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# Hydrogen sulfide protects renal grafts against prolonged cold ischemia-reperfusion injury via specific mitochondrial actions

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**Running Title:** Mitochondrial actions of $H_2S$ reduce renal IRI

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**Abbreviations:** 3-mercaptopyruvate sulfurtransferase (3-MST); Apoptosis inducing factor (Apaf); Acute tubular necrosis (ATN); Blood urea nitrogen (BUN); Cystathionine-beta synthase (CBS); Carbon monoxide (CO); Cystathionine-gamma lyase (CSE); Dihydrorhodamine 123 (DHR-123); Dulbecco's modified eagle medium (DMEM); Electron transport chain (ETC); Ethidium homodimer 1 (EthD-1); Fetal bovine serum (FBS); Hematoxylin and eosin (H&E); Hypoxia/hypercapnia and re-oxygenation (H/R); Hydrogen sulfide ($H_2S$); Hypoxia-inducible factor 1 (HIF-1); Ischemia-reperfusion injury (IRI); Mitochondrial permeability transition pore (MPTP); Nitric oxide (NO); Phosphate-buffered saline (PBS); Post-operative day (POD); Reactive oxygen species (ROS); Renal transplantation (RTx); Sulfide:quinone oxidoreductase (SQR); Tri-phenyl phosphonium cation ($TPP^+$); Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL); University of Wisconsin solution (UW).


## Abstract

Ischemia-reperfusion injury (IRI) is unavoidably caused by loss and subsequent restoration of blood flow during organ procurement and prolonged IRI results in increased rates of delayed graft function and early graft loss. The endogenously produced gasotransmitter, hydrogen sulfide ($H_2S$), is a novel molecule that mitigates hypoxic tissue injury. The current study investigates the protective mitochondrial effects of $H_2S$ during *in vivo* cold storage and subsequent renal transplantation (RTx) and *in vitro* cold hypoxic renal injury. Donor allografts from Brown Norway rats treated with University of Wisconsin (UW) solution + $H_2S$ (150 μM NaSH) during prolonged (24-hour) cold (4ºC) storage exhibited significantly ($p<0.05$) decreased acute necrotic/apoptotic injury and significantly ($p<0.05$) improved function and recipient Lewis rat survival compared to UW solution alone. Treatment of rat kidney epithelial cells (NRK-52E) with the mitochondrial-targeted $H_2S$ donor, AP39, during *in vitro* cold hypoxic injury improved the protective capacity of $H_2S$ >1000-fold compared to similar levels of the non-specific $H_2S$ donor, GYY4137 and also improved syngraft function and survival following prolonged cold storage compared to UW. $H_2S$ treatment mitigates cold IRI-associated renal injury via

mitochondrial actions and could represent a novel therapeutic strategy to minimize the detrimental clinical outcomes of prolonged cold IRI during RTx.

## Introduction

Organ procurement is inherently associated with ischemia-reperfusion injury (IRI), resulting from loss and subsequent restoration of blood flow, which leads to increased rates of delayed graft function, acute graft rejection and early graft loss (1,2). Current methods of limiting IRI during renal transplantation involve cold storage of kidneys in preservation solution during the peri-transplant period (3). However, these strategies have been maximized and prolonged periods (>24 hours) of cold IRI are still associated with increased rates of acute tubular necrosis (ATN), delayed graft function and decreased graft survival (4-9). There are many pathophysiological components of IRI, including reactive oxygen species (ROS) production, inflammation and induction of cellular apoptotic pathways, and it has been well established that mitochondrial damage and subsequent dysfunction is a key mediator of these injurious pathways (10). During ischemia, ATP depletion causes inhibition of mitochondrial $Na^+/K^+$ ion channels, resulting in increased mitochondrial inner membrane permeability, influx of $Ca^{2+}$ ions and subsequent swelling of mitochondria. In addition, prolonged periods of ischemia can permanently damage complexes in the electron transport chain (ETC), which are then prone to electron leak and produce a burst of ROS as oxygen floods cells upon reperfusion. High levels of mitochondrial ROS production combined with $Ca^{2+}$-induced mitochondrial swelling result in severe damage of mitochondrial membranes and formation of mitochondrial permeability transition pores (MPTP), releasing pro-apoptotic factors, cytochrome c and apoptosis inducing factor (Apaf), which then activate the caspase 9/3 apoptotic signaling cascade, initiating cellular apoptosis (11).

Treatment of donor kidneys with gasotransmitters has recently been identified as a potential therapeutic strategy to limit IRI during renal transplantation (12). Gasotransmitters are endogenously produced gaseous molecules that share similar physical and chemical characteristics. This family of molecules currently includes nitric oxide (NO), carbon monoxide (CO) and the most recently characterized member, hydrogen sulfide ($H_2S$) (13). Hydrogen sulfide is an endogenously produced gaseous molecule derived from cysteine by the actions of three major cellular enzymes, cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) (14). In addition to playing important physiological roles in cellular signaling and vasodilation (15,16), $H_2S$ has been found to exhibit anti-oxidant, anti-inflammatory and anti-apoptotic effects that protect kidneys during warm ischemic injury (17-20). Addition of $H_2S$ to organ preservation solution during cold organ storage is a promising therapeutic strategy to reduce graft injury associated with prolonged cold IRI.

We have previously shown that treatment of renal grafts with $H_2S$ mitigates IRI-associated graft injury and improves early graft function and survival in murine models of syngeneic renal transplantation (RTx) following prolonged (24-hour) cold storage (21) and allogeneic RTx following moderate (6-hour) cold storage (22). Therefore, to determine whether $H_2S$ can mitigate IRI in the most unfavourable clinical circumstances, the current study investigates the protective effects of $H_2S$ in the context of allogeneic RTx following prolonged (24-hour) cold organ storage. In addition, it has recently been identified that $H_2S$ may exert protective effects by translocating to mitochondria during hypoxia/ischemia, where it prevents oxidative damage and preserves mitochondrial membrane integrity (23-25). Accordingly, the current study also aims to

characterize renoprotective effects of $H_2S$ specific to mitochondria during *in vitro* hypoxic injury and *in vivo* cold IRI.

## Materials and Methods

*Experimental animals*

Male Brown Norway (n=56) and Lewis rats (n=52) were purchased from Charles River Canada and used at 300 – 350 grams and maintained at Western University according to standard conditions. Animal studies received ethics approval from the Western University Council for Animal Care.

*Allogeneic renal transplant surgical procedure and post-operative monitoring of rats*

Allogeneic renal transplantation was performed using left kidneys from Brown Norway rat donors and Lewis rat recipients, a model which elicits a robust recipient immune response against donor tissue. Rats were randomized into treatment groups, anaesthetized with ketamine (30 mg/kg) and maintained under anaesthesia with 1% isoflurane during surgery. Using aseptic techniques, donor kidneys were procured and flushed using a 28G angiocath with 1 mL of either cold (4 °C) University of Wisconsin (UW) preservation solution (UW group, n=8) or cold UW plus $H_2S$ donor molecule (150 μM NaSH (Sigma-Aldrich®); $H_2S$ group, n=8) until venous effluent was clear. Grafts were subsequently placed in 50 mL of the same perfusion solution and stored at 4 °C for 24 hours, which represents an extreme period of clinical cold storage time. Following bilateral nephrectomy, recipient rats underwent renal transplantation (RTx) with donor kidneys removed from cold storage and transplanted orthotopically into the left renal fossa using 10-0 prolene suture as previously described (26). Lewis sham operated rats (midline

