Supplemental hydrogen sulfide in models of renal transplantation after cardiac death

Background: The increasing use of kidneys from donations after cardiac death (DCD) for renal transplantation is hindered by negative outcomes owing to organ injury after prolonged warm and cold ischemia–reperfusion. Recently, hydrogen sulfide (H$_2$S) has shown cytoprotective effects against ischemia–reperfusion injury; however, its effectiveness in the context of DCD renal transplantation is unknown.

Methods: We tested a novel 30-day in vivo syngeneic murine model of DCD renal transplantation, in which the donor kidney was clamped for 30 minutes and stored for 18 hours in cold University of Wisconsin (UW) solution or UW with 150 µM sodium hydrogen sulfide (UW + NaHS) before transplantation. We also tested a 7-day in vivo porcine model of DCD renal autotransplantation, in which the left kidney was clamped for 60 minutes and preserved for 24 hours using hypothermic perfusion with UW or UW + 150 µM NaHS before autotransplantation. We collected blood and urine samples periodically, and collected kidney samples at the end point for histopathology and quantitative reverse transcription polymerase chain reaction.

Results: Rats that received H$_2$S-treated kidneys showed significantly higher survival, faster recovery of graft function and significantly lower acute tubular necrosis than controls. Pig kidneys perfused with UW + NaHS showed significantly higher renal blood flow and lower renal resistance than control kidneys after 24 hours of perfusion. After autotransplantation, pigs that received H$_2$S-treated kidneys showed significantly lower serum creatinine on days 1 and 7 after transplantation. Rat and pig kidneys treated with H$_2$S also showed more protective gene expression profiles than controls.

Conclusion: Our findings support the potential use of H$_2$S-supplemented UW solution during cold storage as a novel and practical means to improve DCD graft survival and function.

Contexte : Il est difficile d’accroître l’utilisation de reins provenant de dons après décès cardiaque récirculatoire (DDC) aux fins de transplantation rénale en raison des résultats négatifs dus aux lésions d’organe après une ischémie–reperfusion chaude et froide prolongée. On a observé récemment que le sulure d’hydrogène (H$_2$S) exerçait des effets cytoprotecteurs contre les lésions d’ischémie–reperfusion, mais on ignore encore s’il est efficace dans un contexte de transplantation rénale provenant d’un DDC.

Méthodes : Nous avons mis à l’essai un nouveau modèle murin syngénique in vivo de transplantation rénale provenant d’un DCD sur 30 jours, dans lequel le rein du donneur a été clampé pendant 30 minutes et conservé pendant 18 heures dans une solution froide de l’Université du Wisconsin (UW) (UW + 150 µm d’hydrogénosulfure de sodium [NaSH]) avant la transplantation. Nous avons également mis à l’essai un modèle porcin in vivo d’autotransplantation rénale provenant d’un DCD sur 7 jours, dans lequel le rein gauche a été clampé pendant 60 minutes et conservé pendant 24 heures par perfusion hypothermique (avec UW ou UW + 150 µm de NaSH) avant l’autotransplantation. Nous avons recueilli des échantillons de sang et d’urine régulièrement et avons prélevé des échantillons de rein au point final en vue de l’histopathologie et d’un test d’amplification en chaîne par polymérase quantitative coupé à une transcription inverse.

Résultats : On a observé chez les rats ayant reçu des reins traités au H$_2$S un taux de survie significativement plus élevé, une récupération plus rapide de la fonction du greffon et une nécrose tubulaire aiguë significativement plus faible que chez les témoins. Les reins de porc perfusés (UW + NaSH) ont montré un débit sanguin rénal significativement plus élevé et une résistance rénale plus faible que les témoins à la fin de la période de perfusion de 24 heures. Après l’autotransplantation, les porcs ayant reçu des reins traités au H$_2$S présentaient une créatinine sèrique significativement plus faible aux jours 1 et 7 après la transplantation. Les reins de rat et de porc traités au H$_2$S ont également montré des profils d’expression génique plus « protecteurs » que les témoins.

