O$), with a molecular weight of 158.11g/mol. STS is currently on the World Health Organization's list of essential medicines and has numerous other uses including a common food preservation additive, a water dechlorinator, a photographic fixative, and a bleaching agent for paper pulp.

1.5.1 Contemporary uses of STS

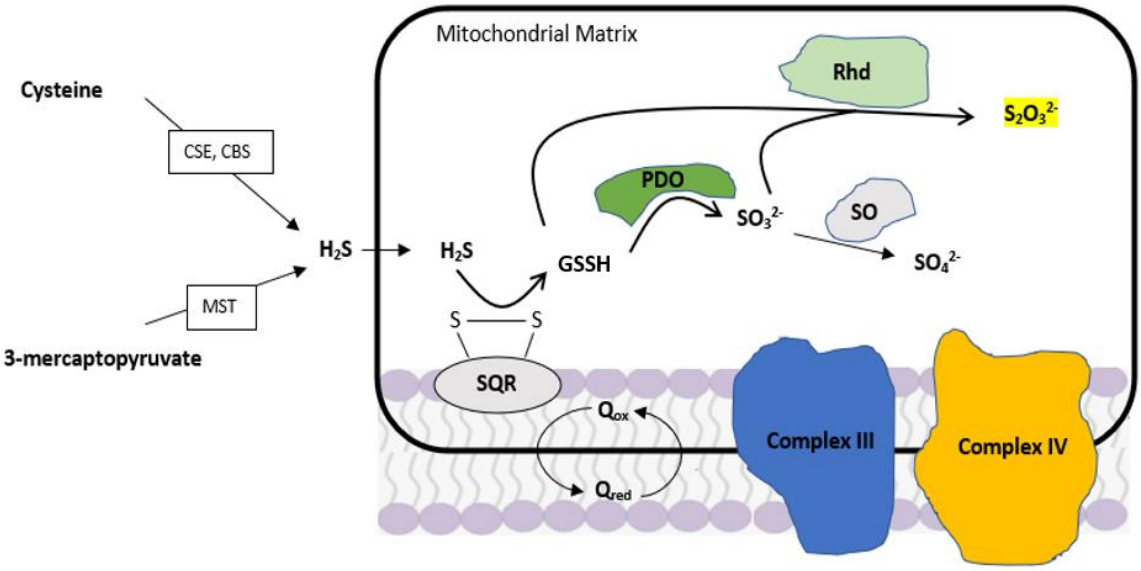
STS has long been known to physicians as STS was used to treat some rare medical conditions, including cyanide poisoning, cisplatin toxicity, and calciphylaxis. Over the past few decades, STS has been used as an antidote for the treatment of cyanide poisoning. STS was shown to serve as a sulfur donor in the reaction catalyzed by rhodanese for detoxification of cyanide to thiocyanate, which is then renally excreted [65]. This occurs after sodium nitrate removes cyanide from the mitochondrial electron transport chain by inducing the formation of methemoglobin [65,66]. The United States has a standard cyanide antidote kit which first uses 10 mL intravenous sodium nitrite, followed immediately by 50 mL (250 mg/mL) intravenous STS.

Another STS use is to protect against cisplatin toxicity. Cisplatin is one of the most widely used agents to treat solid tumors. However, it has adverse effects on renal, neurological, and hematological systems [67]. Laplace *et al*. showed that administering STS protects renal impairment following cisplatin chemotherapy [68]. Efficacy is thought to be related to STS binding to free platinum, resulting in total clearance of inactive metabolite and limiting renal tubular cell necrosis [69]. In addition, the use of cisplatin to effectively treat childhood hepatoblastoma can cause severe and permanent ototoxicity, leading to eventual hearing loss. Studies concluded that addition of STS six hours after cisplatin chemotherapy resulted in lower incidence of cisplatin-induced hearing loss among children with standard-risk hepatoblastoma, without jeopardizing overall and event-free survival [70]. STS was shown to inhibit oxidative stress at the sensitive cochlea that was caused by ototoxicity. After two successful clinical trials, Fennec Pharmaceuticals is currently waiting on FDA approval on the first potential prevention of platinum induced ototoxicity in pediatric patients [71].

Calciphylaxis is yet another condition where STS is used for treatment. Seen in people with ESRD, calciphylaxis is predictor of cardiovascular death in long-term hemodialysis patients [72]. It is characterized by systemic medial calcification of the arterioles leading to ischemia and subcutaneous necrosis. Promising results have been obtained through the use of intralesional STS. Areas of clinically active disease were treated with 250ml/mg STS, resulting in the resolution of calciphylaxis lesions over a period of weeks with no recurrence of the disease [73]. Recently, Peng *et al*. conducted a systemic review of several cases on the use of STS for calciphylaxis and found that STS has a promising role as an effective therapy for calciphylaxis by acting as an anti-calcification agent with vasodilatory and antioxidant properties [74]. Their study results supported previous reports that suggested STS could combine with insoluble tissue calcium salts to form calcium thiosulfate, a salt that can later be dialyzed [74-76].

1.5.2 Biochemical characteristics of STS and its relationship to H_2S

At a physiological level in tissue, thiosulfate can be generated from the mitochondrial sulfide oxidation pathway with H_2S as the substrate. This process involves 3 mitochondrial enzymes: quinone oxidoreductase, sulfur transferase, and sulfur dioxygenase [77]. Using an isolated mitochondria rat model, Hildebrandt *et al*. showed a potential biochemical pathway of H_2S oxidation to thiosulfate [78]. First a membrane bound sulfide, quinone oxidoreductase (SQR), oxidizes H_2S to persulfide, which is transferred to a persulfide group (SQR-SSH). A persulfide dioxygenase (PDO) in the mitochondrial matrix oxides one persulfide molecule to sulfite (H_2SO_3), which is then used in a sulfurtransferase reaction catalyzed by rhodanese (Rhd) to form thiosulfate [77]. Rhodanese is a mitochondrial enzyme that transfers sulfur atom from sulfane-containing donor to thiophilic acceptor substrate [79]. Catalytic activity of rhodanese occurs by a double displacement mechanism, where the active site cysteine residue (Cys247) accepts a sulfur atom from the persulfide intermediate state followed by the transfer of sulfide sulfur from enzyme to the nucleophilic acceptor sulfite to produce thiosulfate [80]. Although human mitochondria also utilize sulfide oxidation pathway, recent evidence suggests that the persulfide intermediate formed by human SQR uses glutathione (GSH) [81, 82]. More recently, *Libiad* et al. found that the kinetic behavior of these enzymes favors SQR using glutathione as an acceptor to form glutathione persulfide (GSSH), which is then converted to thiosulfate by human rhodanese [83]. This is further confirmed by kinetic simulations in previous rat liver mitochondria studies in the absence and presence of GSH, which supports that glutathione persulfide is the first intermediate formed in the flow of the sulfide oxidation pathway [82]. This complex process is graphically depicted in Figure 2.



SQR – sulfide quinone oxidoreductase

CIII/CIV – mitochondrial electron transport complexes 3 and 4

SO – sulfite oxidase

MST-mercaptopyruvate sulfurtransferase

GSH- glutathione

PDO- persulfide dioxygenase

Rhd – Rhodanase

Figure 2: Mitochondrial sulfide oxidation pathway generates thiosulfate from H_2S. Hydrogen sulfide (H_2S) is produced by enzymes of the trans-sulfuration pathway, cystathione γ-lyase (CSE), and cystathionine β-synthase (CBS). A third enzyme, 3-mercaptopyruvate sulfurtransferase (MST) can also produce endogenous H_2S in the presence of the substrate 3-mercaptopyruvate. A membrane bound sulfide, quinone oxidoreductase (SQR), oxidizes H_2S to persulfide, which is transferred to a glutathione (GSH). A persulfide dioxygenase (PDO) in the mitochondrial matrix oxides one glutathione persulfide (GSSH) to sulfite (H_2SO_3), which is then used in a sulfurtransferase reaction catalyzed by rhodanase (Rhd) to form thiosulfate ($S_2O_3^{2-}$) by transferring a second glutathione persulfide from SQR to sulfite. Sulfite can be further oxidized by sulfite oxidase (SO) to form sulfate and is subsequently excreted in the urine.

In addition to the generation of thiosulfate from H_2S via the sulfide oxidation pathway, the reverse reaction can also occur in the mitochondria. Jackson *et al*. [81], using recombinant human SQR in *Escherichia coli*, showed that the metabolism of thiosulfate is catalyzed by thiosulfate reductase as it consumes two glutathione and results in the generation of sulfite, oxidized glutathione, and H_2S. Further evidence of thiosulfate's ability to produce H_2S via a glutathione-dependent reduction was confirmed in a study that showed exogenous thiosulfate treatment effects sulfide levels in a dose dependent manner that impacts glutathione metabolism [84]. On the other hand, other studies do not support the contribution of glutathione in H_2S production from thiosulfate. Olson *et al.* found that H_2S generation from thiosulfate was due to the presence of DTT, an exogenous reducing agent [85]. However, regardless of the exact mechanism of how sulfur is transferred, thiosulfate appears to be a key intermediate. In addition to thiosulfate being a stable, nontoxic metabolite of H_2S [86], it is also a sulfane sulfur which is defined as sulfur atoms covalently bonded to other sulfur atoms, making it unstable and readily oxidizing in air and reducing with thiols [87]. Compounds containing sulfane sulfur are known to possess cell regulatory effects through the activation or inactivation of enzymes and changing protein activities [88]. The functions of sulfane sulfur include tRNA sulfuration, antioxidant regulation, and iron-sulfur protein formation [88-90].

1.5.3 Protective roles of STS

STS has shown some promise in treating other conditions by serving as a H_2S donor molecule. Thiosulfate has two unpaired electrons: one at the single bonded sulfur moiety of the disulfide bond, and the other at the single bonded oxygen [91]. This characteristic allows thiosulfate to donate electrons to unpaired damaging electrons associated with reactive oxygen species (ROS), making it an effective antioxidant [85, 92, 93]. Further evidence of thiosulfate's antioxidant properties was confirmed in a mouse model of congestive heart failure study by Sen *et al*., where their data showed that thiosulfate scavenged superoxide in myocardial tissue [94]. Mishanina *et al*. took a step further and discovered that thiosulfate can react with superoxide to form glutathione, a thiol-dependent

antioxidant system in mammalian cells [95]. Further evidence of STS's antioxidant properties was confirmed in both *in vitro* and *in vivo* models of hyperoxaluria and renal injury, where the authors showed that STS treatment scavenged reactive oxygen species in a dose dependent manner and maintained superoxide dismutase activity in order to reduce cellular hydrogen peroxide levels [96]. A recent study examined the protective properties of STS in angiotensin II-induced renal hypertension in rats where the authors showed that STS treatment induced a lower plasma urea, proteinuria, and improved creatinine clearance [97]. In addition, STS prevented angiotensin II-induced influx of macrophages, demonstrating its anti-inflammatory properties [97]. Their study results agreed with previous reports which demonstrated that suggested H_2S has anti-inflammatory capabilities by down-regulating expression of inflammatory genes such as IL-1β, TNF-α, and MAP-1, and by reducing macrophage recruitment [98].

1.5.4 Physiological roles of STS in IRI

It has been well recognized that mitochondrial damage and its ensuing dysfunction is a key mediator of IRI. Under ischemic and oxidative conditions, mitochondrial permeability transition pore (mPTP) opens, leading to mitochondrial dysfunction and ultimately cell death [99]. However, a recent study by Baldev *et al*. developed an *in vivo* model where rats were induced with nephrolithiasis by administrating ethylene glycol [100]. STS treatment group demonstrated normal renal tissue form, along with normal urea and serum creatinine. STS was found to activate mitochondrial ATP-sensitive potassium (K_{ATP}) channel, suggesting that the opening of these channels may have inhibited mitochondrial permeability transition [94,101]. Further evidence of STS maintaining mitochondrial function was confirmed in a study by Mohan *et al.*, where isolated rat mitochondria were subjected to physiological oxidative stress. The results showed that the pretreated STS group had higher renal mitochondrial enzyme activity due to its increased NADH dehydrogenase activity compared to the non-treated group [102]. In a recent study, Ravindran *et al*. reported that a rat heart IRI model preconditioned with STS exhibited similar ATP synthase activity and mitochondrial enzyme activity compared to sham [103]. In addition, the STS treatment group improved activities of ETC complex enzymes I-IV

and showed significantly increased expression of PGC-1α, a positive regulator of mitochondrial biogenesis, ATP production, and ROS-detoxifying system [104].