incision only; n=5) were also followed to establish a baseline for survival, serum creatinine and histological analysis. After RTx, rats were monitored for a period of 14 days or until sacrifice to assess survival ratios and serum creatinine levels (determined using the enzymatic method performed on the Roche Modular P instrument). An additional subset of animals in the $H_2S$ group had allografts removed pre-emptively at post-operative day (POD) 2 (n=5) for comparison of histological analysis to UW animals that were sacrificed at this time point. Additionally, donor kidneys in each treatment group (n=8 per group) were obtained following cold storage, but prior to transplantation, to assess the protective effects of $H_2S$ immediately following cold storage. Donor kidneys obtained prior to cold storage were used as a baseline for this analysis (Pre-storage group; n=8). Following 24-hour cold storage, donor kidneys were perfused with 10 mL of 5 μM Ethidium Homodimer (EthD-1) at a rate of 1 mL/min and subsequently washed by perfusion with 5 mL phosphate-buffered saline (PBS) at a rate of 1mL/min. EthD-1 fluoresces once bound to double-stranded DNA and is a marker of cellular necrosis as it can only enter cells if the plasma membrane is compromised. In each arm of the study, renal grafts obtained at time of sacrifice were sagitally bi-valved and placed in formalin for histological analysis. All surgeries were performed by the same micro-surgeon and the length of surgery for the recipient was approximately 2-3 hours for both treatment groups. There was no difference in operative times between treatment groups. Renal failure was presumed in animals that required premature sacrifice (>20% weight loss and/or under severe visible distress) while exhibiting highly elevated serum creatinine levels (>300 μmol/L). At time of sacrifice surgical complications were ruled out by visual observation as a cause of death.

*Histological staining*

Histological sections were stained with hematoxylin and eosin (H&E) and were assessed for the presence of necrotic tubules that exhibit loss of nuclei and necrotic casts with a background of congestion, then assigned a score for ATN by a blinded transplant pathologist (ATN scores out of 5; 0 = 0% graft ATN, 1 = <10% graft ATN, 2 = 11-25% graft ATN, 3 = 26-45% graft ATN, 4 = 46-75% graft ATN, 5 = >75% graft ATN). To approximate acute allograft rejection, H&E sections were also assigned tubulitis (T), vasculitis (V) and mononuclear cell interstitial inflammation (I) scores according to the Banff 97 working classification of renal allograft pathology (27). Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was used to assess the degree of apoptosis present in renal allograft sections. For EthD-1 analysis, kidneys were sectioned, stained with the nuclear marker 4',6-diamidino-2-phenylindole (DAPI) and analyzed via fluorescent microscopy.

*Microscopic image analysis*

TUNEL and EthD-1 sections were analyzed using a Nikon Eclipse 90i digital light microscope and an Olympus IX83 Inverted Microscope, respectively. Five images were analyzed per section for TUNEL and EthD-1 analysis and total (+) staining per field of view was quantified by ImageJ software v 1.48 (National Institutes of Health, Bethesda, MD). Background was subtracted from images and the colour threshold (RGB) was adjusted uniformly to accentuate positively stained areas which were then measured digitally.

*In vitro model of renal epithelial cell hypoxia/hypercapnia and re-oxygenation.*

Underlying mechanisms of protective effects of $H_2S$ during renal IRI were assessed using a novel *in vitro* model of cold hypoxia/hypercapnia and warm re-oxygenation (H/R) injury that mimics cellular conditions during *in vivo* cold IRI. Rat kidney epithelial cells (NRK-52E cell line; ATCC) were cultured in DMEM + 5% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin at 37 ºC in room $O_2$ and 5% $CO_2$. During experimentation, control cells were cultured in identical conditions to pre-experimental cells. Experimental cells were treated with either PBS or PBS plus varying concentrations of the non-specific $H_2S$ donor, GYY4137, or the mitochondrial-targeted $H_2S$ donor, AP39, which were synthesised in-house as previously described (28,29). Cells were exposed to cold (12 ºC) hypoxia/hypercapnia (0.1% $O_2$/15% $CO_2$) for 24 hours, followed by replacement of experimental media with control media (no phenol red) and re-oxygenation in conditions identical to pre-experimental cells. We used 12 ºC for our hypothermic condition as this was the lowest temperature that could be technologically achieved while maintaining strict control of $O_2$ levels. Cellular ROS production was assessed via staining of cells with 10 µM Dihydrorhodamine 123 (DHR-123; Molecular Probes$^{TM}$) following 15 min re-oxygenation and mitochondrial membrane permeability was assessed via staining of cells with 5 µM JC-1 dye (Molecular Probes$^{TM}$) following 18 hours re-oxygenation. Following 24 hours re-oxygenation, cellular viability was measured via Annexin-V and 7-AAD staining, which assess cellular apoptosis and necrosis, respectively. Cells were analyzed via flow cytometry using the Beckman-Coulter FC 500 flow cytometer.

*In vivo model of prolonged cold organ storage and syngeneic renal transplantation.*

To assess protective effects of AP39 against renal graft IRI, Lewis rats underwent bilateral native nephrectomy and subsequent RTx with syngeneic donor kidneys flushed with either University of Wisconsin preservation solution (UW group; n=7) or UW + 200 nM AP39 (AP39 group; n=7) and stored for 24 hours at 4°C in the same solution. Sham surgeries (midline incision only; n=5) were also performed and animals were monitored for 14 days to assess graft function and survival. The concentration of AP39 selected for *in vivo* experimentation was the dose that demonstrated cytoprotection *in vitro* and was closest to 1000-fold less than the 150 μM NaSH used in the previous RTx experiment. Syngeneic RTx was utilized to eliminate confounding effects of graft rejection while assessing the novel therapeutic potential of AP39 in this injury model.

*Statistical Analysis*

Survival data were analyzed via Kaplan-Meier survival analysis, all other data were analyzed via ordinary one-way ANOVA and Tukey's post-hoc test performed using the GraphPad Prism statistical software package, version 6.0. Statistical significance was accepted at the 95% confidence interval. All values represented in figures are mean or median with minimum and maximum values.

# Results

*Hydrogen sulfide treatment improves early allograft survival and function.*

Animals that received allografts treated with $H_2S$ during cold storage exhibited significantly improved survival ($p<0.01$) compared to those receiving UW treated allografts (Figure 1A).

While UW treated animals exhibited only 50% survival at POD 2 and 0% survival at POD 6, $H_2S$ treated animals exhibited 50% survival at POD 7 and still maintained ~10% survival by the end of the time course (POD 14) (Figure 1A). $H_2S$ treatment markedly improved allograft function during the early post-transplant period compared to UW treatment. Both treatment groups exhibited significantly increased serum creatinine levels ($p<0.001$) at POD 2 and POD 4 compared to Sham (Figure 1B). However, while UW animals exhibited serum creatinine levels that only increased until POD 6 (0% survival) $H_2S$ treated animals exhibited serum creatinine levels that decreased toward baseline (Sham) until time of sacrifice (Figure 1B).

*Hydrogen sulfide mitigates donor kidney apoptosis and necrosis but not allograft rejection.*

Renal sections stained with TUNEL revealed that while UW treated allografts exhibited significantly elevated ($p<0.05$) levels of apoptosis compared to Sham at POD 2-4, apoptosis levels in $H_2S$ treated kidneys were not significantly different compared to Sham at the same time point (Figure 2). In addition, H&E staining showed that $H_2S$ treated allografts exhibited slightly decreased ATN scores at POD 2-4 compared to UW, which tended to increase as animals began to lose grafts (Figure 3A). To provide a more quantitative measure of whether $H_2S$ treatment prevents tubular necrosis associated with cold IRI, donor kidneys were analyzed via EthD-1 staining immediately following cold storage. Donor kidneys treated with only UW solution during cold storage exhibited significantly increased levels of EthD-1 fluorescence ($p<0.01$) compared to pre-storage kidneys, while $H_2S$ treated kidneys exhibited significantly decreased EthD-1 fluorescence ($p<0.05$) compared to UW kidneys (Figure 4). This indicates that $H_2S$ treated kidneys accumulated fewer necrotic cells compared to UW treated kidneys during prolonged cold storage. To determine whether $H_2S$ improves allograft survival by altering the progression of graft rejection, renal sections were scored for acute rejection using components of

the Banff 97 working classification of renal allograft pathology. $H_2S$ treated allografts exhibited significantly increased ($p<0.05$) inflammation, tubulitis and vasculitis scores at POD 6-14 compared to both UW and $H_2S$ kidneys at POD 2-4 (Figure 3B). Since scores >2 in these categories indicate acute allograft rejection, this observation suggests that UW treated animals lose grafts early in the time period due to renal failure, not rejection, and that $H_2S$ by itself does not modulate allograft rejection.