Conclusion : Nos conclusions appuient une éventuelle utilisation d’une solution UW enrichie en H$_2$S pendant la conservation à froid comme nouveau moyen pratique d’améliorer le taux de survie et la fonction du greffon lors de DDC.
In 2017, 18,136 of 96,120 eligible patients received kidney transplants in the United States.1 As demand for donor kidneys continues to exceed current supply, the use of suboptimal kidneys from deceased donors in renal transplantation is on the rise. Marginal donor kidneys were used in 71% of kidney transplants in 2017.1 Within this category, the most rapidly growing source of transplant worldwide arises from donation after cardiac death (DCD), where death is based on an “irreversible cessation of circulatory and respiratory function.”2 Despite their increased use, DCD organs are at greater risk of poor graft outcomes such as acute tubular necrosis, vasocostriction and delayed graft function, defined as the need for dialysis within the first week after transplantation.3 Delayed graft function is an especially important risk factor for poor patient and graft outcomes, and can potentially lead to extended hospital stays, the need for more difficult immunosuppressive drug regimens and higher health care costs.4,5

This heightened risk of delayed graft function can be attributed to the greater risk for ischemia–reperfusion injury of DCD organs. Upon withdrawal of support, DCD organs face both warm ischemia time (up to 2 hr before the declaration of death) and cold ischemia time (up to 48 hr of cold preservation with University of Wisconsin [UW] solution), followed by reperfusion of the organ within the recipient.3 Collectively, these events contribute to greater risk of ischemia–reperfusion injury, a well-characterized series of events that involves sustained anaerobic metabolism causing cellular death, which manifests histopathologically as acute tubular necrosis and clinically as delayed graft function.6

With prolonged ischemia, cells transition to anaerobic metabolism, thereby limiting production of adenosine triphosphate, while establishing an environment conducive to the production of reactive oxygen species and a generalized inflammatory response upon reperfusion. In conjunction with several other injury mechanisms, sufficient structural damage to the microvasculature can result in a “no-reflow” phenomenon, delaying adequate restoration of blood flow back to the affected areas.7 Overall, the perpetuation of the ischemic state, in combination with reperfusion, acts as a strong proapoptotic trigger that induces cell death in the organ, leading to a greater risk of delayed graft function and graft failure.1 However, as ischemia times are unavoidable,8 the major challenge for the use of DCD transplants is reducing ischemia–reperfusion injury and the risk of delayed graft function to improve graft outcomes.

One strategy to improve graft outcomes is optimizing organ preservation to minimize ischemic injury. Currently, cold preservation is used to reduce metabolic demand within the organ until transplantation. Static cold storage on ice in UW solution is the clinical standard of care for preserving kidneys.8 However, hypothermic machine perfusion (HMP) with UW solution is preferred for preserving DCD grafts because it has been shown to improve post-transplant outcomes compared with static cold storage.9 The advantages of HMP are that it facilitates the circulation of nutrients and removal of waste products; however, it is more expensive and less accessible globally.9 Importantly, the preservation solutions used for static cold storage and HMP, such as UW solution, have not changed in decades. An approach that is gaining momentum is to supplement preservation solutions with therapeutic molecules.9

Recent studies have shown support for the therapeutic use of hydrogen sulfide (H2S) in the context of renal ischemia–reperfusion injury. Hydrogen sulfide plays an important role in vasodilation, inflammation, antioxidation and antiapoptosis events.10–12 In the kidney, H2S has a direct effect on increasing the glomerular filtration rate and urinary excretion of sodium and potassium, thereby decreasing blood pressure. To implement its vasodilatory effect, H2S, an endothelium-derived relaxing factor, uses sulfhydration mechanisms on vascular smooth muscle cells, interferes with renin production and works synergistically with nitric oxide to upregulate the production of cyclic guanosine monophosphate. At the same time, H2S signalling evokes antioxidation and antiapoptosis responses through downstream targets such as nuclear factor κB.14

