**Hydrogen sulfide treatment ameliorates long-term renal dysfunction resulting from prolonged warm renal ischemia-reperfusion injury**

Ian Lobb, BSc;*† Ian Alp, Sener, MD, PhD, FRCSC;*‡ Justin Zhu, MD;§ Weihua Liu, MD;‡ Aaron Haig, MD;‡ Zhu Lan, MD;§
Alp, Sener, MD, PhD, FRCSC*‡†§

*Department of Microbiology and Immunology, Western University, London, ON; †Schulich School of Medicine and Dentistry, Western University, London, ON; ‡Department of Pathology, Western University, London, ON; §Department of Surgery, Western University; †Multi-Organ Transplant Program, London Health Sciences Centre; ‡Matthew Mailing Center for Translational Transplant Studies, London Health Sciences Centre, London, ON

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**Abstract**

**Introduction**: The incidence of renal cell carcinoma (RCC) continues to rise concurrently with the increased prevalence of end-stage renal disease worldwide. Treatment for small renal masses continues to be partial nephrectomy mostly involving the clamping of renal blood vessels. Although necessary, this technique results in warm renal ischemia and reperfusion injury (IRI) to the afflicted kidney. We have recently demonstrated that hydrogen sulfide (H$_2$S), a novel endogenous gaseous molecule, protects against prolonged cold and short-term warm renal IRI. In the current study, we examined whether exogenous H$_2$S has long-term protective effects against warm renal IRI associated with renal surgical procedures.

**Methods**: Uni-nephrectomized Lewis rats underwent 1 hour of warm ischemia induced by clamping of the renal pelvis. Animals underwent either intraperitoneal treatment with phosphate buffered saline (PBS; IRI group) or PBS supplemented with 150 μM NaHS (H$_2$S group), and were compared against Sham-operated rats.

**Results**: H$_2$S treatment improved long-term renal function as serum creatinine at day 7 was significantly decreased in the H$_2$S group compared to IRI animals ($p<0.05$). H$_2$S treatment decreased the expression of pro-inflammatory markers TLR-4, TNF-α, IFN-γ, IL-2 and ICAM-1, increased the expression of pro-survival molecule Bcl-2 and decreased the expression of pro-apoptotic marker BID at postoperative day 1. H$_2$S-treated kidneys also showed a significant decrease ($p<0.05$) in infiltration of macrophages at day 7 post-IRI compared to no treatment.

**Conclusion**: H$_2$S treatment improved long-term renal function and decreased long-term inflammation associated with warm IRI, and may offer a novel therapeutic approach to preventing warm IRI-induced renal injury associated with renal surgical procedures.

**Introduction**

The incidence of renal cell carcinoma (RCC) continues to rise worldwide, constituting a heavy epidemiological and economic burden.¹⁻⁵ Treatment is primarily surgical and often includes partial nephrectomy (PN), which usually involves temporary clamping of the renal pedicle for up to 45 to 60 minutes, resulting in warm renal ischemia-reperfusion injury (IRI).⁶ IRI is a complex event initiated by renal ischemia, leading to adenosine triphosphate (ATP) depletion and impairment of cellular polarity and cytoskeletal structure.⁷ Subsequent reperfusion potentiates this damage by initiating a robust inflammatory response and release of reactive oxygen species (ROS), contributing to necrosis and apoptosis of renal tubular cells and renal dysfunction.⁸⁻⁹ Multiple studies have previously demonstrated that prolonged warm ischemia time (WIT) during PN causes residual renal tissue injury, is severely detrimental to postoperative renal function and can result in new onset chronic kidney disease (CKD).¹⁰⁻¹² Recently, small endogenously produced gaseous molecules, called gasotransmitters, have been shown to exert protective effects against tissue IRI. Hydrogen sulfide (H$_2$S) is the most recently characterized member of the gasotransmitter family, along with nitric oxide (NO) and carbon monoxide (CO), and has been the subject of increased interest due to its significant protective effects against tissue IRI.¹³⁻¹⁴ H$_2$S is endogenously produced by 3 enzymes (cystathionine γ-lyase [CSE], cystathionine β-synthase [CBS] and 3-mercaptopyruvate sulfur transferase [3-MST]); these enzymes play important physiological roles in vasodilation, angiogenesis and neuromodulation.¹⁵⁻¹⁸ More recently, H$_2$S has been shown to be protective in many models of tissue IRI, including brain, intestine, lung, liver and myocardium via a variety of antioxidant, anti-apoptotic and anti-inflammatory effects.¹⁹⁻²³ Using a rodent model of warm renal IRI involving uninephric renal clamping with clinically relevant, prolonged warm ischemic times, we have found that exogenous H$_2$S treatment during warm renal IRI improves renal function and reduces IRI-induced inflammation in the acute recovery period.²⁴ To improve clinical outcomes following PN, H$_2$S treatment must be shown to provide long-term improvement of renal function and resolution of inflammation. The current study examines the effects of exogenous H$_2$S treatment during warm IRI on renal function, injury and inflammation at extended postoperative time points.
Methods