*Targeting of hydrogen sulfide to mitochondria increases potency of protective effects 1000-fold during in vitro cold renal H/R injury.*

NRK-52E cells treated with PBS during *in vitro* cold H/R injury exhibited significantly decreased cellular viability ($p<0.05$) compared to control (normoxic) cells. Treatment of cells with 200 nM and 400 nM AP39 during cold H/R injury significantly improved cellular viability ($p<0.05$) compared to PBS-treated cells, to levels similar to control cells. Cells treated with 100 nM, 200 nM, 400 nM and 100 μM GYY 4137 during cold H/R injury exhibited cellular viability levels that were significantly decreased ($p<0.05$) compared to cells treated with 400 nM AP39 and were not significantly different compared to PBS-treated cells (Figure 5B,C). Only cells treated with 400 uM GYY 4137 exhibited significantly improved cellular viability ($p<0.05$) compared to PBS-treated cells (Figure 5C). As well, cells treated with 400 nM AP39 during cold H/R injury exhibited significantly decreased ($p<0.0001$) levels of ROS production and significantly increased ($p<0.0001$) mitochondrial membrane potential compared to cells treated with either PBS, 400 nM GYY4137 or 400 μM GYY4137 treatments (Figures 6 and 7).

*Treatment of renal grafts with AP39 during cold storage improves graft function and survival following syngeneic RTx.*

Animals that received renal grafts treated with AP39 during cold storage exhibited significantly improved survival ($p<0.01$) compared to UW treated animals. Whereas UW treated animals exhibited ~40% survival at POD 2 and ~15% survival from POD 5 to end of time course (POD 14), AP39 treated animals exhibited 100% survival at POD 2 and retained ~70% survival by end of time course. AP39 treatment also improved graft function (Figure 8A). UW treated animals exhibited significantly increased serum creatinine ($p<0.05$) compared to Sham at POD 1-3 that increased until time of sacrifice, with only one UW animal recovering renal function by POD 14. Conversely, AP39 treated animals exhibited serum creatinine levels that were elevated, but not significantly different, compared to Sham at POD 1-3 (Figure 8B). Only two AP39 treated animals exhibited serum creatinine levels that increased until time of sacrifice, while the remainder reached baseline serum creatinine levels by POD 4-6 (Figure 8B).

## Discussion

Evidence for the therapeutic potential of $H_2S$ in treating pathologies of most major organ systems is continually expanding (30). A number of prior studies have shown that $H_2S$ treatment effectively protects kidneys exposed to warm ischemic injury, however the effect of $H_2S$ during cold IRI has not been as well explored (17-20). Our findings show that treatment of donor kidneys with $H_2S$ during prolonged cold storage significantly improved resultant allograft function and survival compared to kidneys stored in UW solution alone. These functional data were also corroborated by histological findings. Staining of donor kidneys with EthD-1 immediately following cold storage showed that $H_2S$ treatment significantly blunted the progression of tissue necrosis during prolonged cold storage compared to untreated kidneys. Allografts treated with $H_2S$ also showed markedly decreased levels of apoptosis acutely following RTx compared to UW treated allografts. These findings indicate that $H_2S$ limits renal

cellular injury associated with prolonged cold ischemia time, resulting in greater preservation of functioning renal parenchyma following cold storage and improved recovery of allograft function during the acute post-transplant period. Despite the early protective effects of $H_2S$ treatment in our model, only one $H_2S$ treated animal survived until end of time course. While our study utilizes a model of extreme graft injury to explore the maximal protective capacity of $H_2S$-based preservation, we have previously shown that $H_2S$ treatment is also protective during a more moderate period of 6h cold storage (22). Scoring of renal sections using criteria for acute rejection from the Banff 97 working classification of renal allograft pathology indicated that graft loss from POD 6-14 was likely due to acute rejection. This is not surprising as Brown Norway to Lewis RTx is known to illicit a robust acute rejection response in the absence of immune suppression (31). However, it is expected that combination of $H_2S$ treatment with immune suppressive therapy would result in significantly improved long-term allograft function and survival rates compared to untreated kidneys.

Due to the relatively small number of studies investigating $H_2S$-mediated renoprotection during cold IRI, little is known regarding the specific cellular actions of $H_2S$ in this context. Therefore, the second aim of our study was to identify a potential cellular mechanism through which $H_2S$ can protect renal epithelial cells during cold renal IRI. It has recently been suggested that mitochondria are a primary site of $H_2S$ activity. While $H_2S$ exerts toxic effects at high concentrations through inhibition of cytochrome c in the ETC, it has been conversely shown to stimulate ATP production at lower concentrations by donating electrons to the ETC between complexes II and III via the enzyme sulfide:quinone oxidoreductase (SQR) (32). The idea that specific mitochondrial actions are important contributors to $H_2S$-mediated cytoprotection has been substantiated by the observations of several studies that $H_2S$ treatment preserves

mitochondrial membrane integrity and function during cellular injury (23,24,34). It has also been shown that the cytosolic $H_2S$-producing enzyme, CSE, can translocate to mitochondria during cellular injury, presumably to produce $H_2S$ where it is needed most to mediate cellular stress responses (25). Therefore, to investigate whether mitochondrial actions of $H_2S$ are important to mediating its protective effects during cold renal IRI, we treated renal epithelial cells with either the non-specific $H_2S$ donor, GYY4137, or the mitochondrial-targeted $H_2S$ donor, AP39, and exposed them to a physiologically relevant *in vitro* model of cold H/R injury. While GYY4137 and AP39 are both synthetic donors that release $H_2S$ in a controlled manner similar to physiological production, AP39 contains a cationic triphenylphosphonium ($TPP^+$) group that allows the compound to home to mitochondria before releasing $H_2S$ (28,29). Our experiments showed that treatment with AP39 at nM levels drastically improved cellular viability, reduced ROS production and preserved mitochondrial membrane integrity following cold H/R injury compared to untreated cells. Contrastingly, GYY4137 was only able to protect cells at μM levels, though it was not as effective as AP39 even at 1000-fold increased concentrations. This study is the first to investigate the potential protective effects of $H_2S$ using a physiologically relevant *in vitro* model of cold H/R injury rather than artificially simulating warm ischemic injury via chemical induction of hypoxia-inducible factor 1 (HIF-1), ETC inhibition or oxidative stress. Our findings indicate that targeting of $H_2S$ release to mitochondria improves its cytoprotective potency during cold H/R injury by >1000-fold and that the mitochondrial actions of $H_2S$ may be sufficient to confer renoprotection against ischemic injury. We also show that treatment of donor kidneys with AP39 at a similar concentration during prolonged cold storage significantly improves graft function and survival compared to UW alone. This observation demonstrates that AP39 is effective against cold IRI *in vivo* and again confirms the increased

potency of mitochondrial-targeted $H_2S$ release, as our previous RTx models required use of ~1000-fold higher concentrations of the non-specific sulfide salt, NaSH, to achieve a similar protective benefit during prolonged cold storage (21,22).