Studies using endogenous supplementation of H2S (via the addition of the enantiomeric H2S precursor, d-cysteine) have reported attenuation of renal ischemia–reperfusion injury, with greater preservation of glomerular structure in mice.13 Further evidence has shown that exogenous supplementation of H2S can mitigate damage to the mitochondria, preserving membrane integrity and function during ischemia; as such, H2S is believed to exert cytoprotective effects against ischemia–reperfusion injury.10,12 Given its nature as a gasotransmitter, the exogenous supplementation of H2S is possible only by using donor molecules. As has been well established in the literature, the H2S donor, sodium hydrogen sulfide (NaHS), has a short half-life and is protective against ischemia–reperfusion injury in various models of ischemia and transplantation.16–18 Using murine models, we have previously shown that supplementation of UW solution with NaHS enhances recovery of renal function after prolonged (1 h) warm ischemic injury.16 In addition, prolonged static cold storage in UW solution with NaHS improves renal function recovery and overall survival after syngeneic and allogeneic renal transplantation.11,18

However, organ preservation with H2S has yet to be investigated using a model of DCD renal transplantation that mimics both prolonged warm and cold ischemia. Therefore, in this study, we aimed to evaluate the role of H2S in improving ischemia–reperfusion injury in DCD renal transplantation through in vivo models. First, we sought to develop a novel in vivo murine model of DCD...
renal transplantation to evaluate the protective effects of prolonged static cold storage in H$_2$S-supplemented UW solution on DCD renal graft outcomes. Next, as a proof of concept, we used an in vivo porcine autotransplantation model to validate the use of H$_2$S-supplemented UW solution for HMP of DCD renal grafts in higher mammal systems.

**METHODS**

**In vivo murine model**

We used a novel in vivo DCD model to evaluate the effects of H$_2$S on overall survival and graft outcomes of rats. We randomized adult Lewis syngeneic rats (Charles River Laboratories International Limited), weighing 250 g, into sham, untreated or H$_2$S-treated groups (Figure 1A). We used male rats because male rodents are more susceptible to renal ischemia–reperfusion injury than female rodents.$^{19,20}$

Sham rats underwent a midline incision without renal transplantation and were used to establish the baseline for survival ratios and graft function parameters. The untreated and H$_2$S-treated donor kidneys underwent warm and cold ischemia akin to that seen in DCD renal transplantation. We administered an intraperitoneal injection of D-cysteine (Sigma Aldrich), prepared in 0.5 mL of sterile saline solution (0.9% NaCl) at a concentration of 2 mmol per kilogram rat weight to the H$_2$S-treated donor rats at least 1 hour before surgery. Dosage and timing for the D-cysteine injection were based on a previous study.$^{15}$

We induced warm ischemia in both untreated and H$_2$S-treated donor rats via occlusion of the left renal pedicle by atraumatic clamping for 30 minutes to mimic DCD warm ischemia injury. We used aseptic techniques to procure and flush the donor kidney with 10 mL of either cold (4°C) UW preservation solution (Bridge to Life) for the untreated group or UW solution supplemented with 150 μM NaHS (Sigma Aldrich) for the H$_2$S-treated group until venous effluent was clear. After procurement, we

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**Fig. 1.** Summary of the animal models used to evaluate the protective roles of hydrogen sulfide (H$_2$S) in renal transplantation from donation after cardiac death (DCD). Summary of the (A) in vivo murine model and the (B) in vivo porcine model of DCD renal autotransplantation. (C) A porcine kidney being perfused with cold University of Wisconsin (UW) solution using the LifePort Kidney Transporter. IP = intraperitoneal, NaHS = sodium hydrogen sulfide, post-op = postoperative.
induced cold ischemia of the donor organs through 18 hours of static cold storage in 50 mL of the same solution as that used for flushing. Recipient syngeneic rats underwent bilateral nephrectomy and renal transplantation of the left donor kidney. An experienced microsurgeon who was blinded to the treatment group performed all surgeries.