A 2017 study showed that in both *in vitro* and in *vivo models* of heart IRI, STS preconditioning significantly increased NADH dehydrogenase activity and decreased oxidative stress due to increased activities of antioxidant enzymes such as GSH, catalase, and superoxide dismutase [105]. In addition, an *in silico* model in the same study showed that STS has higher binding affinity for caspase 3. Once STS interacts with caspase 3 and binds to its Cys-163 active site via strong hydrogen bonds, it increases persulfidation of the enzyme. This oxidation reaction results in the inactivation of caspase-3 by preventing access of natural substrate to caspase-3 binding site, ultimately halting apoptosis [105]. Further evidence of STS's higher binding affinity for caspase 3 was confirmed in a study by Marutani *et al*., where the authors discovered that STS inhibits caspase 3 via persulfidation of the same active site Cys-163 to protect the mice against neuronal IRI [106]. In addition, STS was shown to activate Erk 1/2 and block the c-jun N-terminal kinase (JNK), which lead to the inhibition of apoptosis by preventing the dephosphorylation of the pro-apoptotic protein Bad and the downregulation of antiapoptotic gene, Bcl-2 [107-109]. In summary, the literature suggested that STS modulates mitochondria potassium ATP channel, possess a calcium chelation effect, mitigates oxidative stresses, has anti-inflammatory properties, involves in mitochondrial ETC regulation, and acts as a sulfide donor, ultimately leading to protection against IRI in various tissues.

1.5.5 Rationale and Hypothesis

We have previously shown that supplementing standard organ preservation solution (UW solution) with H_2S donor molecules (AP39 and GYY147) protects rats and porcine donor kidneys from prolonged warm and cold ischemic injury and improves graft function following transplantation [59,61-63]. However, it is important to note that these H_2S donor molecules, such as AP39, must go through many regulatory steps before they could be available for clinical use. STS on the other hand is an FDA-approved compound for treating diseases such as calciphylaxis and cyanide poisoning [64-68]. In addition, several studies have elucidated the ability of mitochondrial enzymes to generate STS from

H_2S via a sulfide oxidation pathway and the reverse reaction via a glutathione-dependent reduction [77-78]. Emerging data on the biological effects of STS and its close chemical relationship with H_2S support the development of STS-based therapeutics.

Besides its clinical applications, STS has also been shown experimentally to effectively protect against renovascular hypertension, and other models of renal injury via its antioxidant and anti-inflammatory properties [95,97,98]. Additionally, numerous studies suggested that STS can mitigate IRI-induced damage in heart and brain tissues by preserving mitochondrial complex, modulating mitochondrial ATP channel, mitigating oxidative stress, and downregulating pro-apoptotic genes [99-104].

In the context of kidney transplantation, modification of the preservation solutions with STS at the right concentration may be a simple, inexpensive, and nontoxic novel therapeutic strategy to mitigate prolonged cold renal IRI in donor organs to ultimately improve renal graft outcomes and minimize post-transplant complications. However, this novel concept remains to be explored in a clinically relevant model of renal transplantation.

HYPOTHESIS: Supplementation of standard organ preservation solutions with STS will lead to improved graft function and decreased tissue injury in an *in vitro* and *in vivo* rat transplantation model by limiting IRI-induced oxidative stress and by preserving cellular metabolic activity.

1.6 Aims and Objectives

The objective of this study was to elucidate the protective effects of STS organ preservation in cellular and rat models of renal IRI. This study could ultimately help identify STS as a novel therapeutic target for treatment of IRI associated with kidney transplantation.

The specific aims of the work outlined in this thesis were to: 1) Characterize underlying mechanisms of cytoprotective effects of STS during renal IRI *in vitro* using rat kidney epithelial (NRK-52E) cell lines; and, 2) Investigate whether STS improves graft function and reduces tissue injury in an *in vivo* model of rat kidney transplantation.

Chapter 2

2 Materials and Methods

2.1 *In vitro* model of renal epithelial cell IRI

An *in vitro* model of cold hypoxia and warm reoxygenation injury that mimics cellular conditions during *in vivo* cold IRI was used to assess the protective effects of STS during renal IRI. Rat kidney epithelial cells (NRK-52E cell line; ATCC, USA) were used in the *in vitro* experiments because these cells are susceptible to ischemic injury [42] and used for consistency with the rat *in vivo* transplantation model used for the second aim of this study. The cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) inactivated by heat at 60^0C for 20min and 1% penicillin/streptomycin (P/S). Cells were incubated at normal growth conditions of 37^0C, 21% O_2, and 5% CO_2. During experimentation, control cells were in conditions identical to those for pre-experimental cells. Experimental cells were treated with either serum free media (SF), SF plus 200nM AP39, or SF plus varying concentrations of sodium thiosulfate pentahydrate (STS), which were obtained from a 250mg/ml injectable solution of STS (Seacalphyx® [Seaford Pharmaceuticals Inc, Mississauga, ON, Canada]). 200nM AP39 was used because they previously have shown to be cytoprotective against the same cell line in a similar model of cold IRI [62]. Cells were then incubated at 10^0C for 24h in hypoxic growth conditions (5% CO_2, 0.5% O_2, 95% N_2) to simulate ischemic during cold organ preservation conditions. These hypoxic conditions were created using HypOxystation H85 hypoxia chamber ($HYPO_2$YGEN, USA). 10^0C was used for the hypothermic condition because this was the lowest temperature that could be technologically achieved while maintaining a hypoxic 0.5% O_2 level. Following hypoxia, experimental cells media was replaced with control media and were reoxygenated via incubation in normal growth conditions (37^0C, 21% O_2, and 5% CO_2) for 24h to simulate reperfusion and associated injury.

Following 24h reoxygenation, cellular viability was assessed via staining of cells with FITC-conjugated Annexin-V (FITC-Annexin-V; BioLegend, USA) and 7-Aminoactinomycin D (7-AAD; BioLegend, USA), which measures cellular apoptosis and

necrosis, respectively. Cells were analyzed via flow cytometry using the CytoFLEX S (Beckman Coulter, USA) and FlowJo version 11 (FlowJo LLC, USA) was used to appropriately gate the data for statistical analysis.

2.2 Animal description and care

2.2.1 Experimental animals

Male Lewis rats were purchased from Charles River Canada (St. Constant, QC, Canada) and used at 250-300g (n= 30). Rats were maintained in the Animal Care and Veterinary Services facility at Western University (London, ON) under standard conditions. Animal studies were approved by the Western University Council on Animal Care and Animal Use.

2.2.2 Syngeneic renal transplantation surgical procedure and postoperative monitoring of rats

Syngeneic Lewis rat renal transplantation was performed to eliminate any confounding effects of immunosuppression. Rats were randomized into treatment groups, anaesthetized with ketamine (30 mg/kg) and maintained under anesthesia with isoflurane during surgery. Using aseptic techniques, the left donor kidneys were procured and flushed with a 28-G Angiocath Becton-Dickinson with 10mL of either cold (4^0C) University of Wisconsin (IW) preservation solution (UW group, n=8), or cold UW plus sodium thiosulfate pentahydrate (150uM Seacalphyx® [Seaford Pharmaceuticals Inc, Mississauga, ON, Canada]; STS group, n=6) until venous effluent was clear. Grafts were then placed in 50mL of the same perfusion solution and stored at 4^0C for 24h, a period that has previously been shown to result in acute tubular necrosis and inflammation that lead to graft loss and only 5% survival at POD 3 rats treated with UW solution alone [59]. After undergoing bilateral nephrectomy, recipient rats underwent renal transplantation with donor kidneys removed from cold storage via end-to-side anastomosis of donor inferior vena cava to recipient inferior vena cava with 11-0 Prolene suture followed by the donor ureter being anastomosed to recipient ureter using 10-0 PDS sutures. Our aim was to assess the protective effects of STS on graft function and survival after extreme cold storage in a

survival model where the recipient rat was solely dependent upon the transplanted graft for renal function. Sham-operated rats (mid-line incision only; n=5), were also followed to establish a baseline for survival, histological analysis, BUN, and serum creatinine. Additionally, another subset of rats in the STS group had grafts removed pre-emptively at POD 3 (n=5) for comparison of histological analysis to UW animals that were sacrificed at this time point. All surgeries were performed by the same microsurgeon who was blinded to the experimental design with the length of surgery for the recipient being approximately 2-3h for both treatment groups. There was no difference in operating times between the UW and STS treatment groups. Graft failure was presumed in animals that required premature sacrifice (severe visible distress and/or >20% weight loss) or death. At the time of sacrifice, all anastomoses were checked for surgical complications that may cause variations in the results; none were discovered. The experimental model is graphically depicted in Figure 3.

2.3 Creatinine and urine assays

Following renal transplantation, rats were monitored in metabolic cages for a period of 14 days or until sacrifice. The time period of 14 days was picked since animals that survived past 9 days post-transplant were seen to have fully recovered renal function [59,61,62.63]. At various time points (POD 3, 5, 7, 10, and 14) rats were removed from their metabolic cages and the previous 24h worth of urine output were measured and collected for urine osmolality analysis. In addition, 200uL of blood was taken from the tail vein following the ACVS protocol, spun at 2500rpm for 10min to separate the serum and stored at -80^{0}C until analyzed for serum creatinine and BUN levels. Blood urea nitrogen and creatinine and levels of serum obtained from renal transplantation and Sham-operated rats were determined using the enzymatic method performed on the IDEXX Catalyst One Chemistry Analyzer machine (IDEXX, Markham, ON). Urine osmolality levels were determined by freezing-point osmometry using the 3320 Osmometer machine (Advanced Instruments, Norwood, MA) and compared to company provided standards.

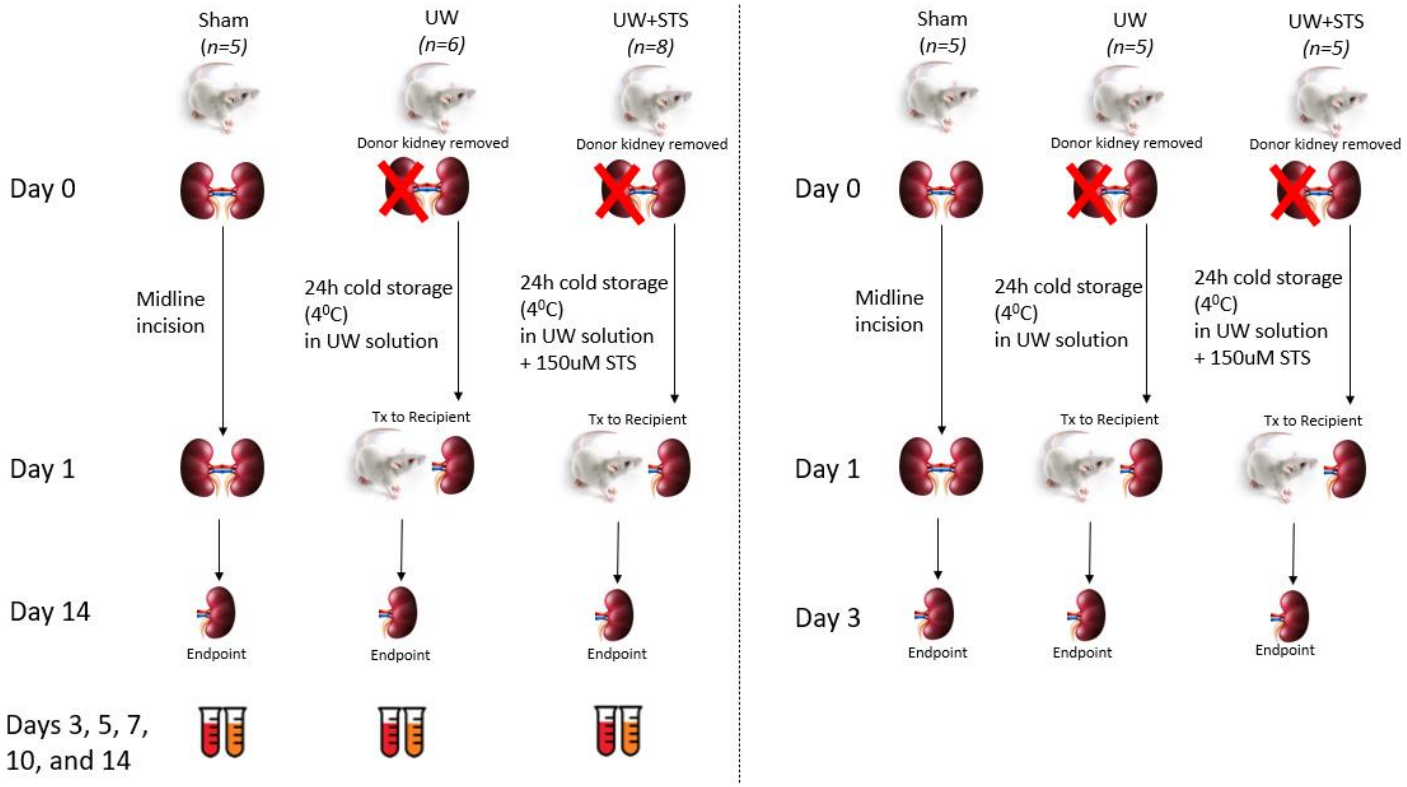


Figure 3: Renal transplantation model in rats.

2.4 Histological staining

In each arm of the study, renal grafts obtained at time of sacrifice were sagitally bivalved, where half of the sagitally bivalved kidneys was placed in 10% formalin for paraffin embedding and sectioning, while the other half was flash-frozen in liquid nitrogen and stored at -80^{0}C for quantitative PCR (qPCR) analysis. Histological sections were stained with Hematoxylin and Eosin (H&E) and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to determine the level of acute tubular necrosis and apoptosis respectively. H&E sections were assigned a score 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% graft ATN. Histological sections also underwent immunohistochemical staining, with n=5 for each treatment group. Sections were incubated with antibodies against kidney injury marker (KIM-1), macrophage surface marker CD68, and neutrophil-specific enzyme myeloperoxidase (MPO; Abcam®, Toronto, Canada) and visualized with secondary antibodies and DAB substrate chromogen using the Dako Envision System (Dako, Glostrup, Denmark) as per manufacturer's protocol.