Animal description and care

Adult male Lewis rats (200-250 g; Charles River Laboratories International Ltd.) were maintained in accordance with the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The experimental protocol followed the guidelines of the Council on Animal Care of our institution.

Warm IRI surgical procedure and postoperative monitoring

Rats were anesthetized by inhalation of 5% isoflurane and maintained under anesthesia with 2% isoflurane during surgery and 1% isoflurane during reperfusion. A right nephrectomy was initially performed via a midline abdominal incision to remove confounding protective effects of a functioning contralateral kidney and renorenal reflex. The left renal pedicle was subsequently occluded viaatraumatic clamping for 60 minutes followed by reperfusion. During occlusion, the abdomen was filled with 10 mL of either phosphate buffer saline (PBS; IRI group, n = 6) or with PBS plus 150 μmol/L NaHS (H₂S group, n = 8), after which the abdominal incision was closed. Sham animals were also followed (n = 4). Each surgery was performed by the same experienced micro-surgeon, blinded to treatment groups. Animals were monitored for 7 days post-IRI and blood samples were obtained at day 3 and day 7. Serum was analyzed at London Health Science Centre Laboratories for levels of creatinine (Cr), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Previous biochemical data at 2 hours post-reperfusion were used as baseline.²⁴

Histological evaluation

At the time of sacrifice, the kidney was removed and divided sagittally, with each half used for either histological or qRT-polymerase chain reaction (PCR) analysis. Tissues fixed in 10% formalin were embedded in paraffin, sectioned and stained with both hematoxylin and eosin (H&E) and terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) to determine levels of renal necrosis and apoptosis, respectively. Histological sections also underwent immunohistochemical staining with antibodies against macrophage surface marker CD68 (Abcam Inc.) visualized with secondary antibodies and DAB substrate chromogen using the Dako Envision System (Dako Inc.) as per the manufacturer’s protocol. All H&E and TUNEL sections were scored by an experienced clinical renal pathologist in a blinded fashion. IHC sections were quantified using median cell counts of positive cells contained in 5 random areas of each section (100× magnification) in a blinded fashion.

Quantitative RT-PCR analysis

Total RNA was isolated from homogenized renal tissue using TRIzol (Invitrogen, Inc.) and reverse transcribed into cDNA using Super Script II Reverse Transcriptase (Invitrogen, Inc.) in conjunction with Oligo(dT)₁₂₋₁₈ primers as per the manufacturer’s protocol. Isolated RNA and cDNA consistently had A₂₆₀/₂₈₀ ratings >1.95 and >1.8, respectively. All qPCR assays were performed using SYBR Green PCR Master Mix (Quanta Biosciences Inc.) and analyzed via StepOnePlus Real-Time PCR thermal cycler and software package (Applied Biosystems Inc.). Primer sequences were designed using Primer-BLAST software (NCBI) against hypoxanthine ribosyltransferase (HPRT-1), toll-like receptor 4 (TLR-4), tumour necrosis factor alpha (TNF-α), intercellular cell adhesion molecule 1 (ICAM-1), interleukin 2 (IL-2), B-cell lymphoma 2 (Bcl-2) and gene BH3 interacting-domain death agonist (BID) genes. Fold-change in gene expression was calculated via the ΔΔCt method, using HPRT-1 as a reference gene.

Statistical analysis

Data in figures are presented as mean ± standard deviation (SD). ANOVA and Student’s t-tests were performed to statistically analyze independent groups (GraphPad Software Online). Statistical significance was accepted at p < 0.05.