There are a number of mitochondrial processes that could be influenced by $H_2S$ during cold IRI. As previously described, low levels of $H_2S$ can stimulate ATP production through donation of electrons to the ETC (32). In the absence of $O_2$ and nutrients during ischemia, this action of $H_2S$ could potentially minimize the detrimental impact of ATP depletion on cellular function and viability. Two recent studies by the same research group have shown that nM levels of AP39 can stimulate mitochondrial respiration and ATP production and are cytoprotective against oxidative stress in both endothelial and renal epithelial cells (35,36). The authors also showed that treatment of rats with AP39 during warm bilateral renal ischemia improved renal function and decreased oxidative stress and inflammation following reperfusion (36). These studies establish that stimulation of mitochondrial bioenergetics could be a viable protective mechanism through of $H_2S$ during cellular injury. However, another possibility is that $H_2S$ modulates mitochondrial ion channels during ischemic injury. It is well known that $H_2S$ can both activate and inhibit ion channel activity through persulfidation of various ion channel subunits (37). One recent study has shown that treatment of rats with AP39 can inhibit T-type $Ca^{2+}$ channel activity on myocardial cell membranes (38), which could provide the basis for a similar effect on mitochondrial $Ca^{2+}$ channels in our model. Considering the importance of mitochondrial $Ca^{2+}$ influx and subsequent mitochondrial swelling in the pathogenesis of IRI, $H_2S$ potentially prevent MPTP formation during IRI via modulation of mitochondrial $Ca^{2+}$ channel activity to pump out incoming $Ca^{2+}$ ions or blunt initial $Ca^{2+}$ influx. Due to its pleiotropic effects, the specific

mitochondrial mechanisms of $H_2S$ during ischemic injury will require much further study to definitively characterize.

In order to translate our findings to improve clinical outcomes for renal transplant patients, studies that demonstrate the safety and efficacy of delivery of $H_2S$ to human donor kidney tissue should be pursued. Impactful studies could be designed that obtain discarded human donor kidneys and assess whether preservation in solution supplemented with $H_2S$ could improve the health of these kidneys to such a degree that they could be utilized for transplantation. Another potential strategy to speed clinical translational of $H_2S$-based preservation techniques is to investigate the renoprotective effects of $H_2S$-releasing compounds derived from organic sources already approved for treatment of unrelated conditions. Two such potential substances are thiosulfate and aged garlic extract (AGE). Thiosulfate is a naturally occurring intermediate of sulfur metabolism and is currently used as a part of treatment regimens for clinical conditions including calciphylaxis and cyanide poisoning (39,40). However, thiosulfate has also recently been shown to produce $H_2S$ under physiological conditions and mediate protection of neurons from ischemic injury (41,42). Aged garlic extract is known to exert potent anti-oxidant effects and has a wide variety of clinical applications, including reduction of blood pressure in hypertensive patients and slowing the progression of atherosclerotic plaques (43-45). While the most well studied component of AGE is the anti-oxidant S-allylcysteine (SAC), it also contains polysulfide compounds diallyl-disulfide (DADS) and diallyl-trisulfide (DATS) that have been shown to be sources of $H_2S$ production (46). A few studies have shown that application of garlic and it $H_2S$-producing derivatives can mitigate injury associated with warm IRI, particularly in renal and myocardial tissue (47,48). Given their ability to produce $H_2S$ and mitigate warm ischemic injury in a variety of tissues, along with their current use as part of standard clinical

treatments, these two therapeutic substances appear to have considerable potential for improving clinical preservation of donor kidneys.

In conclusion, treatment of donor kidneys with $H_2S$ mitigates ATN and apoptosis associated with prolonged cold storage and improves allograft function and survival in the acute phase following *allogeneic* RTx. Targeting of $H_2S$ release to mitochondria improves the potency of $H_2S$-mediated protection in both a physiologically relevant *in vitro* model of cold renal H/R injury and an *in vivo* model of prolonged cold storage and *syngeneic* RTx. Our findings provide a strong base of evidence to support the notion that treatment of donor kidneys with $H_2S$ could represent a novel, cost-effective strategy to minimize the deleterious impact of prolonged cold IRI during RTx and ultimately improve clinical outcomes for renal transplant patients.

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## Disclosure

The authors of this manuscript have conflicts of interest to disclose as described by the American Journal of Transplantation. MW, MEW and the University of Exeter have intellectual property

(patent filings) related to slow release hydrogen sulfide donors, including AP39, and their use. The other authors have no conflicts of interest to disclose.

## Figure Legends

**Figure 1. $H_2S$ improves renal allograft survival and function following 24-hour cold organ storage and allogeneic renal transplantation.** Survival rates (A) and serum creatinine levels (B) of renal transplant recipient Lewis rats receiving donor Brown Norway kidneys perfused and stored in UW solution only (UW; n=8) or UW solution plus 150 µM NaSH ($H_2S$; n=8) for 24 hours at 4°C as well as sham-operated Lewis rats (Sham; n=5). Survival data analyzed via Kaplan-Meier survival analysis and log rank test. Serum creatinine data analyzed via one-way ANOVA and Tukey's post-hoc test. Lines indicate mean serum creatinine. *p<0.05; **p<0.01; ***p<0.001.

**Figure 2. $H_2S$ mitigates renal allograft apoptosis following 24-hour cold organ storage and allogeneic renal transplantation.** (A) Representative images (10X magnification) of renal allograft sections perfused and stored in UW solution only (UW) or UW solution plus 150 µM NaSH ($H_2S$) for 6 hours at 4°C as well as sham-operated Brown Norway rats (Sham) stained with TUNEL showing staining for fragmented DNA, a marker of cellular apoptosis. Arrows indicate cells positive for staining of fragmented DNA. (B) Corresponding digital analysis of absolute numbers of apoptotic cells in TUNEL sections. Background was subtracted from images and the colour threshold (RGB) was adjusted uniformly for each section so that only positively stained areas would be measured. Sections were obtained at POD 2-4 (Sham, n=3; UW, n=7; $H_2S$, n=8) and POD 6-14 (UW, n=1; $H_2S$, n=7). *p<0.05.

**Figure 3. $H_2S$ may mitigate renal allograft necrosis scores but does not modulate acute allograft rejection following 24-hour cold organ storage and allogeneic renal transplantation.** (A) Acute tubular necrosis (ATN) scores of renal allograft sections perfused and stored in UW solution only (UW) or UW solution plus 150 μM NaSH ($H_2S$) for 24 hours at 4°C as well as sham-operated Brown Norway rats (Sham) stained with Hematoxlyin & Eosin. Sections were assessed for the presence of necrotic tubules that exhibit loss of nuclei and necrotic casts with a background of congestion and assigned a score for ATN out of 5, (0 = 0% graft ATN, 1 = <10% graft ATN, 2 = 11-25% graft ATN, 3 = 26-45% graft ATN, 4 = 46-75% graft ATN, 5 = >75% graft ATN) (B) Acute rejection scores of renal allograft sections stained with H&E and scored for tubulitis, vasculitis and inflammation according to the Banff 97 working classification of renal allograft pathology. Sections were scored by a blinded transplant pathologist and tubulitis, vasculitis and inflammation scores were out of 3. Sections were obtained at POD 2-4 (Sham, n=5; UW, n=7; $H_2S$, n=7) and POD 6-14 ($H_2S$, n=7). Lines indicate median pathological score. *p<0.05; **p<0.01; ****p<0.0001.