2.5 Microscopic image analysis

TUNEL and immunohistochemically stained sections were analyzed using an Eclipse 90i digital light microscope at 10x magnification (Nikon® Instruments, New York) and were read by a trained renal pathologist who was blinded to the treatment groups. Five images were analyzed per section of TUNEL, KIM-1, CD68, and MPO analysis and total (+) staining per field of view was quantified by ImageJ software v. 1.8 (National Institutes of Health, Bethesda, MD).

2.6 Quantitative PCR analysis

Homogenized renal graft tissue obtained at POD 3, containing equal portions of cortex and medulla, was analyzed to determine relative expression levels of genes of interest using qPCR, with n=5 for each treatment group and Sham-operated rats. Renal tissues stored at -80^{0}C were thawed and then immediately homogenized with a mechanical homogenizer. Total RNA was isolated using RNeasy® Mini Kit (Qiagen, Toronto, Canada) and reverse transcribed into cDNA using OneScript® Plus cDNA synthesis Kit (ABM, Canada) in

conjunction with oligo(dT)$_{12-18}$ primers as per manufacturer protocol. Isolated RNA were analysed via nanodrop (DeNovix DS-11 Spectrophotometer, Canada) before use, with $A_{260/280}$ ratings consistently > 1.95 and > 1.8 respectively. The reaction mixture of each qPCR sample had a volume of 20uL and was made as per Blastaq® Green 2X qPCR Master Mix (ABM, Canada) protocol and analyzed using CFX Connect Real-Time PCR Detection System machine (Bio-Rad, Canada). Primer sequences were designed using Primer-BLAST software (NCBI) against beta-actin, poly [ADP-ribose] polymerase 1 (PARP1), interferon gamma (IFN-y), tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6), B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (BAX), caspase 3, BH3 interacting-domain death agonist (BID), c-Jun N-terminal kinase 1/2 (JNK1/2), peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α), mitochondria complex I (NDUFB8), mitochondria complex II (SDHB), mitogen-activated protein kinase 1/2 (ERK1/2), nuclear factor-erythroid factor 2 (Nrf2), lipocalin-2 (NGAL), and kidney injury molecule-1 (KIM-1) genes are shown in Table 1. All genes of interest were normalized against beta-actin. Fold changes of gene expression were compared to Sham-operated rats and were calculated using the ΔΔCt method.

2.7 Statistical analysis

Survival data were analysed using Kaplan-Meier survival analysis and all other data were analysed using one-way ANOVA followed by Tukey's post-hoc test performed using the GraphPad (La Jolla, CA) Prism statistical software package, version 9.0. Statistical significance was accepted at $p < 0.05$.

Table 1. List of qPCR primer sequences

Primer	Sequence (5' ⟶ 3')
β-actin Forward	CCGCGAGTACAACCTTCTTG
β-actin Reverse	CGTCATCCATGGCGAACTGG
PARP Forward	TTGACTATGGCCAGGACGAA
PARP Reverse	CCAAAGTCATGGGGGATGAG
IFN- γ Forward	AGTTCGAGGTGAACAACCCACAG
IFN- γ Reverse	ATCAGCACCGACTCCTTTTCCG
TNF- α Forward	TTCGGGGTGATCGGTCCCAAC
TNF- α Reverse	TGGTGGTTTGCTACGACGTGG
IL-6 Forward	TTTCTCTCCGCAAGAGACTTC
IL-6 Reverse	GGTCTGTTGTGGGTGGTATC
BCL-2 Forward	TCATGTGTGTGGAGAGCGTC
BCL-2 Reverse	AGTTCCACAAAGGCATCCCAG
BAX Forward	CGTCTGCGGGGAGTCAC
BAX Reverse	CGATCCTGGATGAAACCCTGT
Caspase-3 Forward	GAGCTTGGAACGCGAAGAAA
Caspase-3 Reverse	GCCCATTTCAGGGTAATCCA
BID Forward	TGTCGGTCGGCAAACCTCTG
BID Reverse	GCCATTGCTGACCTCAGAGTCC

JNK1 Forward	AGCCGGCCATTTCAGAATCA
JNK1 Reverse	CTCTCGCCTGACTGGCTTTA
JNK2 Forward	CAAGGAATTGTTTGTGCTGCTT
JNK2 Reverse	ACAGGACTTTATGGAGGACCA
PGC-1α Forward	GAGGGACGAATACCGCAGAG
PGC-1α Reverse	CTCTCAGTTCTGTCCGCGTT
NDUFB8 Forward	GGAGAGCCTTCCACATGACC
NDUFB8 Reverse	ATCTGGGTACGGCTCGTAGT
SDHB Forward	GCAGGCTTATCGCTGGATGA
SDHB Reverse	CTGGATTCAGACCCTTGGGG
ERK1 Forward	GCCCGAAACTACCTACAGTC
ERK1 Reverse	CTCAGCCACTGGTTCATCTG
ERK2 Forward	GATCTTAAATTGGTCAGGACAAGG
ERK2 Reverse	CCAACAGATGGCGTGTAAGT
NGAL Forward	GACTACGACCAGTTTGCCAT
NGAL Reverse	CCCCTTGGTTCTTCCGTACA
KIM1 Forward	GTGCTATTTTACCCAGGAGGA
KIM1 Reverse	GGACTTGTGGGAATTTCTGGTTTA
Nrf2 Forward	TGTAGATGACCATGAGTCGC

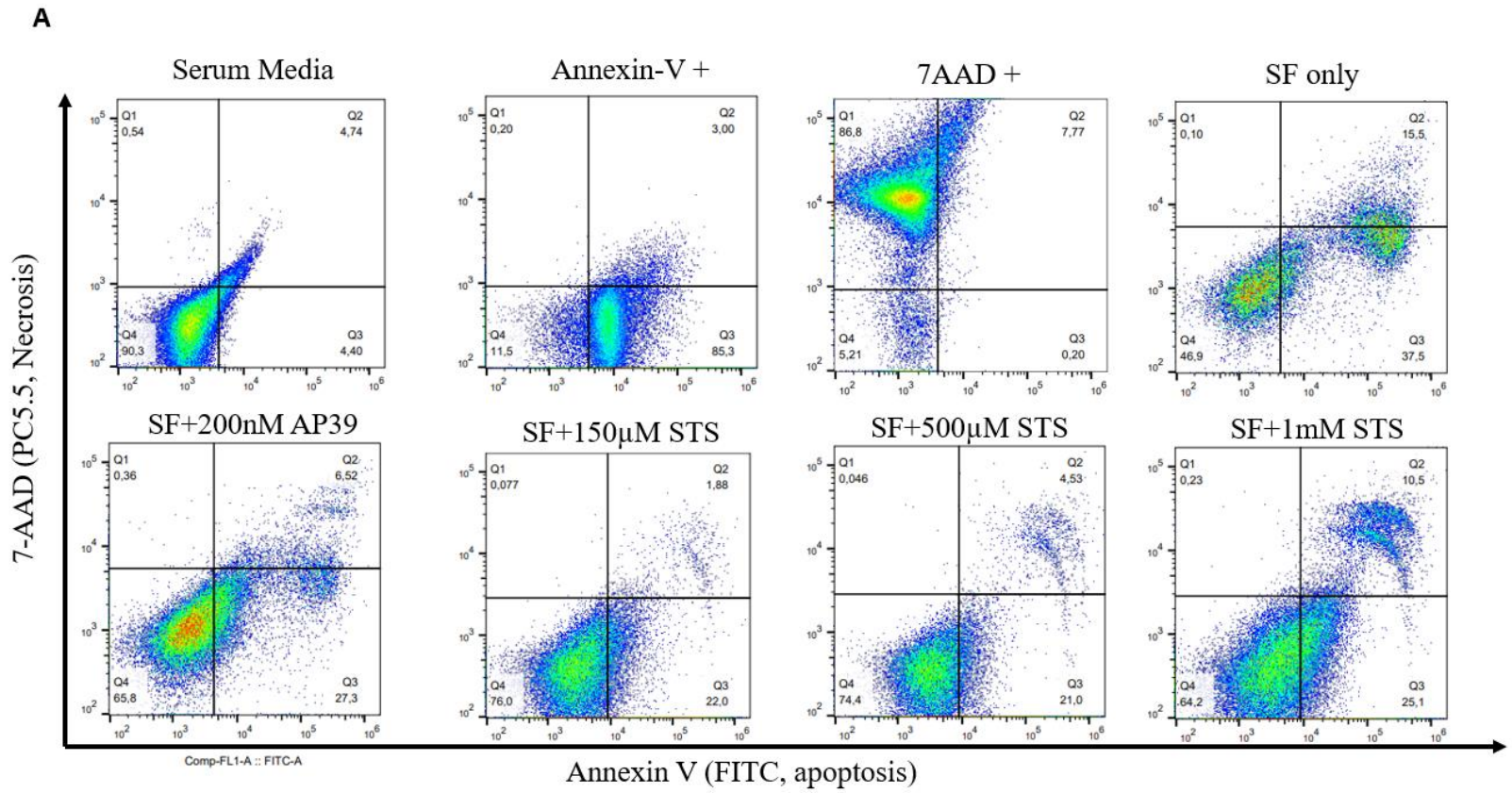
Nrf2 Reverse	TCAGGGGTGGTGAAGACTGA

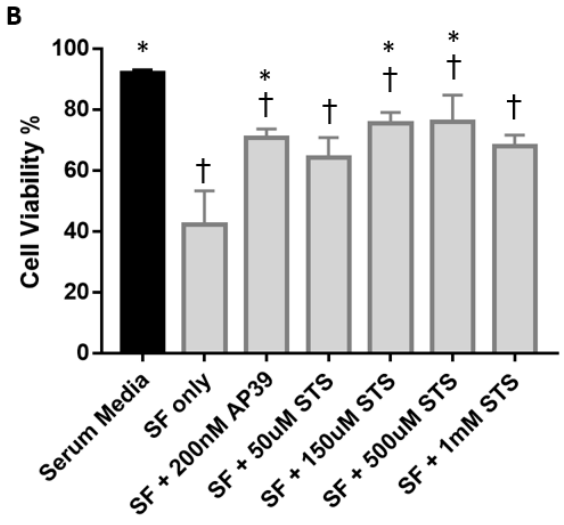
Chapter 3

3 Results

3.1 STS-supplemented serum free media improves renal tubular epithelial cell survival in a dose-dependent manner during cold hypoxia and reoxygenation

Flow cytometry analysis after staining for apoptosis and necrosis showed that NRK-52E cells treated with SF during in vitro cold IRI exhibited significantly decreased cellular viability ($p < 0.05$) compared to control (normoxic) cells. A dose-dependent increase in the percentage of unstained viable cells was evident as the concentration of STS added to serum free media was increased (Figure 4A and 4B). While all the experimental samples exhibited lower renal tubular epithelial cell viability ($p < 0.05$) than cells grown in normoxic conditions, cells treated with SF supplemented with 150µM and 500µM STS exhibited significantly higher viability levels ($p < 0.05$) to 77 +/- 3% and 78 +/ 6% compared to serum free only treated cells whose viability levels remained at 41 +/- 8% (Figure 4B). The reverse trend was observed with apoptosis levels, where cells treated with SF supplemented with 150µM and 500µM STS exhibited significantly lower apoptosis levels ($p < 0.05$) to 26 +/- 3% and 24 +/ 7% compared to serum free only treated cells whose apoptosis levels remained at 57 +/- 9% (Figure 4C). Additionally, the dose-dependent increase in viability and decrease in apoptosis appear to reach optimum levels with 150µM STS and the addition of higher doses of STS begins to reverse these trends (Figure 4B and 4C).