After renal transplantation, rats were monitored for 30 days or until sacrifice. We collected blood and urine samples from the recipient rats on postoperative day 3, 5, 10, 20 and 30 to assess graft function and survival. Serum and urine samples were analyzed for serum creatinine, serum urea and urine protein-to-creatinine ratio at the Core Pathology Laboratories (University Hospital, London, Ont.). At the time of death or sacrifice, the kidney grafts were removed and bivalved sagittally such that half was stored at –80°C and used for analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR), and the other half was used for histological staining.

In vivo porcine model

As a proof of principle, we studied the effects of cold organ preservation using H2S-supplemented UW solution in an in vivo porcine model of DCD renal autotransplantation. We obtained adult male pigs weighing 30–65 kg from a local farm. A midline incision was used to expose the left kidney. The left kidney was dissected free from the retroperitoneum while leaving the blood supply to the ureter intact, and then the ureter was divided. The left renal vascular pedicle was left as long as possible by freeing the renal artery and vein up to the aorta and vena cava. The left renal pedicle was clamped for 60 minutes to induce warm ischemia to mimic DCD kidney injury after intravenous infusion of 3000 units of intravenous heparin injection. Subsequently, the left kidney was nephrectomized, the abdomen was closed and the pigs were allowed to recover. The donor kidneys were flushed and mechanically perfused for 24 hours with cold UW solution or UW solution supplemented with 150 µM NaHS using the LifePort Kidney Transporter (LifePort Inc.), which is a clinically approved HMP device (Figure 1B and 1C). Pressure, flow rate and resistance were monitored throughout the perfusion period.

After the 24-hour preservation period, the left kidneys were autotransplanted back into the donor animal and a right nephrectomy was performed after a laparotomy. The left kidney was autotransplanted onto the common iliac artery and vein using 7–0 polypropylene sutures in a running fashion. The ureter was stented with a 14 cm double-J stent and the ureter was anastomosed to the bladder with 5.0 polydioxanone sutures in a refluxing fashion. A 2-layer abdominal closure was performed, and the pigs were allowed to recover in our mini-intensive care unit. Intramuscular injection of buprenorphine and transdermal fentanyl patches were used for pain management during recovery.

Under sedation, we collected 10 mL of blood from the ear vein on postoperative day 1, 3 and 7. On postoperative day 7, the kidneys were collected and the animals were euthanized. We stored collected kidneys at -80°C before qRT-PCR analysis. Serum samples were analyzed for creatinine at the Core Pathology Laboratories (University Hospital, London, Ont.).

Renal histopathology

For histopathological analysis of samples from rat models, the kidney sections were fixed in 10% formalin and stained with hematoxylin and eosin to detect any signs of necrosis. All stained sections were imaged using a Nikon Eclipse 90i microscope. A renal pathologist (Dr. Aaron Haig, London Health Sciences Centre), blinded to treatment group, scored slides for acute tubular necrosis on a scale of 0–5, where 0 represents absence, and 1 through 5 denote involvement of 1%–10%, 11%–25%, 26%–45%, 46%–75% and more than 75% of the glomeruli or cortical area.

Quantitative reverse transcription polymerase chain reaction

We used frozen kidney samples from the rat and pig models to evaluate relative mRNA expression levels for specific inflammation and apoptosis genes using qRT-PCR. A small part of renal tissue (containing both the cortex and medulla) was thawed and homogenized after adding cell disruption buffer, provided in the PARIS kit (Life Technologies). We isolated the total RNA content using the kit, as per the manufacturer’s protocol. We measured the concentration and purity of mRNA using the NanoDrop spectrophotometer (Thermo Fisher Scientific). For reverse transcription, we added SuperScript VILO MasterMix (Invitrogen) to 500 ng of mRNA and used manufacturer’s instructions to generate complementary DNA (cDNA) using an Eppendorf Mastercycler Gradient. For qRT-PCR, each sample used 2X SensiFAST SYBR Hi-ROX Mix (BIOLINE) and contained 20 ng of cDNA. We generated the forward and the reverse primer sequences using the National Center for Biotechnology Information database and the Integrated DNA Technologies primer quest tool. We ordered the primers from Sigma Aldrich (Table 1 and Table 2).