**Figure 4. $H_2S$ decreases renal allograft necrosis immediately following 24-hour cold organ storage.** (A) Representative fluorescent microscopic images (40X magnification) of kidney sections taken from donor Brown Norway kidneys perfused and stored in UW solution only (UW; n=8) or UW solution plus 150 μM NaSH ($H_2S$; n=8) for 24 hours at 4°C as well as kidneys obtained prior to cold storage (Pre-storage; n=8). Kidneys were perfused with 5 μM EthD-1 following cold storage and subsequently placed in formalin for histological analysis. Sections were also stained with the nuclear marker DAPI. (B) Digital quantification of EthD-1 fluorescence levels. Sections digitally quantified via ImageJ analysis and values are mean EthD-

1 fluorescence of 5 random fields of view (20X magnification) taken per sample. Lines indicate mean EthD-1 fluorescence per treatment group. *p<0.05, **p<0.01.

**Figure 5. Targeting of $H_2S$ release to mitochondria improves potency of protective effects during *in vitro* cold hypoxia/hypercapnia and re-oxygenation (H/R) injury.** Cellular viability levels of rat kidney epithelial (NRK-52E; ATCC) cells following *in vitro* cold H/R injury. Control cells were cultured in DMEM + 5% fetal bovine serum (FBS) at 37 ºC in room $O_2$ and 5% $CO_2$. Experimental cells were treated with either phosphate-buffered saline (PBS) alone or PBS plus varying concentrations of GYY4137 (non-specific $H_2S$ donor) or AP39 (mitochondrial-targeted $H_2S$ donor) and exposed to cold (12 ºC) hypoxia/hypercapnia (0.1% $O_2$/15% $CO_2$) for 24 hours (n=5 per treatment group). Cells were then re-oxygenated for 24 hours in conditions identical to control cells and viability was assessed via flow cytometry using Annexin-V/7-AAD staining, indicating apoptosis and necrosis, respectively. (A) Representative FACS plots of healthy cells vs. injured cells. (B) Quantification of % viable (Annexin-V(-)/7-AAD(-)) cells treated with either DMEM, PBS or PBS (+) 100-400 nM AP39 or GYY4137. (C) Quantification of % viable (Annexin-V(-)/7-AAD(-)) cells treated with either DMEM, PBS or PBS (+) 100-400 μM GYY4137. Bars indicate mean ± SEM. *p<0.05 vs. DMEM, δ p<0.05 vs. PBS, ϕ p<0.05 vs. 400 nM AP39.

**Figure 6. AP39 is more potent at reducing ROS production during *in vitro* cold hypoxia/hypercapnia and re-oxygenation (H/R) injury compared to GYY4137.** Levels of ROS production in rat kidney epithelial (NRK-52E; ATCC) cells following *in vitro* cold H/R injury. Control cells were cultured in DMEM + 5% fetal bovine serum (FBS) at 37 ºC in room

$O_2$ and 5% $CO_2$. Experimental cells were treated with either phosphate-buffered saline (PBS) alone or PBS (+) 400 nM or 400 µM GYY4137 (non-specific $H_2S$ donor) or 400 nM AP39 (mitochondrial-targeted $H_2S$ donor) and exposed to cold (12 ºC) hypoxia/hypercapnia (0.1% $O_2$/15% $CO_2$) for 24 hours (n=5 per treatment group). Cells were then re-oxygenated for 15 min in conditions identical to control cells and ROS production was assessed via flow cytometry using DHR-123 staining, which becomes fluorescent when oxidized by ROS. (A) Representative FACS plots of DHR-123 fluorescence in cells exposed to H/R injury. Quantification of (B) DHR-123 MFI and (C) %DHR-123 (+) cells in each treatment group. Bars indicate mean ± SEM. ****p<0.0001.

**Figure 7. AP39 preserves mitochondrial membrane potential following *in vitro* cold hypoxia/hypercapnia and re-oxygenation (H/R) injury.** JC-1 staining in rat kidney epithelial (NRK-52E; ATCC) cells following *in vitro* cold H/R injury. Control cells were cultured in DMEM + 5% fetal bovine serum (FBS) at 37 ºC in room $O_2$ and 5% $CO_2$. Experimental cells were treated with either phosphate-buffered saline (PBS) alone or PBS (+) 400 nM or 400 µM GYY4137 (non-specific $H_2S$ donor) or 400 nM AP39 (mitochondrial-targeted $H_2S$ donor) and exposed to cold (12 ºC) hypoxia/hypercapnia (0.1% $O_2$/15% $CO_2$) for 24 hours (n=5 per treatment group). Cells were then re-oxygenated for 18 hours in conditions identical to control cells and mitochondrial membrane potential was assessed via flow cytometry using JC-1 staining, which emits green fluorescence in monomer form, but emits red fluorescence when aggregated in polarized mitochondria. (A) Representative FACS plots of JC-1 staining in cells exposed to H/R injury. (B) Ratio of Red (+):Green (+) cells in each treatment group. Higher values indicate more cells with polarized (healthy) mitochondria. Bars indicate mean ± SEM. ****p<0.0001.

**Figure 8. AP39 improves renal allograft function and survival and reduces renal injury following 24-hour cold organ storage and allogeneic renal transplantation.** Survival rates (A) and serum creatinine levels (B) of renal transplant recipient Lewis rats receiving donor Lewis kidneys perfused and stored in University of Wisconsin (UW) solution only (UW; n=5) or UW solution plus 200 nM of the mitochondrial-targeted $H_2S$ donor AP39 (AP39; n=3) for 24 hours at 4°C as well as sham-operated Lewis rats (Sham; n=4). Lines indicate mean serum creatinine. *$p<0.05$.

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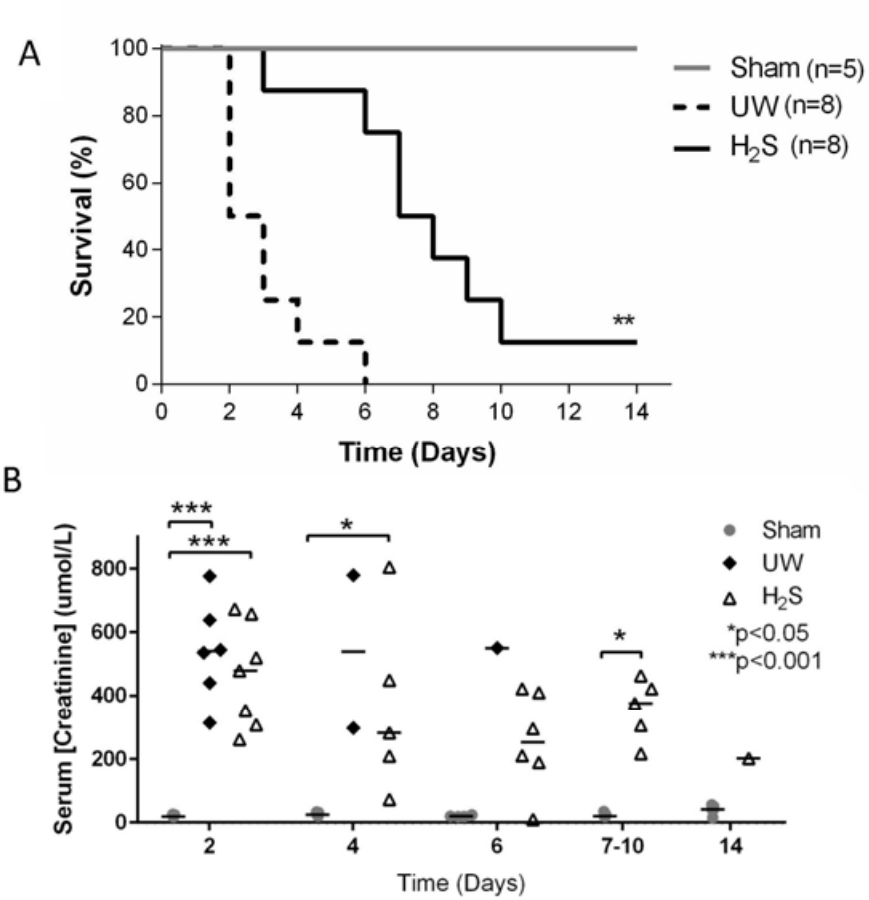



**Figure 1. $H_2S$ improves renal allograft survival and function following 24-hour cold organ storage and allogeneic renal transplantation.** Survival rates (A) and serum creatinine levels (B) of renal transplant recipient Lewis rats receiving donor Brown Norway kidneys perfused and stored in UW solution only (UW; n=8) or UW solution plus 150 µM NaSH ($H_2S$; n=8) for 24 hours at 4°C as well as sham-operated Lewis rats (Sham; n=5). Survival data analyzed via Kaplan-Meier survival analysis and log rank test. Serum creatinine data analyzed via one-way ANOVA and Tukey's post-hoc test. Lines indicate mean serum creatinine. *p<0.05; **p<0.01; ***p<0.001.