A
Serum Media
Annexin-V +
7AAD +
SF only
SF+200nM AP39
SF+150µM STS
SF+500µM STS
SF+1mM STS
7-AAD (PC5.5, Necrosis)
Annexin V (FITC, apoptosis)
Comp-FL1-A :: FITC-A


B
Cell Viability %
Serum Media
SF only
SF + 200nM AP39
SF + 50uM STS
SF + 150uM STS
SF + 500uM STS
SF + 1mM STS

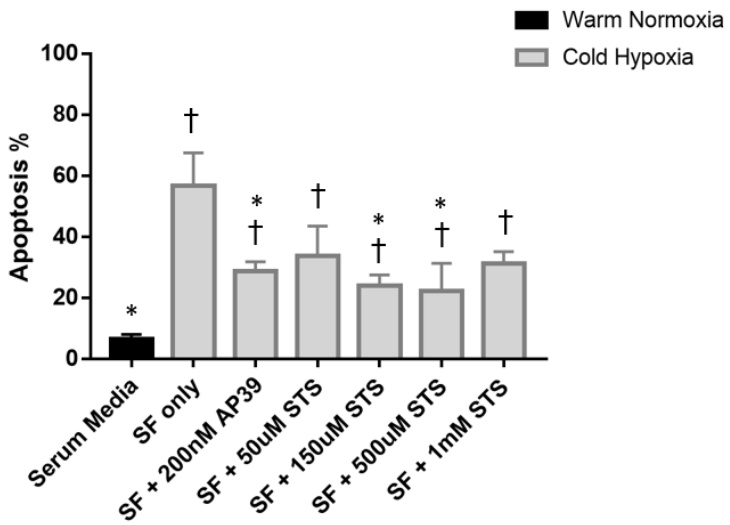
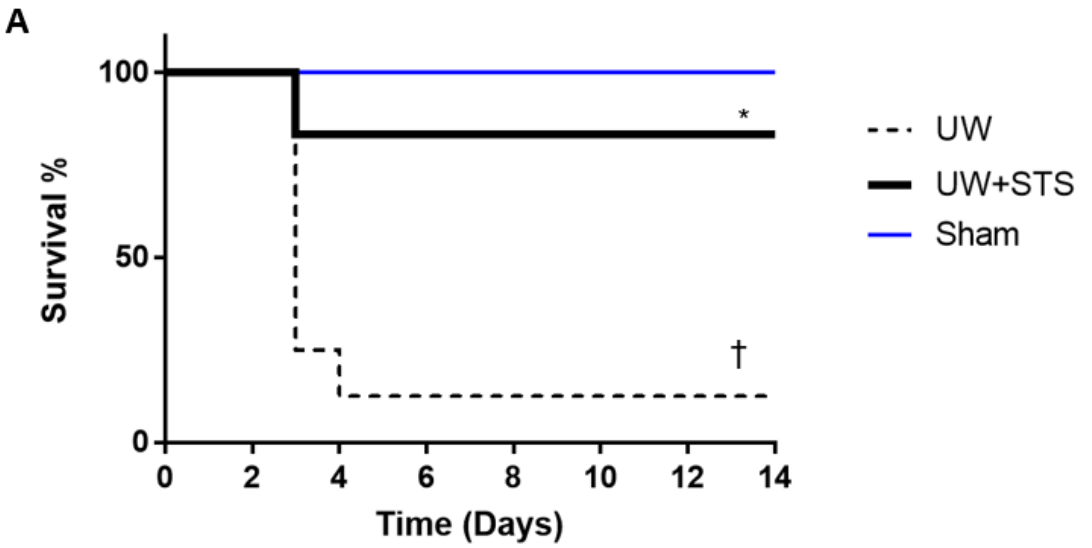
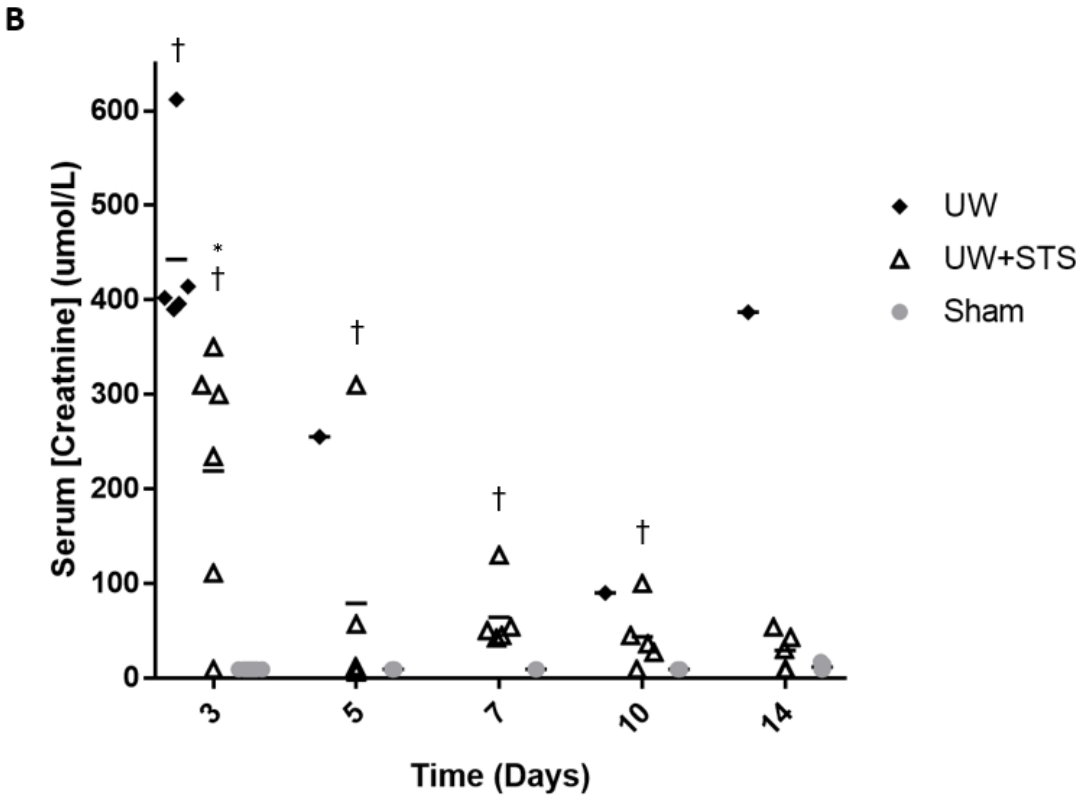

C
Warm Normoxia
Cold Hypoxia
Apoptosis %
Serum Media
SF only
SF + 200nM AP39
SF + 50uM STS
SF + 150uM STS
SF + 500uM STS
SF + 1mM STS

Figure 4: **STS supplementation improves renal tubular epithelial cell survival in a dose-dependent manner during a model of cold ischemia reperfusion injury (IRI).** Cellular viability levels of rat kidney epithelial (NRK-52E; ATCC) cells following cold IRI. Control cells (Control: Serum Media) were cultured in DMEM containing 10% FBS and 1% P/S at normal growth conditions of 37^0C, 21% O_2, and 5% CO_2. Experimental cells were treated with either DMEM without FBS (SF alone), SF supplemented with 200nm AP39 or SF supplemented with different concentrations of STS. The experimental cells were exposed to cold (10^0C) hypoxia for 24h, followed by reoxygenation for 24h in conditions identical to control cells. Following reoxygenation, cells were stained with 7-AAD and FITC-Annexin-V for flow cytometry to quantify necrosis and apoptosis respectively. All data were analyzed using FlowJo V11. (A) Representative images of flow cytometry results. (B) Mean cell viability % (n=5) as determined by ratio of cells negative for 7-AAD and FITC-Annexin-V staining. (C) Mean apoptosis % (n=5) determined by ratio of cells stained positive for FITC-Annexin-V and negative for 7-AAD. Bars indicate mean ± SEM. Means were analyzed using one-way ANOVA and Tukey's post-hoc test. * $P < 0.05$ vs SF only, † $P < 0.05$ vs Serum Media. ANOVA, analysis of variance; STS, sodium thiosulfate; SF, serum free media.

3.2 STS treatment improves early graft survival and function

To understand the protective potential of STS against cold IRI *in* vivo, we injected 150μM STS into UW solution during cold storage before undergoing rat renal transplantation. Animals that received grafts treated with STS during 24h cold storage exhibited significantly improved survival ($p < 0.05$) compared to those receiving UW-treated grafts, particularly in the first 3 days (Figure 5A). While UW-treated animals exhibited only 12.5% survival at POD 3, STS-treated animals exhibited 83% survival at POD 3 and still maintained the same survival rate by the end of the 14-day time course (Figure 5A). Blood and urine samples were collected on days 3, 5, 7, 10, and 14. STS treatment markedly improved graft function during the early post-transplant period compared to UW treatment. Both treatment groups exhibited significantly increased serum creatinine levels ($p < 0.05$) at POD 3 compared to Sham (Figure 5B). STS-treated animals showed a decline in serum creatinine levels to 219μmol/L compared to UW-treated animals whose serum creatinine remained at 442μmol ($p<0.05$) on POD 3. Interestingly, the serum creatinine level of STS-treated animals decreased towards baseline (Sham) until time of sacrifice at day 14, when it was comparable to that of Sham (Figure 5B). Both treatment groups exhibited significantly increase blood urea nitrogen levels ($p < 0.05$) at POD 3 compared to Sham (Figure 5C). STS-treated animals showed a decline in blood urea nitrogen levels to 56 mmol/L /L compared to UW-treated animals whose blood urea nitrogen level remained at 106 mmol/L ($p<0.05$) on POD 3. . Notably, the blood urea nitrogen level of STS-treated animals decreased towards baseline (Sham) until time of sacrifice at day 14, when it was comparable to that of Sham at POD 7 and onwards (Figure 5C).


A
100
50
0
Survival %
0
2
4
6
8
10
12
14
Time (Days)
*
†
UW
UW+STS
Sham


B
600
500
400
300
200
100
0
Serum [Creatnine] (umol/L)
3
5
7
10
14
Time (Days)
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*
UW
UW+STS
Sham

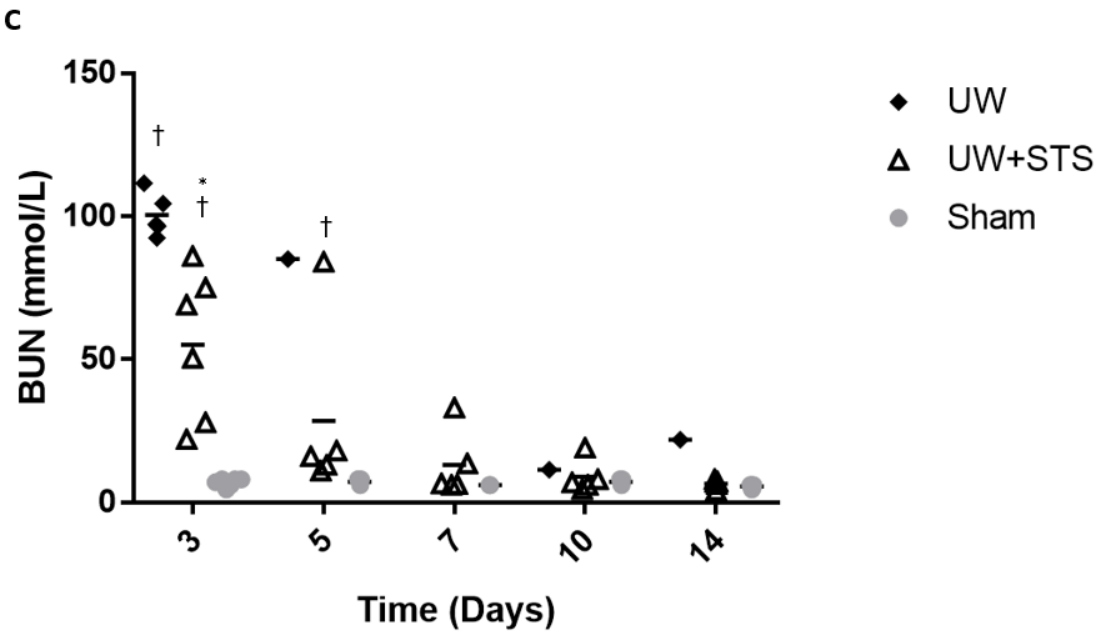
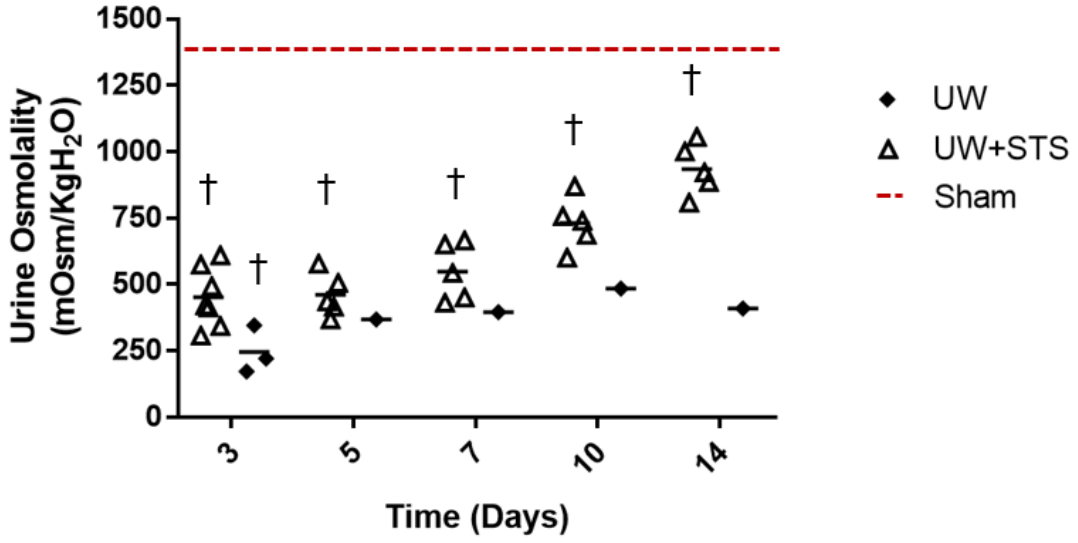


Figure 5: STS improves renal graft survival and function following 24h cold organ storage and syngeneic renal transplantation. Survival rates (A) of renal transplant recipients receiving donor kidneys perfused and stored in UW solution only (UW; black dashed line) or UW solution plus 150uM STS (STS; blue line) as well as Sham-operated rats (Sham; black line). UW (n=8), UW+STS (n=6), Sham (n=5). Serum creatinine levels (B) and blood urea nitrogen levels (C) of renal transplant recipients receiving donor kidneys perfused and stored in UW solution only (UW; black diamond) or UW solution plus 150uM STS (UW+STS; white triangle) as well as Sham-operated rats (Sham; gray circle). Survival data analyzed via Kaplan-Meier survival analysis and log rank test. Serum creatinine and BUN data analyzed via one-way ANOVA and Tukey's post-hoc test. Lines indicate mean serum creatinine and BUN. Note: only one UW rat survived to day 14. *$p<0.05$ vs UW, †$p<0.05$ vs Sham. ANOVA, analysis of variance; STS, sodium thiosulfate; UW, University of Wisconsin.