For the murine model, the target gene signals were normalized against β-actin; mRNA expression for the untreated and H2S-treated groups was expressed as a ratio relative to the sham group. For the porcine model, the target gene signals were normalized against β-actin. We performed all qRT-PCR assays using a Bio-Rad thermal cycler (Bio-Rad Laboratories).
Ethics approval

All surgical and monitoring procedures received ethics approval from the Western University Council for Animal Care, which ensures adherence to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guideline.

Statistical analysis

We analyzed 30-day survival of the 3 murine groups using the Mantel–Cox and Gehan–Breslow–Wilcoxon test. For murine models, we analyzed serum creatinine, serum urea and protein-to-creatinine ratios for animals that survived until postoperative day 30 using 2-way analysis of variance (ANOVA). We compared scores for acute tubular necrosis using 1-way ANOVA and used Student t tests to compare relative gene expression in the untreated and H2S-treated groups. For porcine models, we analyzed flow rate, resistance, serum creatinine and relative gene expression by treatment group using Student t tests. We considered p values of less than 0.05 as significant.

Results

In vivo murine model

We included 5 rats in each of the sham, untreated and H2S-treated groups, for a total of 15 rats. Both the untreated and H2S-treated groups exhibited similar decreases in survival by postoperative day 3 (Figure 2A), but the survival rate of the H2S-treated group was statistically comparable to the survival rate of the sham group by the end of the 30-day study period. In contrast, the untreated group showed significantly worse survival than the sham group by the end of the 30-day period, with only 50% of animals surviving past postoperative day 10.

At postoperative day 3, both untreated and H2S-treated rats showed a faster recovery of serum urea and creatinine levels than the untreated rats before both groups plateaued slightly above baseline at postoperative day 30. However, no significant differences were observed after postoperative day 10. The urine protein-to-creatinine ratio of the untreated rats was significantly higher than the sham group at postoperative day 5 and 10, and we did not observe any significant differences between the H2S-treated and sham groups (Figure 2D).

The histological analysis showed that the H2S-treated kidneys (n = 6) had scores for acute tubular necrosis that were comparable to those of the sham kidneys (n = 5) and that were significantly lower than those of untreated kidneys (n = 8) (Figure 3).

Compared with the untreated kidneys, the H2S-treated kidneys showed a more protective gene expression profile, with some downregulation of proapoptotic (caspase-3, BAX, BID) and proinflammatory (tumour necrosis factor-α) genes (Figure 3E).

In vivo porcine model

To evaluate the effects of cold preservation in H2S-supplemented preservation solutions using a higher mammal model, pig kidneys were preserved for 24 hours using HMP with UW solution (UW, n = 3) or UW solution and 150 μM NaHS (UW + H2S, n = 3) after 60 minutes of warm ischemia to mimic DCD injury. We found that the presence of H2S in the preservation solution led to a significantly higher renal flow rate and significantly lower renal resistance after 24 hours of perfusion (Figure 4A and 4B).

On postoperative day 1 and 7, UW + H2S kidneys showed significantly lower serum creatinine levels than UW kidneys (Figure 4C). In addition, UW + H2S kidneys showed downregulation of proapoptotic and inflammatory genes (Table 2).
markers (interferon-γ, intercellular adhesion molecule-1, caspase-3), and upregulation of the BCL2 prosurvival gene, compared with UW kidneys (Figure 4D).

**Discussion**

In this study, we found that H₂S supplementation through endogenous (via the addition of d-cysteine) and exogenous (via prolonged static cold storage in UW solution with NaHS) means improved DCD renal graft outcomes in an in vivo murine model of syngeneic renal transplantation. In a proof of concept trial, supplementation of UW solution with a NaHS improved DCD renal graft function and induced protective gene expression in an in vivo porcine model of autotransplantation.