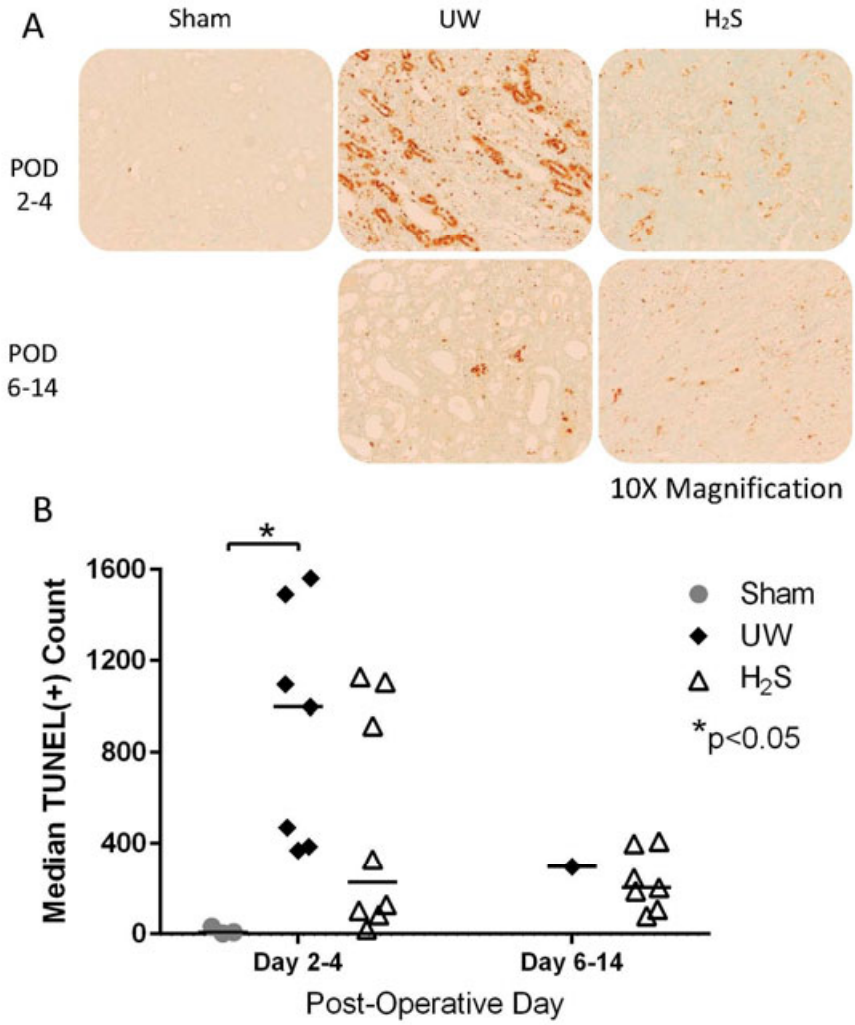
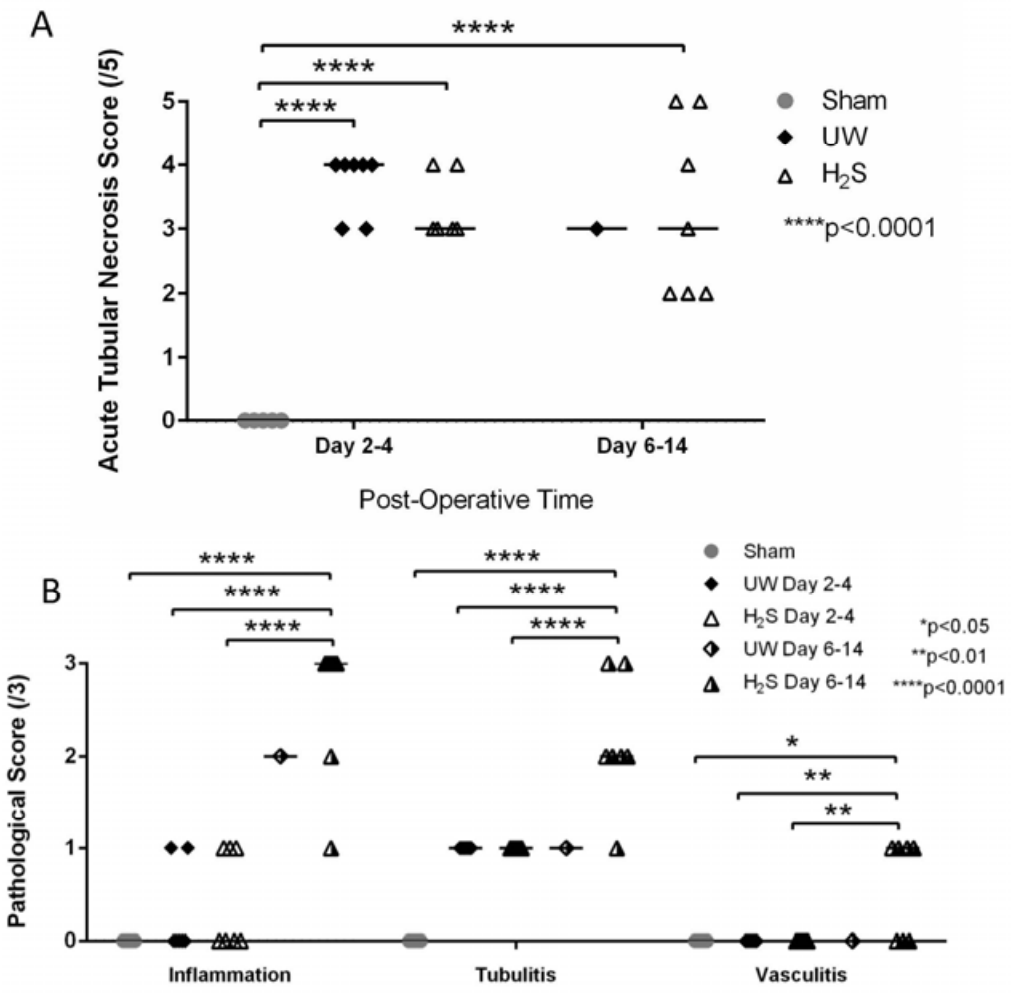



**Figure 2. $H_2S$ mitigates renal allograft apoptosis following 24-hour cold organ storage and allogeneic renal transplantation.** (A) Representative images (10X magnification) of renal allograft sections perfused and stored in UW solution only (UW) or UW solution plus 150 µM NaSH ($H_2S$) for 6 hours at 4°C as well as sham-operated Brown Norway rats (Sham) stained with TUNEL showing staining for fragmented DNA, a marker of cellular apoptosis. Arrows indicate cells positive for staining of fragmented DNA. (B) Corresponding digital analysis of absolute numbers of apoptotic cells in TUNEL sections. Background was subtracted from images and the colour threshold (RGB) was adjusted uniformly for each section so that only positively stained areas would be measured. Sections were obtained at POD 2-4 (Sham, n=3; UW, n=7; $H_2S$, n=8) and POD 6-14 (UW, n=1; $H_2S$, n=7). *p<0.05.