3.3 STS supplementation improves urine osmolality and induces diuresis after renal transplantation

To investigate the role of STS in total urine output and urine concentration, urine was collected on days 3, 5, 7, 10, and 14 after a cold IRI renal transplantation model *in vivo*. Both treatment groups UW and STS exhibited significantly decreased urine osmolality ($p < 0.05$) at POD 3 compared to Sham (Figure 6A). Animals that received grafts treated with STS during 24h cold storage exhibited urine osmolality levels that increased towards baseline (Sham) until time of sacrifice on day 14, although it was still statistically significant compared to Sham. STS led to an increase in urine output from 10 +/- 1mL to 32 +/- 5mL, 23 +/- 4mL, and 17 +/- 5mL following transplantation ($p<0.05$) during the first three collection periods compared with Sham-operated rats (Figure 6B). Interestingly, the mean urine output of STS-treated animals decreased towards baseline (Sham) until time of sacrifice at day 14, when it was comparable to that of Sham (Figure 6B).

A


1500
1250
1000
750
500
250
0
Urine Osmolality (mOsm/KgH2O)
†
3
5
7
10
14
Time (Days)
UW
UW+STS
Sham

B

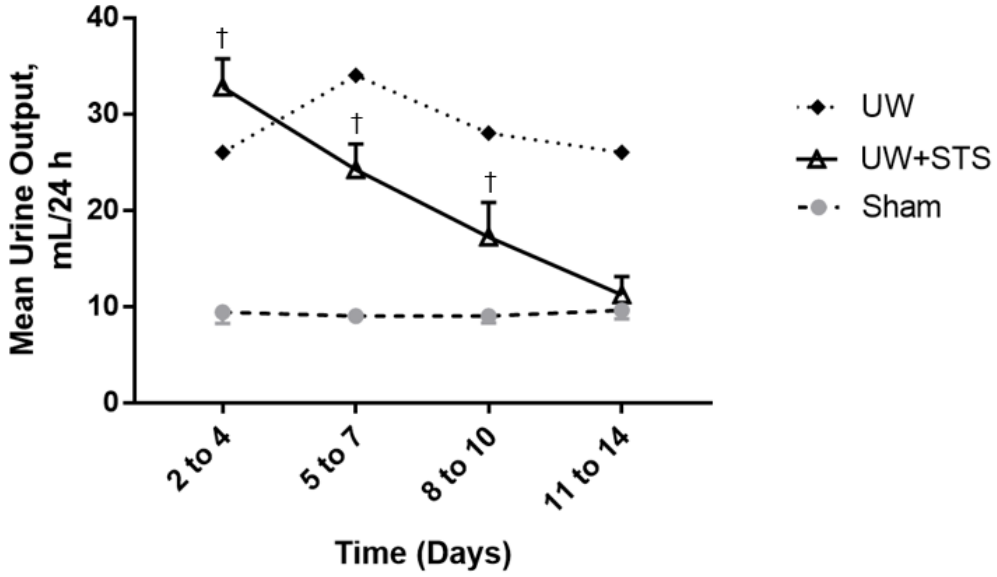

40
30
20
10
0
Mean Urine Output, mL/24 h
†
2 to 4
5 to 7
8 to 10
11 to 14
Time (Days)
UW
UW+STS
Sham

Figure 6: STS supplementation improves urine osmolality and induces diuresis after renal transplantation. Urine osmolality levels (A) of renal transplant recipients after receiving donor kidneys perfused and stored in UW solution only (UW: black diamond) or UW solution plus 150uM STS (UW+STS: white triangle). Osmolality levels were measured using the Model 3320 Osmometer and normalized to company provided standards. Red dash line indicates mean Sham osmolality levels. Urine output levels (B) of renal transplant recipients after receiving donor kidneys perfused and stored in UW solution plus 150uM STS (UW+STS: white triangle) and sham-operated rats (Sham: gray circle). Urine outputs were measured through use of metabolic cages. Note: only one UW rat (UW: black diamond) survived to day 14 and produced urine. Values are mean (SEM). † $P < 0.05$ vs Sham. STS, sodium thiosulfate; UW, University of Wisconsin.

3.4 STS treatment mitigates donor kidney apoptosis and necrosis after renal transplantation

Renal sections obtained at POD 3 and 14 were stained with TUNEL as a measure of apoptotic cell death and scored by a blinded pathologist (Figure 7A). It was revealed that while UW-treated grafts exhibited significantly elevated ($p< 0.05$) levels of apoptosis as indicated by higher TUNNEL score compared to Sham at POD 3, apoptosis level in UW+STS kidneys were not significantly different compared to Sham at the same time point and at POD 14 (Figure 7B). In addition, UW+STS grafts exhibited significantly decreased ($p < 0.05$) apoptosis levels to 5% compared to UW-treated grafts whose apoptosis levels remained at 24% following transplantation at POD 3 (Figure 7B).

Renal sections obtained at POD 3 and 14 were stained with H&E and showed that UW+STS grafts exhibited decreased ATN scores at POD 3 ($p < 0.05$) compared to UW (Figure 8). Both treatment groups showed significantly increased ATN scores ($p < 0.05$) at POD 3 compared to Sham (Figure 8). Also, whereas only one recipient of UW grafts survived to POD 14, and hence their ATN scores could not be determined on POD 14, those of UW+STS grafts survived on POD 14 but showed significantly increased ATN scores compared to Sham group (Figure 8; $p<0.05$).

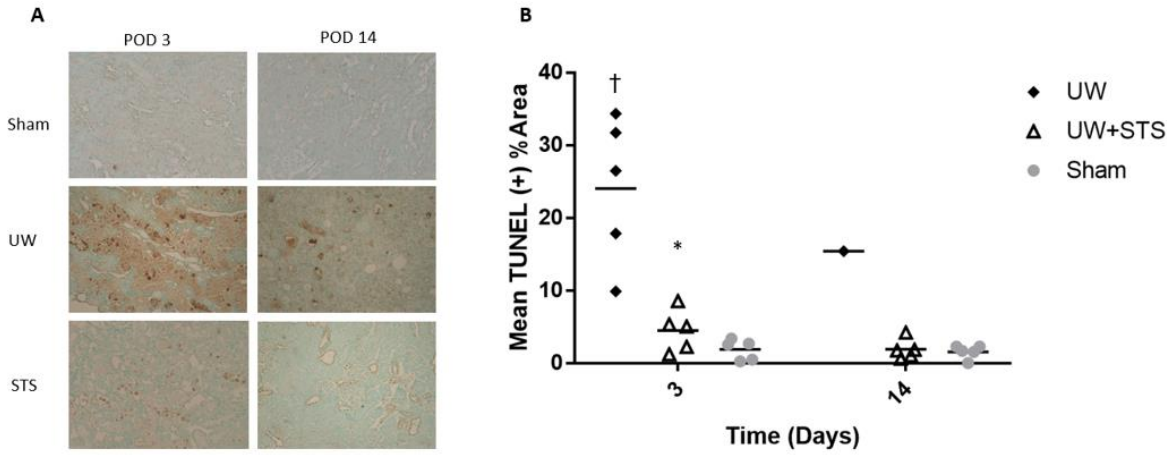


Figure 7: STS mitigates renal graft apoptosis after 24h cold organ storage and syngeneic renal transplantation. (A) Representative images show apoptosis in renal graft sections stored in UW only and UW + 150uM STS for 24 hours at 4C, and sham operated sections. Brown areas indicate fragmented DNA; 10x magnification. (B) Corresponding digital analysis show percent area of sections positive for TUNEL. Threshold limits were 0 lower/130 upper bytes. Sections were obtained at POD 3 (UW, n=5; UW+STS, n=5; Sham, n=5) and at POD 14 (UW, n=1; UW+STS, n=5; Sham, n=5). Line indicates mean TUNEL % area. * $P < 0.05$ vs UW day 3, † $P < 0.05$ vs Sham day 3. POD, postoperative day; STS, sodium thiosulfate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; UW, University of Wisconsin.

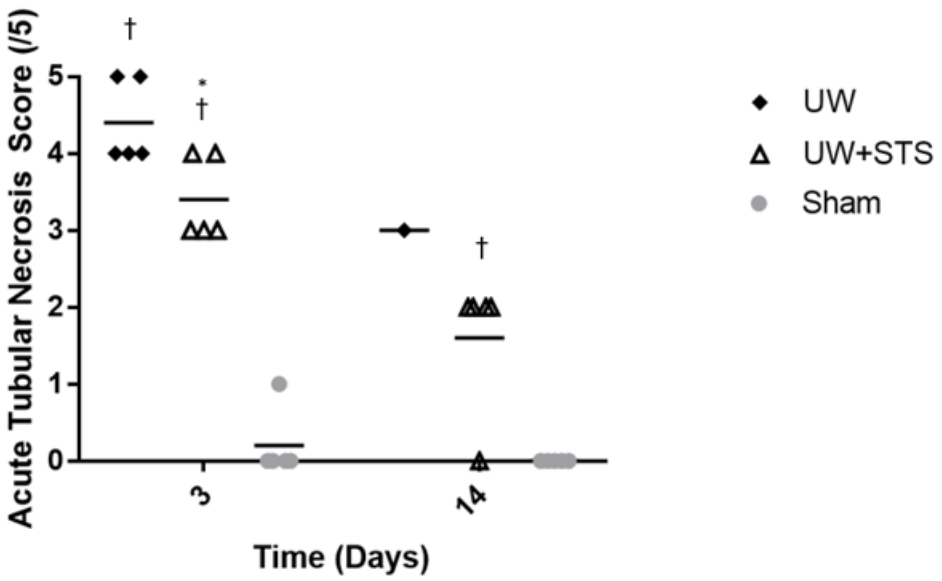


Figure 8: STS mitigates renal graft necrosis scores after 24h cold organ storage and syngeneic renal transplantation. Representative graph show ATN in renal graft sections stored in UW only and UW + 150uM STS for 24 hours at 4C, and sham operated sections. ATN scores were assigned to H&E sections, including 0 - 0% graft ATN, 1 – less than 10%, 2 – 11% to 25%, 3 – 26% to 45%, 4 – 46% to 75%, and 5 – greater than 75%. Sections were obtained at POD 3 (UW, n=5; UW+STS, n=5; Sham, n=5) and at POD 14 (UW, n=1; UW+STS, n=5; Sham, n=5). Line indicates mean H&E score. * $p < 0.05$ vs UW day 3, † $p < 0.05$ vs Sham POD 3. ATN, acute tubular necrosis; POD, postoperative day; STS, sodium thiosulfate; UW, University of Wisconsin.

3.5 STS treated kidneys exhibited decreased injury markers and inflammatory infiltrate after renal transplantation

To determine the effect of STS treatment on renal graft injury, renal sections obtained at POD 3 and 14 were stained with kidney injury molecule-1 (KIM-1) (Figure 9A) to detect proximal tubular injury and revealed that while UW-treated grafts exhibited significantly elevated ($p < 0.05$) levels of KIM-1 expression compared to Sham at POD 3, KIM-1 expression level in STS-treated kidneys were not significantly different compared to Sham at the same time point and at day 14 (Figure 9B). In addition, STS-treated grafts exhibited significantly decreased ($p < 0.05$) KIM-1 levels to 3% compared to UW-treated grafts whose KIM-1 levels remained at 21% following transplantation at POD 3 (Figure 9B).

Renal grafts obtained at POD 3 and 14 were immunohistochemically stained with antibodies against macrophage marker CD68 (Figure 10A) and neutrophil marker myeloperoxidase (MPO) (Figure 11A). UW-treated grafts exhibited significantly increased ($p < 0.05$) numbers of both CD68-positive and MPO-positive cells compared with Sham-operated rats at POD 3, while STS-treated grafts showed significantly fewer ($p < 0.05$) numbers of CD68-positive and MPO-positive cells at day 3 post transplantation, compared with UW-treated grafts (Figure 10B and 11B). STS led to a decrease in CD68 area stain from 20% to 4% and a decrease in MPO area stain from 18% to 3% following transplantation on POD 3.