Continual growth in the field of gasotransmitters has led to evidence supporting the therapeutic potential of H₂S in treating pathologies of many major organs. Previous studies have shown that H₂S supplementation of UW solution during cold preservation before renal transplantation mitigates ischemia–reperfusion injury in renal grafts, improving early graft function and survival. However, although previous studies show that H₂S treatment protects donor kidneys against prolonged warm or cold ischemia, the effects of H₂S in the context of DCD renal transplantation, which involves both warm and cold ischemia, have not been explored. Given that DCD kidneys represent the fastest growing pool of organ donors worldwide, strategies that mitigate ischemia–reperfusion injury in these kidneys are of special interest, as they represent an avenue for expanding the pool of transplantable kidneys. The present study confirms that supplementing UW solution with H₂S during cold organ preservation mitigates renal graft injury associated with a sequence of prolonged warm and cold ischemia followed by reperfusion, as seen in DCD renal transplantation.

The novelty of this study lies in the successful establishment of a novel in vivo murine model of DCD renal transplantation that combines clinically relevant durations of warm and cold ischemia. The warm ischemia time of 30 minutes aligns with existing research on surgical renal ischemia times in animal models. A review of current literature on this subject determined that 40 minutes of warm ischemia time was the maximal limit for...
functional recovery in rats.\textsuperscript{21} For human DCD kidneys, the warm ischemia time can range from 30 to 120 minutes.\textsuperscript{22} Although it is impossible to correlate rat and human warm renal ischemic injury, it is likely that the warm ischemia time in our model reflects the higher end of the warm ischemic injury spectrum, as we are closer to the maximal warm ischemia time limit for functional recovery in rats. Additionally, we have incorporated a cold storage time of 18 hours in our model, which is reflective of renal graft preservation times in clinical practice,\textsuperscript{1} as the demand for transplantable kidneys leads to procurements from distant destinations. We used static cold storage for this model because the size of murine kidneys makes perfusion unfeasible. In future studies, this model could be useful for evaluating therapeutics that can be added to preservation solutions or injected into the circulation before or upon reimplantation of the DCD renal graft.

Fig. 3. Effect of hydrogen sulfide (H\textsubscript{2}S) on acute tubular necrosis (ATN) scores and relative gene expression in an in vivo murine model of renal transplantation upon donation after cardiac death. (A) Histopathology of kidney samples from the (A) sham, (B) untreated and (C) H\textsubscript{2}S-treated groups, stained with hematoxylin and eosin. (D) ATN scores by group (0 = 0%, 1 = 1%–10%, 2 = 11–25%, 3 = 26%–45%, 4 = 46%–75%, 5 = > 75%). (E) Relative mRNA expression of inflammatory and apoptotic genes. Target gene signals for the untreated and H\textsubscript{2}S-treated groups were normalized against β-actin and expressed as a ratio relative to the sham group.*p < 0.05.
The higher survival, faster recovery of graft function and lower tissue injury observed with the rats in the treatment group builds on our previous work establishing the protective effect of NaHS during warm and cold ischemic injury in other murine models.11,16 We have previously shown that prolonged static cold storage in NaHS-supplemented UW solution improved renal graft outcomes in a syngeneic model of murine renal transplantation.11 This study takes our previous work a step forward by establishing the protective effects of NaHS-supplemented UW solution in DCD renal transplantation. To confirm whether these effects translate to higher mammals, we used a porcine model of DCD renal transplantation as a proof of concept. The warm ischemia time in our porcine autotransplantation model is comparable to the warm ischemia time used in several other porcine studies evaluating novel organ preservation approaches.23,24 With the porcine kidneys, we chose to evaluate the use of NaHS-supplemented solution for HMP instead of static cold storage because HMP improves the outcomes of DCD renal grafts8 and is used by many transplant centres across North America. We chose a longer duration of cold preservation (24 h) for this model because HMP confers advantages, like nutrient circulation and waste removal, to static cold storage.9 The improved flow rate and lower resistance exhibited by the treatment group during HMP is likely owing to the vasodilatory properties of H2S.25,26 Furthermore, the improved recovery of graft function exhibited by the treatment group in the porcine model matches the outcomes of the treatment group in the murine model, which suggests that our approach translates to higher mammal systems.