Results

Exogenous H₂S treatment improved long-term renal function

At 2 hours post-ischemia, exogenous H₂S treatment decreased Cr after warm renal IRI from 72.8 ± 5 μmol/L (IRI) to 62.8 ± 3.1 μmol/L (H₂S group), compared to Sham (11 ± 0.7 μmol/L) (Fig. 1). Cr in the H₂S group continued to be decreased compared to the IRI group at day 3 (45.2 ± 12.5 μmol/L vs. 53.25 ± 7.6 μmol/L) and significantly decreased by day 7 (36.8 ± 2.2 μmol/L vs. 49 ± 8.9 μmol/L, p < 0.05) (Fig. 1). Cr in Sham animals was consistently significantly lower than both IRI and H₂S groups at both days 3 (23.5 ± 2.1 μmol/L) and 7 (20.75 ± 2.9 μmol/L) (p < 0.05), except with H₂S at day 3.

Serum ALT and AST levels unaffected in the long-term by H₂S treatment

The elevated ALT and AST in IRI group at 2 hours, indicative of a systemic inflammatory response, returned to baseline levels by day 3 and remained at baseline levels until day
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7 (Fig. 1). Both ALT and AST levels from IRI or H₂S groups had no significant differences compared to Sham (Fig. 1).

H₂S did not improve long-term renal necrosis and apoptosis scores

Upon histological analysis both IRI and H₂S groups had increased tubular necrosis and apoptosis compared to Sham (Fig. 2). However, while H₂S kidneys revealed slightly lower necrosis and apoptosis scores compared to IRI, these differences were not significant (Fig. 2).

Renal expression of pro-inflammatory and apoptotic markers was determined via qRT-PCR on kidneys obtained at both day 1 and day 7 post-IRI. While both treatment groups exhibited increased expression of pro-inflammatory markers TLR-4, TNF-α, ICAM-1 and IL-2 at day 1, H₂S treated kidneys exhibited a marked decrease in the expression of these molecules compared to IRI (Fig. 3). As well, H₂S treated kidneys showed a marked increase in the expression of anti-apoptotic molecule BCL-2 and a marked decrease in the expression of pro-apoptotic molecule BID compared to IRI (Fig. 3). Expression levels of these genes decreased towards baseline by day 7 and no differences in expression were found between treatment groups at this time point (data not shown).

H₂S treated kidneys exhibited decreased long-term inflammatory infiltrate

Kidneys were obtained at day 7 and immunohistochemically stained with antibodies against macrophage marker CD68. IRI kidneys showed a marked increase in the numbers of CD68-positive cells compared to Sham animals, while H₂S treated kidneys showed significantly fewer (p < 0.05) numbers of these cells at day 7 compared to IRI (Fig. 4).

Fig. 1. Exogenous H₂S reduces initial and long-term renal dysfunction and initial systemic inflammation following warm renal IRI. Serum levels of creatinine (A), alanine aminotransferase (ALT; B) and aspartate aminotransferase (AST; C) collected from Lewis rats at 2 hours, 3 days and 7 days following Sham operations (Sham, white), 1-hour ischemia (ischemia and reperfusion injury [IRI, grey]), and IRI plus H₂S treatment (150 μmol/L of NaHS i.p. at time of renal pedicle clamping and during reperfusion; H₂S, black). Creatinine levels used as metabolic marker of kidney function; ALT and AST used as markers of liver injury secondary to systemic inflammatory response induced by warm renal IRI. Data are expressed as mean ± standard deviation. *p < 0.05.

Fig. 2. H₂S treatment does not affect long-term renal necrosis and apoptosis following warm renal ischemia and reperfusion injury (IRI). Pathological necrosis and apoptosis scores of kidneys following Sham operations (Sham, white), 1-hour ischemia (IRI, grey), and IRI plus H₂S treatment (150 μmol/L of NaHS i.p. at time of renal pedicle clamping and during reperfusion; H₂S, black) obtained at 7 days post-ischemia. Renal necrosis and apoptosis assessed upon hematoxylin and eosin (H&E) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, respectively. Scores (amount of change per total slide) are out of 2: 0 = no change; 0.5 = 0-25% change; 1 = 25-50% change; 1.5 = 50-75% change; 2 ≥75% change. Values are mean ± standard deviation.
A recent flood of research has identified H$_2$S as an important player in many physiological processes and its unique attributes have also shown to provide a protective benefit against various forms of tissue IRI, including renal IRI.\textsuperscript{25,26} Previously, we have demonstrated that H$_2$S protects against periods of prolonged cold IRI, as well as from shorter periods of warm IRI.\textsuperscript{24,27} Recently, H$_2$S has also been shown to be reno-protective against warm IRI in large-animals.\textsuperscript{28} However, long-term studies on the effects of H$_2$S have been limited. In this current study, we found that the short-term protection of kidneys against warm IRI via H$_2$S has a long-term protective benefit on renal function and inflammation.