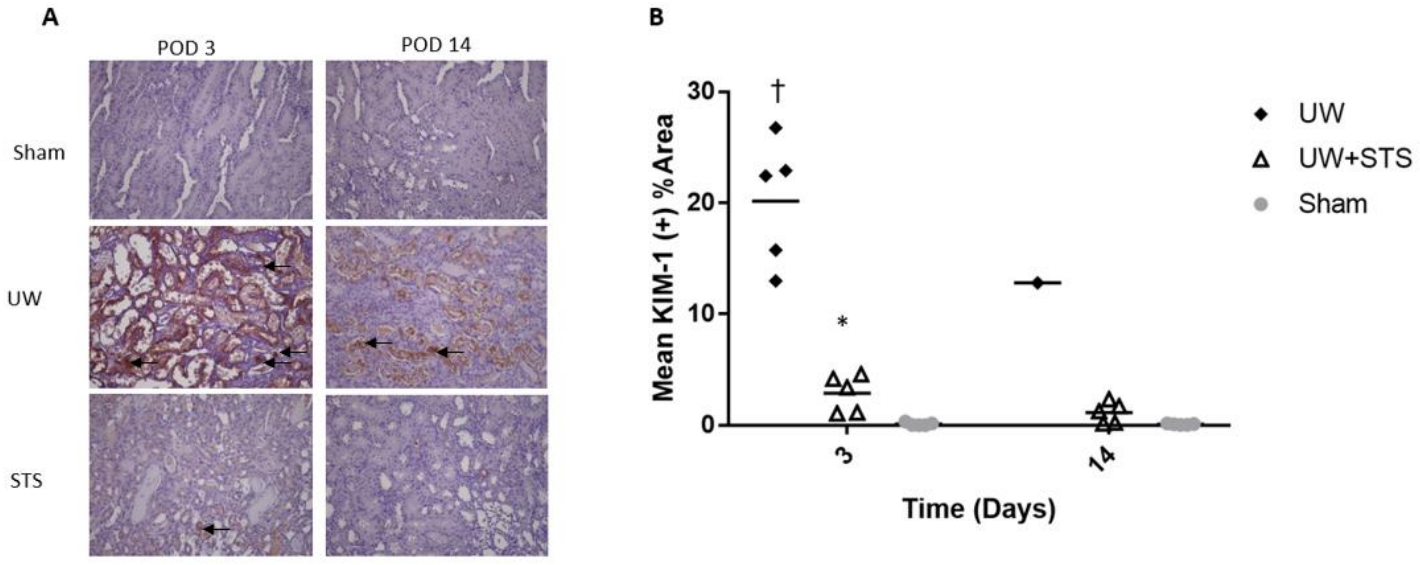


Figure 9: STS mitigates renal graft expression of KIM-1 after 24h cold organ storage and syngeneic renal transplantation. Representative images (A) show immunohistochemical staining for renal biomarker of injury KIM-1 (brown areas) of renal graft sections stored in UW only and UW + 150uM STS for 24 hours at 4C, and sham operated sections; 10x magnification. (B) Corresponding digital analysis show percent area of sections positive for KIM-1. Threshold limits were 0 lower/110 upper bytes. Sections were obtained at POD 3 (UW, n=5; UW+STS, n=5; Sham, n=5) and at POD 14 (UW, n=1; UW+STS, n=5; Sham, n=5). Line indicates mean KIM-1 % area. * $P < 0.05$ vs UW day 3, † $P < 0.05$ vs Sham day 3. POD, postoperative day; STS, sodium thiosulfate; UW, University of Wisconsin.

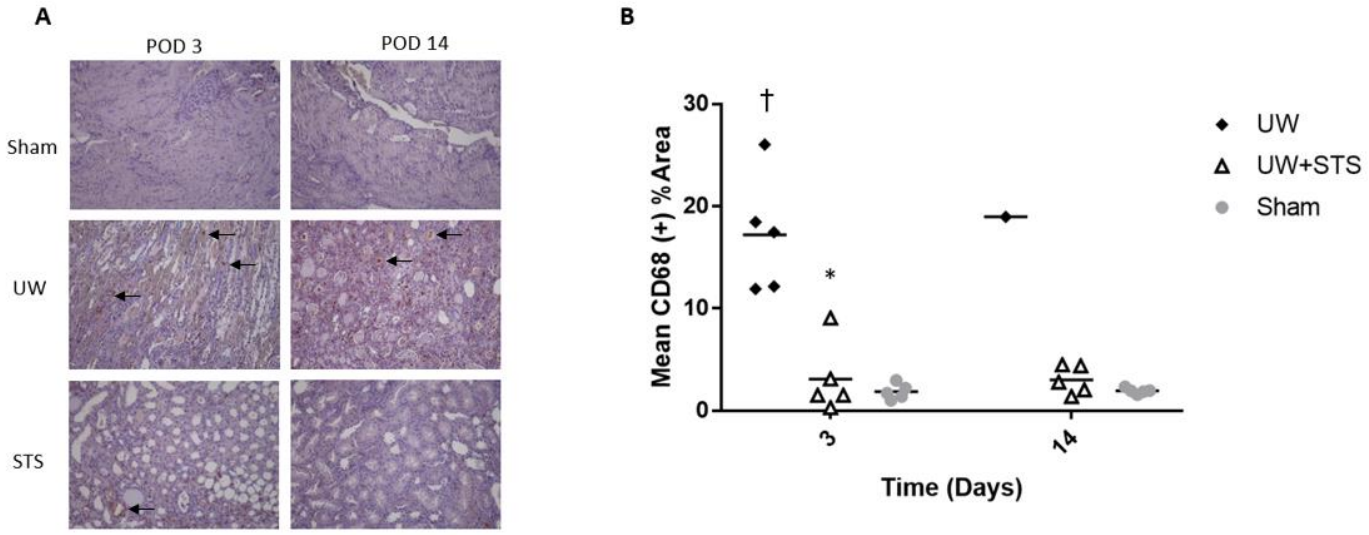


Figure 10: STS decreases macrophage infiltrate in renal grafts after 24-hour cold organ storage and syngeneic renal transplantation. Representative images (A) show immunohistochemical staining for macrophage marker CD68 of renal graft sections stored in UW only and UW + 150uM STS for 24 hours at 4C, and sham operated sections; 10x magnification. (B) Corresponding digital analysis show percent area of sections positive for CD68. Threshold limits were 0 lower/110 upper bytes. Sections were obtained at POD 3 (UW, n=5; UW+STS, n=5; Sham, n=5) and at POD 14 (UW, n=1; UW+STS, n=5; Sham, n=5). Line indicates mean CD68 % area. * $P < 0.05$ vs UW day 3, † $P < 0.05$ vs Sham day 3. POD, postoperative day; STS, sodium thiosulfate; UW, University of Wisconsin.

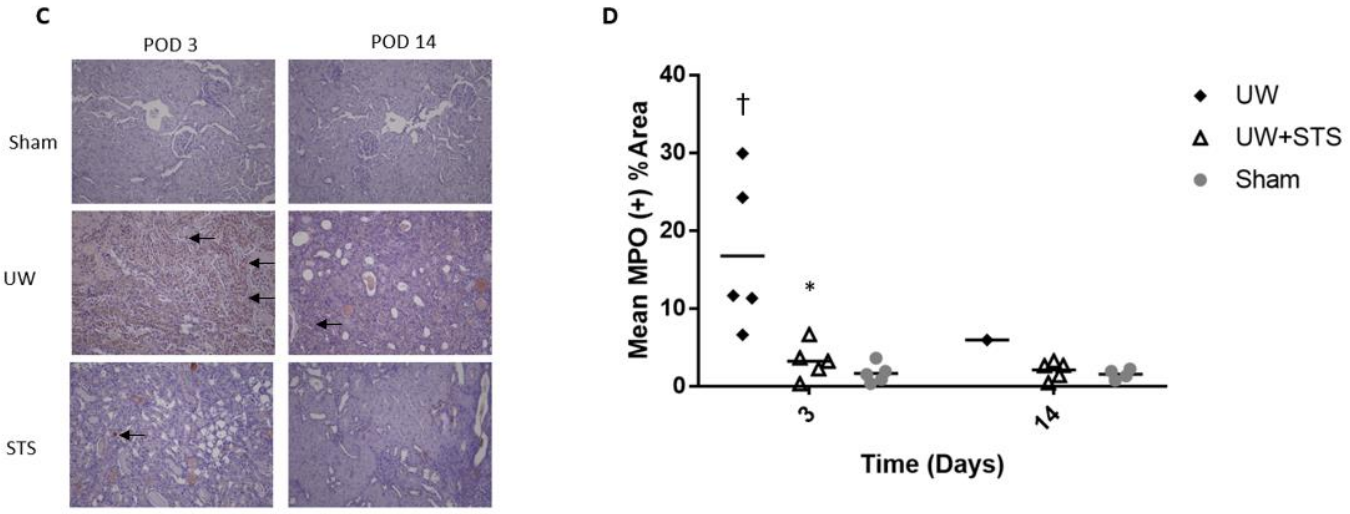


Figure 11: STS decreases neutrophil infiltrate in renal grafts after 24-hour cold organ storage and syngeneic renal transplantation. Representative images (A) show immunohistochemical staining for macrophage marker MPO of renal graft sections stored in UW only and UW + 150uM STS for 24 hours at 4C, and sham operated sections; 10x magnification. (B) Corresponding digital analysis show percent area of sections positive for MPO. Threshold limits were 0 lower/110 upper bytes. Sections were obtained at POD 3 (UW, n=5; UW+STS, n=5; Sham, n=5) and at POD 14 (UW, n=1; UW+STS, n=5; Sham, n=5). Line indicates mean MPO % area. * $P < 0.05$ vs UW day 3, † $P < 0.05$ vs Sham day 3. POD, postoperative day; STS, sodium thiosulfate; UW, University of Wisconsin.

3.6 STS treated grafts modified renal expression of pro-inflammatory, pro-apoptotic, and mitochondrial markers

Renal expression of pro-inflammatory, pro-apoptotic, mitochondria targeted, and kidney injury markers was determined via qRT-PCR on kidneys obtained at day 3 post-transplantation. Genes were normalized against beta-actin and fold changes of gene expression were compared with Sham-operated rats. Expression of pro-inflammatory genes PARP, IFN-y, TNF-α and IL-6 were markedly decreased in STS-treated grafts, with IFN-y, TNF-α and IL-6 expression being significantly decreased compared with UW-treated grafts ($p< 0.05$, Figure 12A). Relative expression of anti-apoptotic Bcl-2 was markedly increased in the STS group compared with that in the UW group, although this increase did not reach statistical significance (Figure 12A). Expression of pro-apoptotic genes BAX, caspase 3, BID, JNK1, and JNK2 were markedly decreased in STS-treated grafts, with BAX, caspase 3, and JNK2 expression being significantly decreased compared with UW-treated grafts ($p< 0.05$, Figure 12A). Expression of mitochondria genes peroxisome proliferator-activated receptor gamma coactivator (PGC) 1α, NDUFB8 (complex I), SDHB (complex II), and nuclear factor erythroid 2-related factor 2 (Nrf2) in STS-treated grafts were significantly increased compared with UW-treated grafts ($p < 0.05$, Figure 12B). Expression of anti-apoptotic genes ERK1 and ERK2 in STS-treated grafts were significantly increased compared with UW-treated grafts ($p < 0.05$, Figure 12B). Expression of kidney injury molecule KIM-1 in STS-treated grafts were significantly decreased compared with UW-treated grafts ($p < 0.05$, Figure 12C). Relative expression of kidney injury marker NGAL showed a decreasing trend in the STS group compared with that in the UW group, although this decrease did not reach statistical significance (Figure 12C).