![Fig. 4. Effect of hydrogen sulfide (H2S) in an in vivo porcine model of autotransplantation upon donation after cardiac death, including (A) flow rate and (B) resistance at the 1-hour and 24-hour time points during preservation perfusion, (C) serum creatinine and (D) relative mRNA expression of inflammatory and apoptotic genes. Target gene signals were normalized against β-actin. UW = University of Wisconsin solution. *p < 0.05.](image-url)
It is important to note that we induced a complete cessation of renal blood flow in our ischemic models by clamping the renal pedicle. This is an established technique used to mimic the warm ischemic injury experienced by DCD organs. However, it likely causes greater renal injury than the clinical DCD scenario because human DCD kidneys are retrieved after a period of reduced blood flow caused by the withdrawal of life support. Although other techniques could have been used to mimic DCD conditions, the chosen approach allowed for a vigorous evaluation of our novel kidney preservation approach. The improved outcomes of the treatment groups in our study would likely be amplified in a clinical setting where there is reduced renal perfusion rather than a complete absence of it. In addition, the consistent use of renal pedicle clamping in both experimental models strengthens the findings of this study.

**Limitations**

Our results add to a growing body of literature supporting the use of H$_2$S as an additive to preservation solutions during cold preservation, specifically showing the effects of this approach in the context of DCD renal transplantation. However, more research is needed to address the limitations of this study. In the murine model, we administered d-cysteine, a precursor to H$_2$S, to the donors in the treatment group before nephrectomy and graft preservation in UW and NaHS, which may be contributing to the improved outcomes of the treatment group. We administered d-cysteine to facilitate endogenous H$_2$S production and limit renal ischemia–reperfusion injury during warm ischemia and subsequent cold preservation. However, considering the results of the porcine model and our previous study of murine renal transplantation, which did not include d-cysteine injections, the improved outcomes of the treatment group in the murine model can largely be attributed to graft preservation with UW and NaHS. However, future research looking at the effects of d-cysteine alone may be beneficial. As both models used in this study are syngeneic, preservation with H$_2$S also needs to be evaluated using an allogeneic model of DCD renal transplantation because acute rejection is a key clinical concern in renal transplantation, especially for DCD renal grafts. Additionally, investigating the effects of slow-releasing H$_2$S donors would be beneficial because NaHS leads to an immediate and robust increase in H$_2$S levels, which is not ideal for clinical purposes.

**Conclusion**

This study shows that prolonged cold preservation in H$_2$S-supplemented UW solution improves DCD renal graft outcomes. We have established an in vivo murine model of DCD renal transplantation, with clinically relevant warm and cold ischemia times, that can be used for the evaluation of other therapeutics. In addition, we used an in vivo porcine model of renal autotransplantation to evaluate the effect of H$_2$S-supplemented UW solution in HMP, confirming the improved outcomes observed with the murine model. Although more research is needed to facilitate clinical translation, supplementing preservation solutions with H$_2$S is a promising avenue for improving the outcomes of renal grafts, including marginal DCD organs, thereby expanding the pool of transplantable kidneys.

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**Competing interests:** None declared.

**Contributors:** Masoud Akbari and Alp Sener contributed to the conception and design of the work. All authors were involved in the acquisition, analysis, and interpretation of data. Smriti Juriasingani, Vicky Vo and Alp Sener drafted the manuscript. All authors revised it critically for important intellectual content, gave final approval of the version to be published and agreed to be accountable for all aspects of the work.

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**Funding:** This work was supported by Physicians Services Incorporated (R4398A10) and the University of Western Ontario (R4398A12).

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