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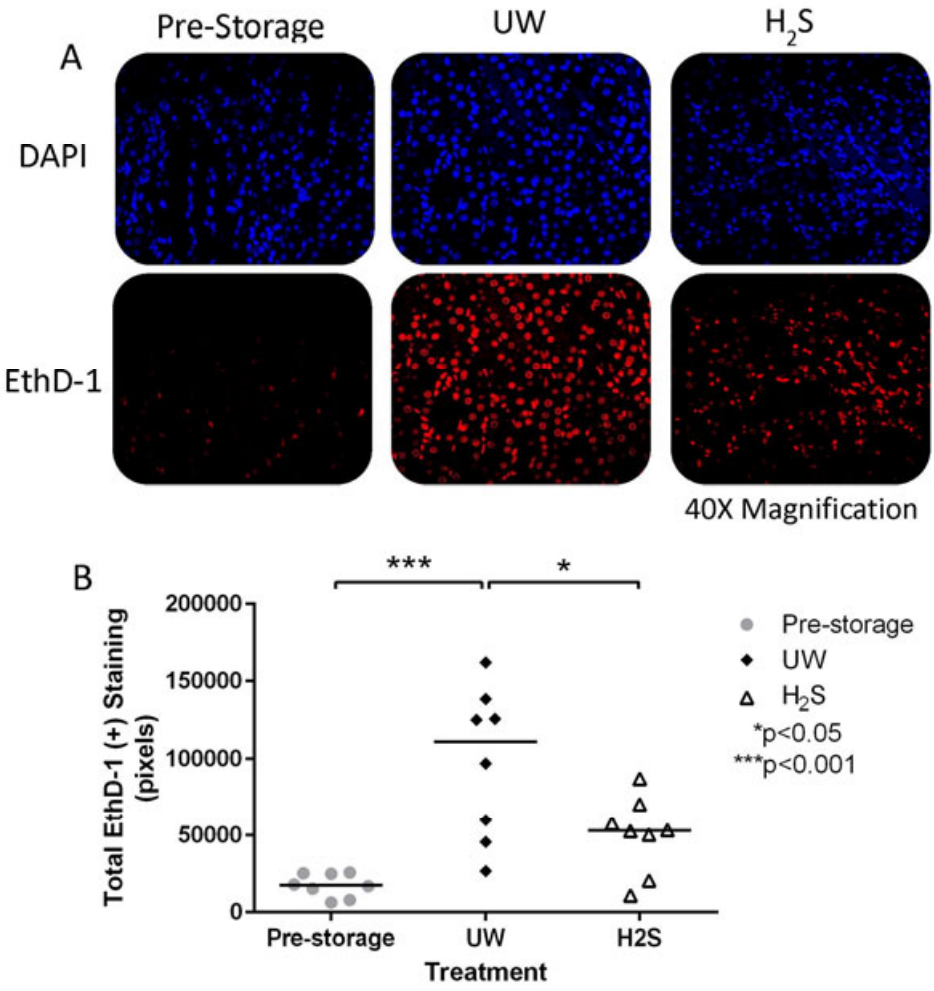


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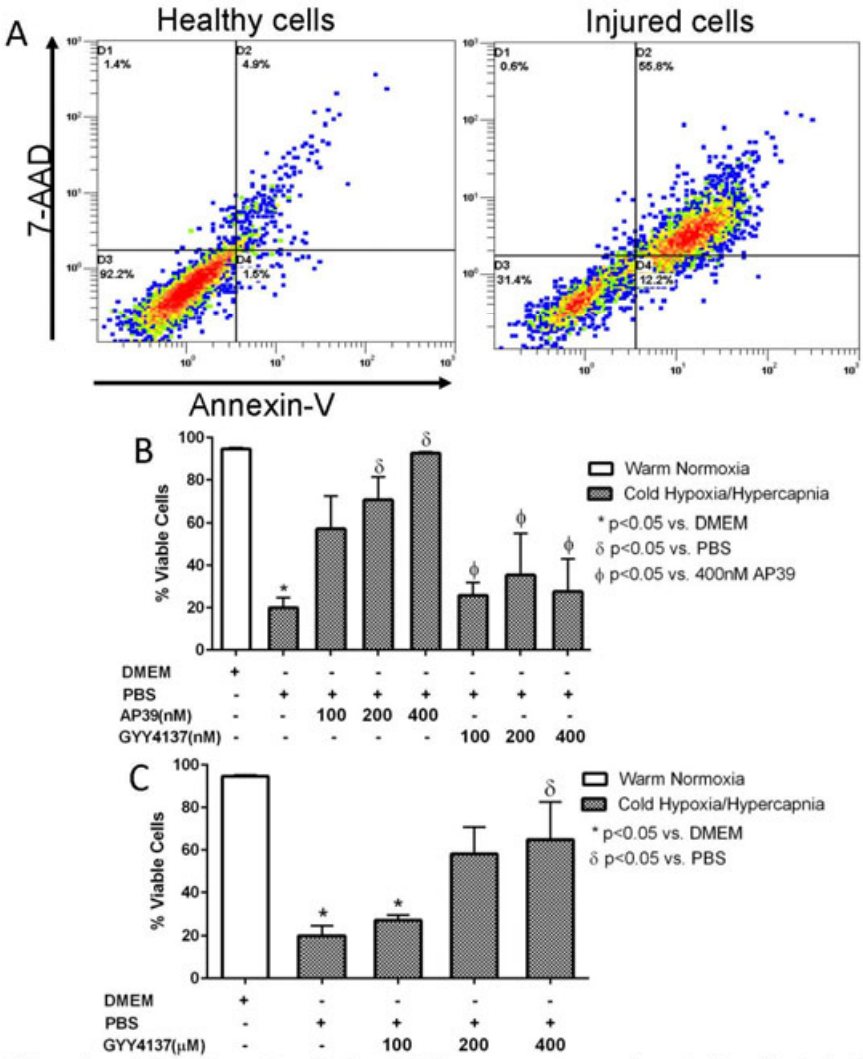