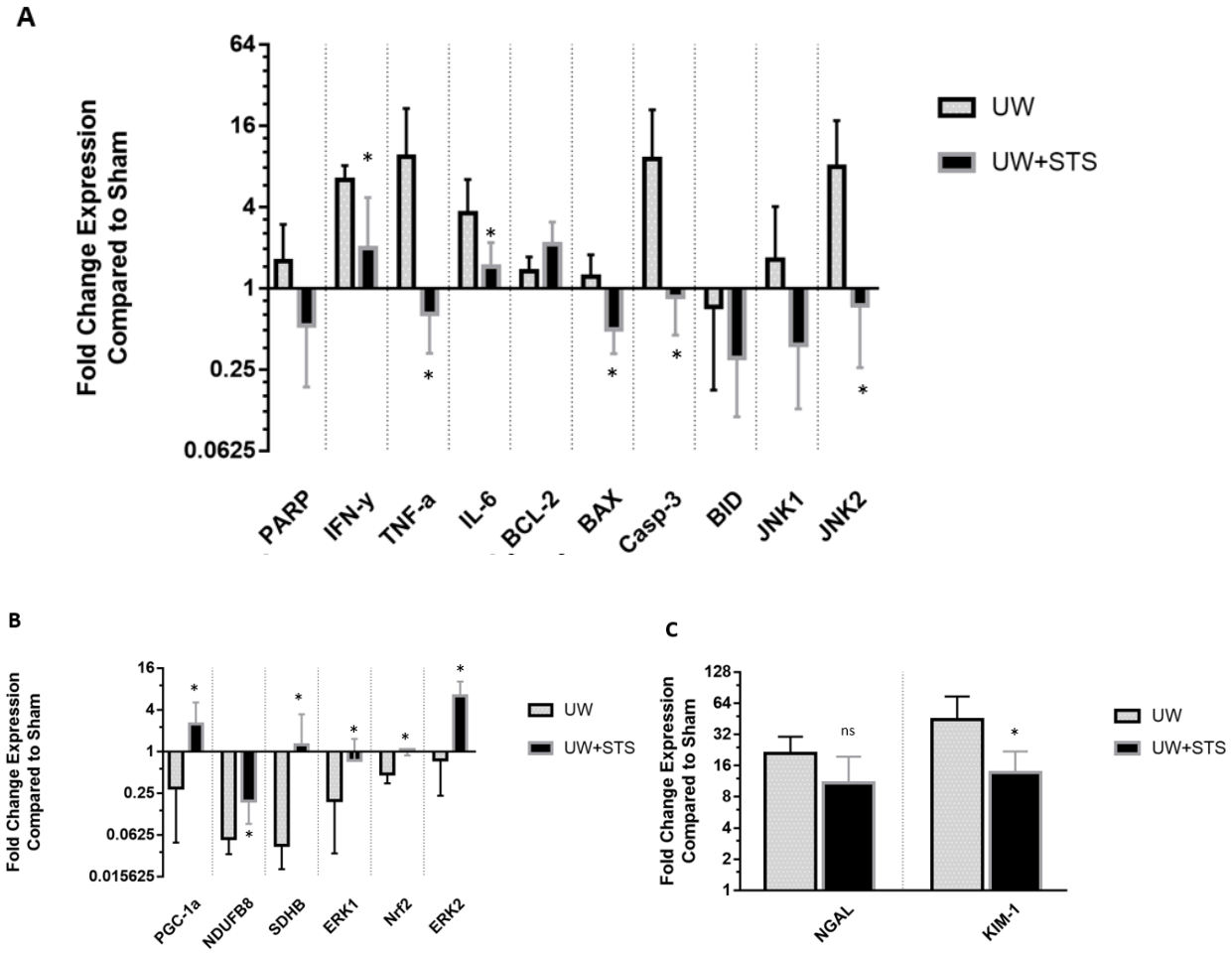


Figure 12: STS modulates renal graft expression of inflammatory and apoptotic genes. qPCR analysis of renal graft homogenates for expression levels of (A) pro-inflammatory genes (PARP, IFN-y, TNF-a, and IL-6), anti-apoptotic genes (Bcl-2, ERK1, and ERK2), pro-apoptotic genes (BAX, caspase-3, BID, JNK1/2), (B) mitochondrial genes (PGC-a, complex I, complex II, Nrf2) (C) and kidney injury markers (NGAL, KIM-1). Genes were normalized against beta-actin and fold changes of gene expression were compared with Sham-operated rats. Renal grafts were perfused and stored in UW solution only (UW) or UW solution plus 150uM STS (UW+STS) and obtained at post-operative day 3. Values are mean log2 fold change (SD). * $p < 0.05$ vs UW POD 3. POD, postoperative day; qPCR, quantitative polymerase chain reaction; STS, sodium thiosulfate; UW, University of Wisconsin.

Chapter 4

4 Discussion

When prolonged, ischemia reperfusion injury (IRI) is a substantial concern because renal IRI has been associated with acute graft rejection, delayed graft function, and early graft loss following renal transplantation [8,9]. This study establishes supplementation of standard preservation solution with sodium thiosulfate (STS), a clinically viable FDA-approved H_2S donor, to mitigate transplant-induced cold renal IRI, improve graft function and prolong graft survival. Using an *in vitro* model of renal IRI and rat model of syngeneic orthotopic kidney transplantation, we demonstrate for the first time that supplementation of University of Wisconsin (UW) solution with STS during prolonged cold storage is cyto- and organ protective.

The primary findings in our *in vitro* model are that STS supplementation to serum free media protects renal epithelial cells from cold hypoxia and warm reoxygenation-induced apoptosis in a dose dependent manner (Figure 3A). Our findings were in agreement with previous studies where they showed the protective effects of the mitochondria-targeting H_2S donor drug, AP39, in an *in vitro* model of cold IRI [19]. Interestingly, a higher STS concentration of 1mM reversed the beneficial effects, implying that STS exhibits a biphasic dose-response phenomenon referred to as hormesis, in which a higher concentration is cytotoxic while a lower concentration is cytoprotective. Mitochondrial damage is a major consequence of renal IRI, as mitochondrial permeability can inhibit ATP production and increase ROS production, a devastating mediator of tissue injury [110]. It has been recently suggested that mitochondria are a primary site of STS activity. Not only is STS known to generate H_2S in the mitochondria via glutathione-dependent reduction and vice versa via the sulfide oxidation pathway, recent studies have shown that STS can preserve mitochondrial ATP synthesis, decrease ROS production, and improve ETC complex enzyme activities [80,84,96,103]. In addition, recent studies showed that STS significantly increased the expression of PGC-1α, a positive regulator of mitochondrial biogenesis and ATP production [77, 104]. The improved mitochondrial enzyme activity and antioxidant protectiveness was supported by an increased expression of PGC-1α in the rat hearts treated with STS, compared to non-treated hearts [104]. These

findings are consistent with our qPCR results, which showed that STS treated grafts had significantly elevated expression of PGC-1α compared to UW grafts. Interestingly, both STS and PGC-1α can activate nuclear factor erythroid-related factor 2 (Nrf2), a transcription factor known to have antioxidant characteristics [77, 111]. Under normal circumstances, Keap1 proteins captures Nrf2 and holds it in the cytoplasm [111]. However, the reactive sulfur atom of STS triggers a sulfhydration reaction and forces Keap1 to undergo a structural change, leading to the release of Nrf2 and its translocation into the nucleus in order to bind to antioxidant response elements (ARE) to induce expression of numerous antioxidant gene clusters [112-114]. The elevated antioxidants are responsible for protecting against lipid peroxidation and maintaining redox balance [115]. Therefore, Nrf2 can be considered as a negative regulator of ferroptosis and promoter of resistance against ferroptotic cell death. These findings reflect our qPCR results, where STS-treated grafts exhibited significantly elevated expression of Nrf2 compared to UW grafts. These observations may be the reason why STS treated grafts had significantly increased expression of mitochondrial ETC complex I- II as well has increased graft survival and function compared to UW grafts. Overall, these results show that STS therapy is effective against cold IRI *in vitro*, which may be due to its numerous protective characteristics of mitigating ROS, enabling mitochondrial biogenesis and mitigating cellular apoptosis.

Based on the *in vitro* results, we decided to investigate the effects of STS in an *in vivo* murine model of cold renal syngeneic transplantation-associated IRI. Our findings show, for the first time, that treatment of donor kidneys with STS during prolonged cold (4^0C) storage significantly improved graft function, shown by decreased serum creatinine and blood urea nitrogen (BUN) levels, higher urine output and prolonged recipient survival compared to kidneys stored in UW solution alone. The observed improvement in renal function after transplantation of grafts preserved in STS-supplemented UW solution conferred a significant survival advantage. Quantitatively, STS treatment prolonged the life of the graft such that 83% of STS treated animals survived from POD 6 and onwards compared to only 12.5% of UW treated animals. Our model of renal transplantation IRI involved an extreme (24 hours) period of cold ischemia, which previous studies have shown to be associated with a survival rate of 14% when stored in UW solution alone and is shown by the observation that only one UW animal in our study survived until POD 14

[62]. Our study showing that STS increases renal graft survival after an extreme period of cold organ storage when UW solution alone was not sufficient to preserve the graft organ, is an important clinical observation. The length of cold IRI is proportional to the incidence of DGF as well as decreased long-term graft survival [35]. Considering that previous studies have shown that the risk for DGF rose significantly for donor kidneys experiencing >22 hours of cold ischemia, the consequences of lengthy cold storage time on renal graft survival and function are a real clinical concern [116]. It is important to note that the immediacy of urine output after clinical transplantation is a critical outcome which determines whether dialysis is required to address delayed graft function (DGF). Thus, our findings that STS increases urine output immediately after transplantation that is comparable to the Sham group on POD 14 is a promising finding. Thus, the future benefit of adding STS to preservation solutions may present a potential solution to this ongoing issue.

Histological staining studies showed that STS treatment significantly decreased apoptosis and acute tubular necrosis (ATN) compared with UW treatment alone, which is likely a mechanism by which STS improves graft survival and function. We also found that STS treatment significantly improved the up-regulation in renal graft expression of the anti-apoptotic gene ERK1/2 and significantly decreased expression of pro-apoptotic gene BAX, caspase 3, and JNK2. This observation may explain the decreased levels of apoptosis in STS-treated renal grafts compared to UW treated grafts observed via TUNEL staining. Our findings also concur with recent evidence that showed how STS treatment can counteract multiple pro-apoptotic mechanisms. Mechanistically, STS can inactivate caspase-3 by preventing access of natural substrate to the active site by attaching to the active site of caspase-3 via strong hydrogen bonds, ultimately halting apoptosis [106]. STS also inhibits phosphorylation of JNK, a protein that is upregulated by inflammatory cytokines plays a critical role in apoptotic signaling [112]. Although it was not statistically significant, treatment with STS slightly reduced pro-apoptotic genes BID and JNK1 and slightly mitigated anti-apoptotic gene BCL-2. A recent study by Chou *et al*. investigated the role of STS on renal apoptosis-related mechanism and found that STS treatment significantly reduced renal BAX expression compared to the non-treatment group, which aligns with our findings [117]. Interestingly, the renal Bcl-2 expression in STS treatment

group had an elevated trend compared to non-treatment group, which also supports our finding [117]. Other studies showed that STS treatment prevented the reduction of Bcl-2 compared to non-treatment groups and could also elevate Bcl-2 expression via activation of Nrf2-dependent signaling, contrasting our observations [106, 112]. This discrepancy may be attributed to insufficient time for the modulation of Bcl-2 transcription to show as increased protein translation prior to analyzing it. Further immunohistochemical analysis of renal graft injury showed that STS reduced expression of KIM-1, an important biomarker that provide additional sensitive assessment of early renal injury along with apoptosis and ATN [118, 119]. This observation also concurs with our findings of significantly decreased expression of KIM-1 in STS treated grafts compared to UW grafts. Being a type 1 transmembrane protein, KIM-1 expression level is significantly elevated in the proximal tubule in patients with post-ischemic kidney [120, 121]. Mechanistically, ischemia initiates phosphorylation and translocation of STAT3 to the nucleus, which binds to the KIM-1 promoter and elevates its mRNA and protein levels [118, 122]. During cold IRI, the KIM-1 ectodomain, which is comprised of an immunoglobulin and a mucin domain, sheds and is subsequently secreted into the urine and blood [121]. In a recent study by Han *et al.,* they showed that ischemic ATN was the most common predisposing factor for elevation of KIM-1 protein [123], which supports our findings that STS-treated kidneys exhibited lower ATN scores and thus, a lower KIM-1 expression level. Although it was not statistically significant, grafts treated with STS exhibited slightly reduced expression of NGAL, another important kidney injury biomarker, compared to UW grafts [124]. These findings are not surprising as previous studies have shown that decreased levels of KIM-1 and NGAL are associated with less renal graft injury and longer graft survival [124].

As discussed earlier, a potential mechanism by which STS improves graft survival and function is the reduction of apoptosis and necrosis during cold IRI. A mediator of such apoptotic and necrotic tissue injury is the subsequent surge in inflammatory cytokines and activation of immune system [125]. This study found significant reduction in CD68-positive macrophages and MPO-positive neutrophils present in STS treated renal grafts compared to UW grafts as well as significant downregulation of pro-inflammatory genes IFN-y and TNF- α. These inflammatory cytokines and recruitment of the innate immune system are known to be mediators of cell death during cold IRI [17], and their reduction in

UW+STS grafts is likely attributed to the well-known characteristics of STS to decrease the endothelial permeability in vascular endothelial monolayer, attenuate TNFα-induced cytokine production, and elicit production of anti-inflammatory cytokines [90]. In addition, the downregulation of pro-inflammatory gene IL-6 was observed in the STS group relative to the UW group, which matches the findings of a previous study on the anti-inflammatory activity of STS by reducing levels of TNF-α and IL-6 in neurological diseases [126, 127]. IL-6 is a key proinflammatory cytokine in its role in acute kidney injury [128], and its downregulation by STS may contribute to the improved graft survival and function of the STS treated grafts compared to the UW grafts. A proposed mechanism of how STS exerts its cytoprotective effects in prolonged cold renal IRI in transplantation is shown in Figure 13.