Following warm IRI, we observed a consistent reduction of Cr in the H$_2$S-treated group compared to the IRI group at days 3 and 7. While Cr in both groups continued to approach baseline with time, H$_2$S treatment allowed animals to regain renal function more rapidly compared to no treatment. This is important clinically as patients are often presented with various postoperative analgesics and antibiotics that are renally cleared. We also observed that the acute rise in liver enzymes quickly resolved to baseline levels by day 3 at similar rates in both treatment groups. While the dampening of the acute systemic inflammatory response to renal IRI via H$_2$S treatment may be important for short-term renal protection, H$_2$S did not exhibit long-term effects on resolution of systemic inflammation.

Previous studies, including our own have demonstrated that H$_2$S is able to protect against renal IRI via limiting acute tubular necrosis (ATN).\textsuperscript{24,25} However, we report that tissue necrosis and apoptosis scores, while still increased compared to Sham, are nearly equal between treatment groups by day 7 post-IRI. This indicates that while H$_2$S treatment appears to protect against necrosis and apoptosis associated with warm IRI in the short-term, kidney injury seems to have been resolved to a somewhat similar degree by day 7 regardless of treatment. NaHS is one of many experimentally available hydrogen sulfide donor molecules. We used it in this study as it delivers a predictable and safe dose of H$_2$S; however, it is limited by its rapid rate of release (about 5-minute half-life in open systems).\textsuperscript{29} This phenomenon may explain why the protective effects of H$_2$S in our current model are blunted compared to previous studies. Future work in this area should include longer acting donor molecules or precursor molecules to either CBS or CSE.

The anti-inflammatory and anti-apoptotic properties of H$_2$S in the context of IRI have previously been well-documented. Multiple studies have demonstrated that H$_2$S is capable of limiting leukocyte migration, and cytokine expression and release.\textsuperscript{22-24,30-32} We found that H$_2$S treatment during warm IRI initially decreased the expression of pro-inflammatory markers, increased the expression of pro-survival marker Bcl-2 and decreased the expression of pro-apoptotic marker BID. While the expression of these markers decreased toward
baseline by day 7, H$_2$S treatment also resulted in decreased long-term renal inflammation as evidenced by the diminished numbers of infiltrating macrophages in H$_2$S treated kidneys at day 7. While it is unclear whether macrophages are players in the initial inflammatory pathogenesis of IRI or merely involved in the tissue healing process, their chronic presence is a strong indicator of renal inflammation. The early effects of H$_2$S on inflammatory and apoptotic gene expression could possibly facilitate this long-term decrease in renal inflammation, which may be an important factor in preserving long-term renal function in H$_2$S treated kidneys following warm IRI.

While the unilateral renal clamping plus contralateral nephrectomy model used in this study is commonly accepted, it is not necessarily representative of actual clinical situations. However, since many patients requiring PN already have diminished overall renal function, our data offer a possible protective solution. Therefore, future studies should also investigate the protective effects of H$_2$S during both PN of the left kidney in varying proportions and concomitant warm ischemia. Previous studies investigating the protective effect of H$_2$S against warm renal IRI have generally looked at renal function immediately following ischemia, while we have established that this improvement is sustained for 7 days. While this is an important finding, longer-term studies are required to determine whether H$_2$S treatment permanently improves graft function following warm IRI and whether this effect reduces long-term morbidity associated with renal surgical procedures.

**Conclusion**

Our findings demonstrate that H$_2$S treatment protects kidneys from injury during prolonged warm renal IRI, which translates to improved long-term renal function during the recovery phase. More research is needed to identify the specific mechanisms of H$_2$S-mediated protection against warm renal IRI, as well as the effective methods of delivery of H$_2$S during renal surgical procedures. However, H$_2$S-based treatments could provide a novel method of protecting already damaged kidneys against warm IRI during renal surgical procedures, resulting in improved long-term clinical outcomes and patient quality of life. Considering the increasing incidence of RCC, especially in patients who have other concomitant risk factors for CKD, novel methods of limiting IRI during renal surgical procedures, such as H$_2$S treatment, will be critical in ensuring the best possible outcomes for patients.

**Competing interests:** Dr. Lobb, Dr. Zhu, Dr. Liu, Dr. Haig, Dr. Lan and Dr. Sener all declare no competing financial or personal interests.

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**References**


Correspondence: Dr. Alp, Sener, Department of Surgery, Western University, LHSC, University Hospital C4-208, 339 Windermere Rd, London, ON N6A 5A5; alp.sener@lhsc.on.ca