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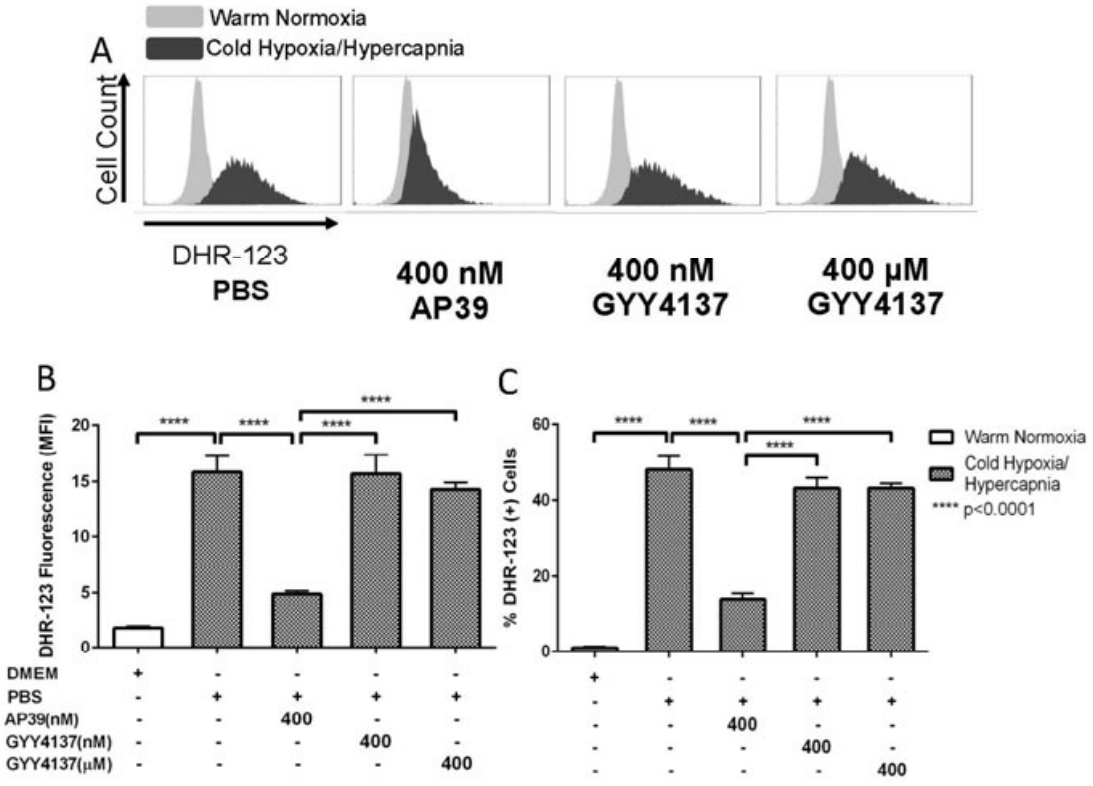


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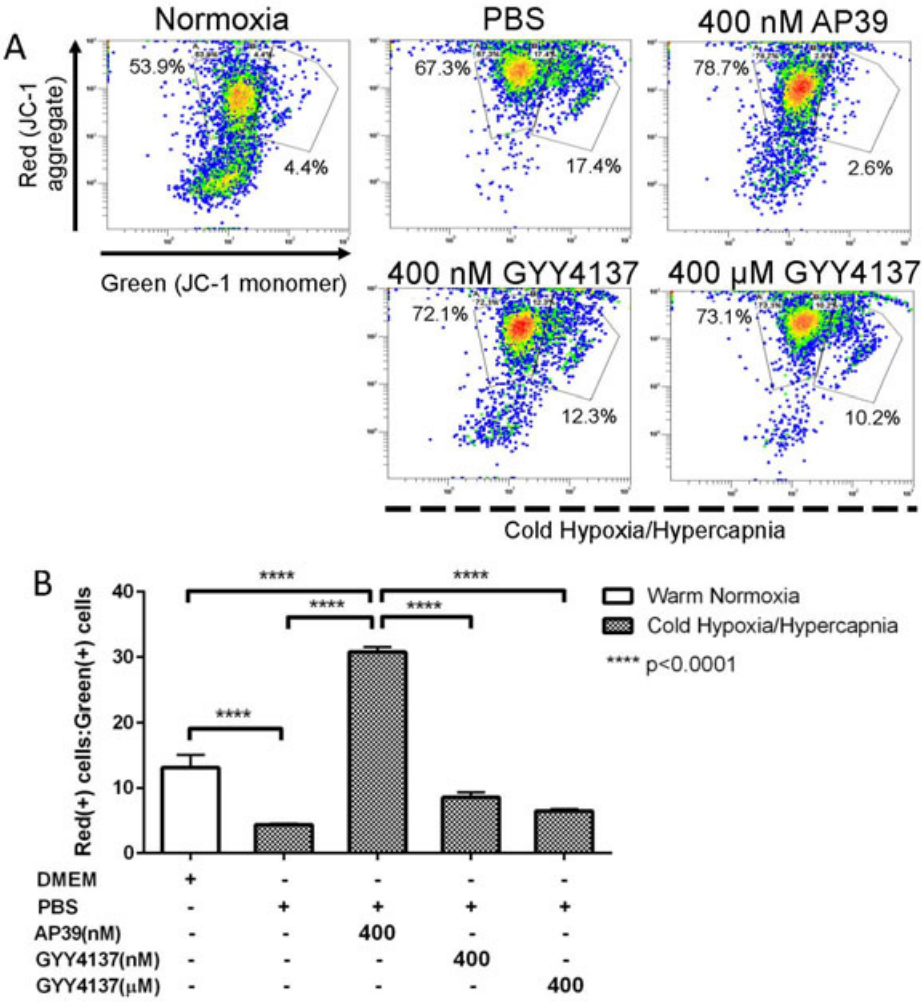


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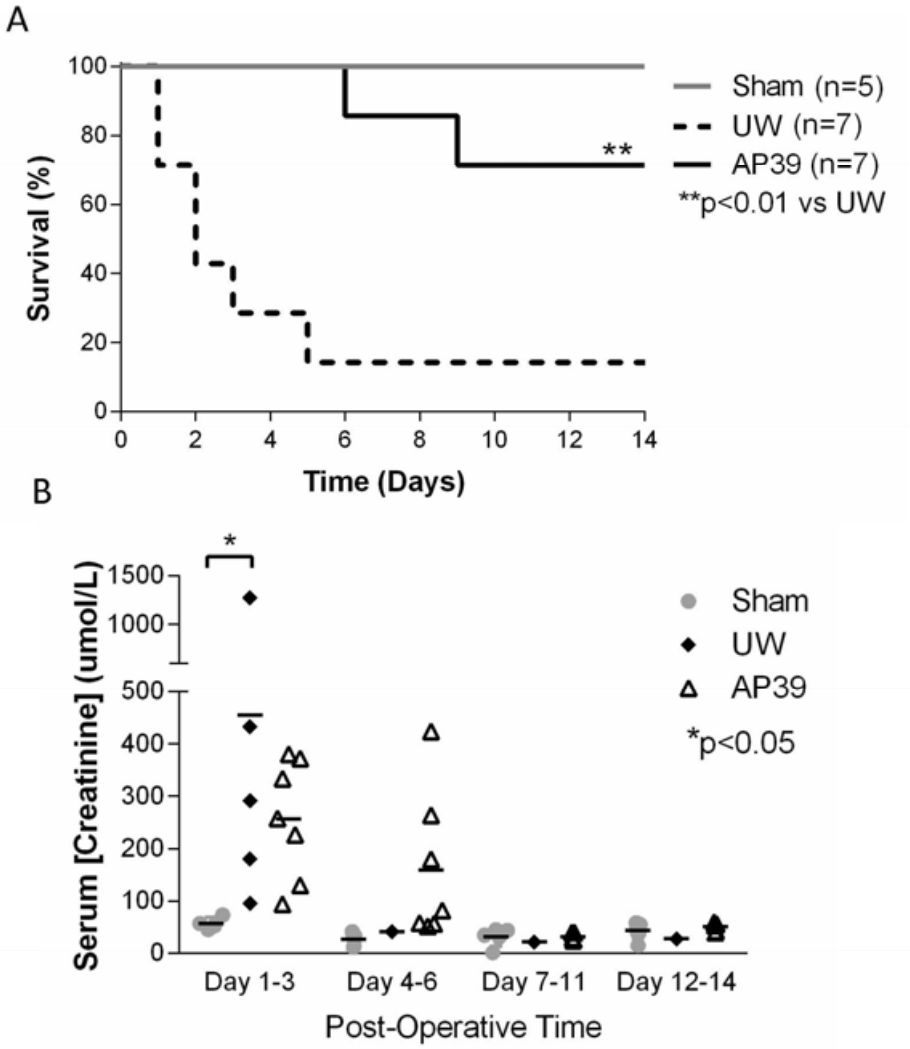


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