The *in vitro* cold renal IRI model is a relevant model to investigate the protective effects of STS-supplemented UW solution during hypoxic storage, followed by reperfusion [129]. The use of this model allowed us to induce a hypoxic (0.05% O_2) environment with the use of a hypoxic chamber (HypOxystation H85, USA) for 24 hours to mimic a prolonged cold storage time that we might see clinically today. A major limitation of this model is that the temperature of cold storage is 10^0C, whereas the current standard preservation method for SCS utilizes 4^0C [34]. However, 10^0C was the lowest temperature that could be technologically achieved without jeopardizing the hypoxic environment. A potential solution is to use chemically induced hypoxia in a plastic bag and place it in a 4^0C fridge in order to reflect clinical settings of SCS. However, these anaerobic atmosphere generation bags were designed to be used at warm temperatures (21^0C-37^0C) since the chemical compounds that induce the hypoxic environment functions under that condition [130]. Future studies will be aimed at optimizing the temperature in the hypoxia chamber to mimic clinical SCS settings. Achieving a consistent temperature of 4^0C without sacrificing a hypoxic environment will allow us to determine whether the observed phenotypes in the experimental *in vitro* cold IRI model are consistently expressed. Another limitation to this model was adding STS to serum free (SF) media for treating cold ischemia, which does not reflect the current gold standard preservation solution for SCS of kidneys: UW solution [48]. Interestingly, other studies with a similar *in vitro* model of SCS against renal IRI also chose to utilize other solutions for preservation, such as SF or PBS

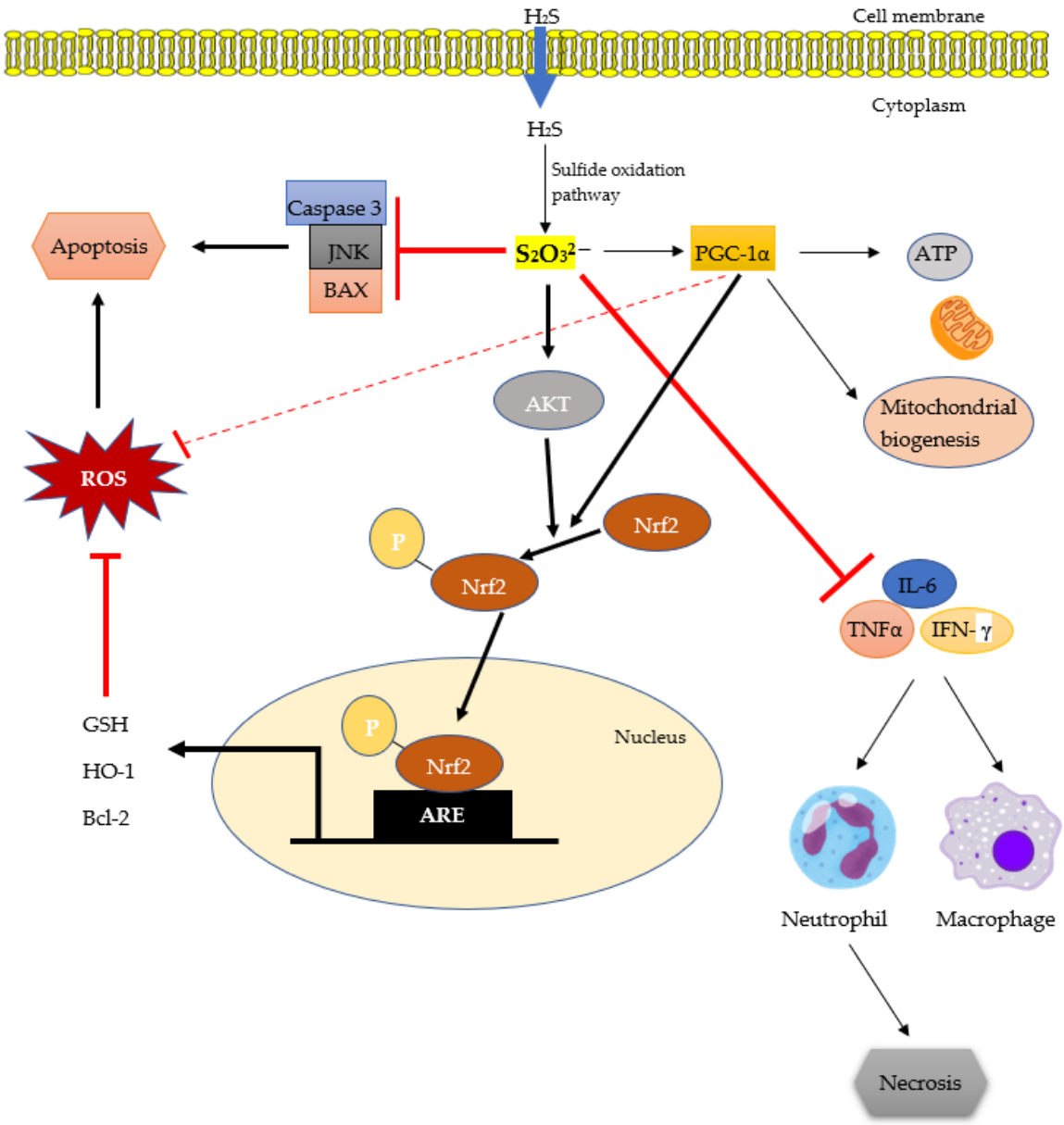


Figure 13: Proposed mechanism of action of STS in prolonged cold renal IRI in transplantation. Thiosulfate ($S_2O_3^{2-}$) is produced from hydrogen sulfide (H_2S) via the sulfide oxidation pathway. We propose that thiosulfate acts by attenuating inflammatory cytokines and pro-inflammatory genes, leading to decreased recruitment of innate immune system. Thiosulfate also increase expression of PGC-1α, a positive regulator of mitochondrial biogenesis and ATP production. Nuclear translocation of phosphorylated Nrf2 binds ARE and promotes expression of anti-apoptotic and antioxidative genes, ultimately leading to decreased ROS production and cell injury.

[62]. This could be due to the irony that UW solution is doing its protective job too well during SCS against renal IRI, thus it's hard to display a change in cell viability from treatment groups vs. non-treatment groups. In addition to measuring apoptosis and necrosis with flow cytometry, future studies will use JC-1, a stain that quantifies mitochondrial membrane permeability [131] since mitochondrial dysfunction is an fundamental part of renal IRI.

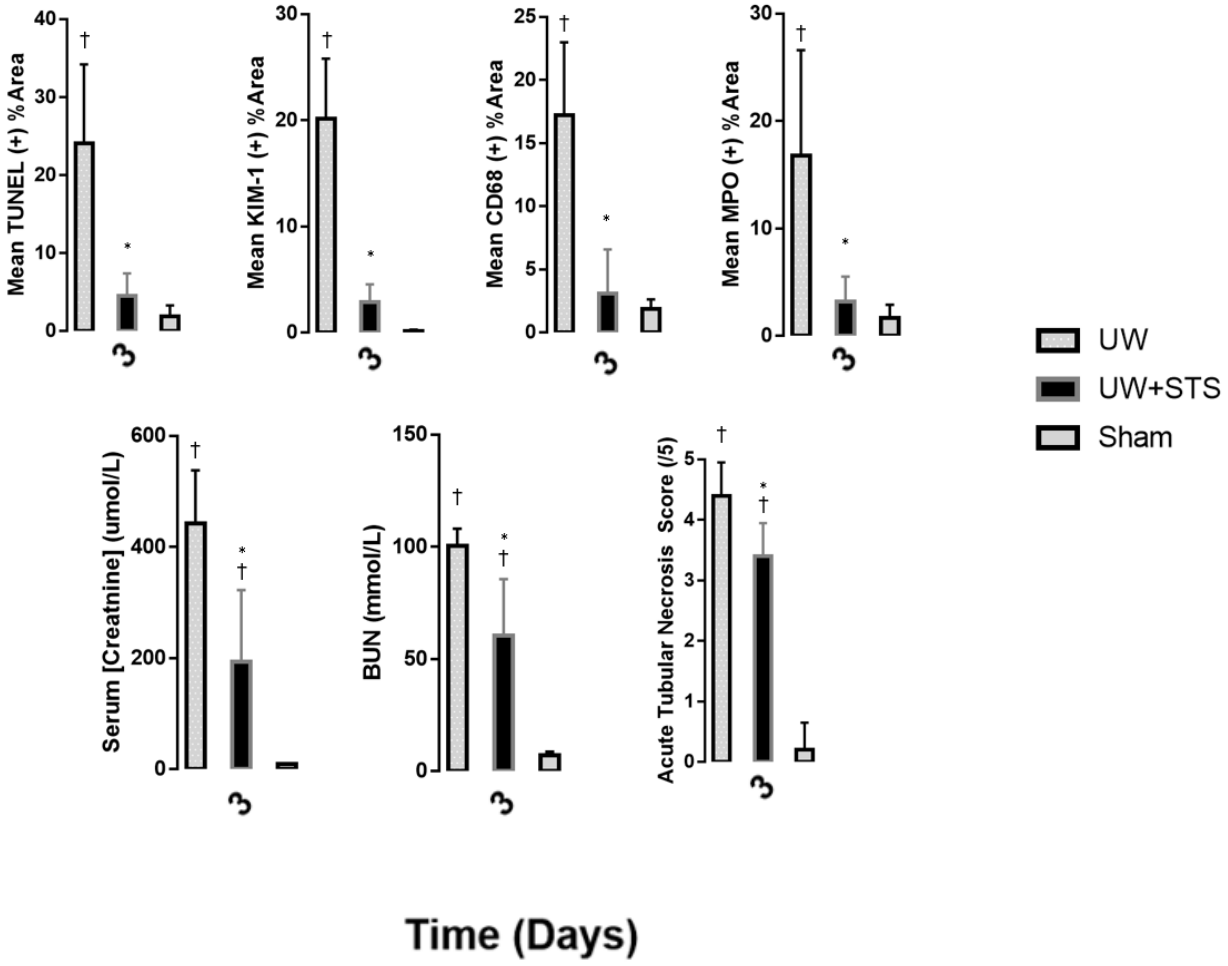
The rat *in vivo* model of cold IRI is a a relevant model to investigate the protective effects of STS on kidney survival and function following renal transplantation. It mimics a human kidney donor-recipient transplantation in a clinical setting and allows us to analyze urine and blood data. However, a drawback is that our model involves syngeneic transplantation, meaning that the donor and recipient rats are genetically identical and are thus, immunologically compatible as to allow for transplantation. This syngeneic concept only applies to identical twins, whereas most of the renal transplantations that are done clinically have the donor and recipient being genetically different. This forces patients to undergo immunological tests prior to transplantation to identify the donor's HLA antigens and determine if the donor and recipient are compatible [132]. These tests are done to avoid the recruitment of both the innate and adaptive recipient immune response against the kidney allograft due to mismatched antigens present on donor tissue, ultimately resulting in increased graft injury and lowered graft survival compared to syngeneic transplantation [133]. Future studies will be aimed at determining the effect of STS on an *in vivo* cold IRI model of renal allogeneic transplantation. Another limitation to this model was the duration of SCS being 24 hours, which represented a prolonged cold IRI period reflecting the organ being transported from one transplant center to another. On the other hand, if the living donor and recipient were in the same building, the cold storage time would be diminished to 4-6 hours. Thus, future studies should explore the protective effects of STS on cold renal IRI with various timepoints for SCS prior to transplanting the kidney in the recipient to mimic clinical settings. Finally, another limitation in this model is that it only focused on living donors, whereas in the clinical setting sub-optimal grafts from donation after cardiac death (DCD) donors are being accepted for usage more frequently to increase the supply of donor kidneys [134]. DCD donors exposes donor organs to various periods of warm ischemia in addition to cold ischemia times during SCS and is associated with increased

rates of delayed graft function (DGF) and decreased graft survival compared to living donors [2]. Future studies will be aimed at incorporating a warm ischemic time in the *in vivo* rat kidney transplantation model to assess STS on treating DCD kidneys that are more susceptible to damage associated with IRI.

In conclusion, our results show a novel discovery which demonstrates that supplementation of standard preservation solution with a clinically viable H_2S donor drug during prolonged SCS of renal grafts protects against transplant-induced cold renal IRI. We have shown that STS treatment improves overall graft quality and graft function and prolongs transplant recipient survival. Furthermore, these protective effects of STS appear to be mitochondria-targeted and mediated by antioxidant, anti-apoptotic, and anti-inflammatory mechanisms. Considering that the risk of DGF increases with prolonged cold ischemic time in clinical kidney transplantation, which raises a major clinical concern, the discovery that STS protects renal grafts during prolonged SCS and prevents DGF after transplantation provides a great clinical promise that could decrease or prevent the incidence of DGF in clinical kidney transplantation. Thus, the future benefit of adding STS to preservation solutions may present a potential solution to this ongoing issue. Overall, this study adds to the growing body of literature that supports the cytoprotective effects of STS against organ IRI, particularly improving graft outcomes in prolonged transplant-induced cold kidney IRI. These strategies could facilitate the use of more grafts exposed to prolonged cold ischemic times, which could increase the pool of transplantable organs.

Appendices

Appendix A 1: STS helps improve early renal graft function and decreases apoptosis and inflammation following prolonged SCS and kidney transplantation.



Supplemental Figure 1: STS helps improve early renal graft function and decreases apoptosis and inflammation following prolonged SCS and kidney transplantation. Summary of data containing urine, blood, and graft sections obtained on POD 3. Serum creatinine and blood urea nitrogen (BUN) levels were obtained from blood on day 3. Kidney sections obtained on day 3 were stained with TUNEL, H&E, KIM-1, CD68, and MPO to measure apoptosis, necrosis, and inflammation respectively. Data are mean ± SEM. * $p < 0.05$ vs UW day 3, † $p < 0.05$ vs Sham POD 3.

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