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Subnormothermic Oxygenated Perfusion Optimally Preserves Donor Kidneys *Ex Vivo*

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Introduction: The current methods of preserving donor kidneys in nonoxygenated cold conditions minimally protect the kidney against ischemia-reperfusion injury (IRI), a major source of complications in clinical transplantation. However, preserving kidneys with oxygenated perfusion is not currently feasible due to the lack of an ideal perfusion mechanism that facilitates perfusion with blood at warm temperature. Here, we have designed an innovative renal pump circuit system that can perfuse blood or acellular oxygen carrier under flexible temperatures, pressures, and oxygenation. We have tested this apparatus to study optimal conditions of storage of our porcine model of donation after cardiac death (DCD) kidneys.

Methods: Porcine kidneys were retrieved after 30 minutes of cross-clamping renal pedicles *in situ*. Cessation of blood mimics postcardiac death in humans and simulates DCD warm ischemic injury. Procured kidneys were flushed and subjected to static cold storage (SCS) for 4 hours. For warm perfusion, kidneys were cannulated for pulsatile oxygenated perfusion with blood:PlasmaLyte for 4 hours at 15 °C, 22 °C, and 37 °C. To mimic posttransplant scenario, all kidneys were reperfused with blood for an additional 4 hours at 37 °C.

Results: Compared with all other groups, 22 °C perfusion resulted in significant reduction of acute tubular necrosis (ATN), apoptosis, kidney damage markers, Toll-like receptor signaling, and cytokine production. It was associated with maximal renal blood flow and urine output. Kidneys stored at 15 °C thrombosed within 2 hours under this condition. Martius Scarlet Blue staining confirmed that 22 °C was the optimal temperature to minimize hemorrhage and blood clots.

Conclusion: Our novel study shows that oxygenated perfusion at near-room-temperature provides optimal donor kidney storage conditions.

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Kidneys obtained DCD are prone to IRI.[1] Currently, cold static and machine-based pulsatile perfusion at 4 °C are the most commonly used methods to reduce metabolic activity and preserve the allograft in clinical kidney preservation. Due to cold injury and prolonged anoxia, these methods fail to optimally protect kidneys from preservation-related damage. Prolonged hypothermia induces interstitial expansion, edema, and capillary compression. As a result, tubular epithelial cells undergo injury and, if unchecked, death by apoptosis and ATN with compromised functional capacity, electrolyte impairment, reduced excretion, and decreased survival rate after transplantation.[2] Furthermore, DCD kidneys sustain additional warm ischemia, which leads to greater ATN and pronounced delayed graft function compared with kidneys obtained from donation after brainstem death.[3,4] Because DCD is becoming the fastest growing source of organs in North America, higher delayed graft function rates have become a considerable problem. Therefore, there is a need to develop a novel

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method for superior protection and preservation for vulnerable organs such as DCDs.

Recently, Nicholson and Hosgood[5] perfused deceased donor kidneys with oxygenated red cell–based solution at 36 °C *ex vivo* for 1 hour to improve transplant outcomes. Although this study demonstrated that delayed graft function rates were reduced with normothermic perfusion of the kidney, the optimal temperature and perfusion time were not explored. With regard to optimal temperatures for organ preservation, Niemann *et al.*[6] showed that organ donors cooled to 34 °C before retrieval may yield superior organs. Therefore, although oxygenation of organs during storage appears to improve graft outcomes, the optimal temperature at which the organ is perfused and availability of a renal pump system that can perform oxygenated perfusion in a regulated way during *ex vivo* storage has not yet been established.

In addition, the specific role of proinflammatory innate immune pathways associated with IRI has not been addressed decisively in larger animals such as pig. Toll-like receptors (TLRs) are the main sources of inflammation in IRI in which high-mobility group box 1 (HMGB1) and nuclear DNA (collectively called DAMPs) released from the necrotic cells or organs undergoing prolonged ischemia act as TLR ligands.[7–9] Therefore, inhibiting TLR directly or their signaling molecules such as HMGB1, MyD88, or nuclear factor (NF)–κB may have an impact in controlling IRI-related proinflammatory consequences.

As an alternative to cold perfusion, we have primarily focused on developing a pump circuit system that can perfuse blood, blood products, or blood-like products. To achieve perfusion-reperfusion in relatively warm conditions, our group has reengineered a clinically used pulsatile perfusion system originally developed for hypothermic machine perfusion (RM3 Renal Preservation System; Waters Medical System, Rochester MN) to deliver oxygenated blood or perfusate at different temperatures, pressures, and degrees of oxygenation. This *ex vivo* apparatus can also mimic *in vivo* function by maintaining similar fluid dynamics, pressure, resistance, and urine-producing capacity. By using this system, we will ultimately optimize conditions to preserve, protect, and repair DCD kidneys during prolonged periods of *ex vivo* storage to improve current logistical constraints and to improve posttransplant outcomes.

In this article, our main objective was to compare the current standard of SCS preservation of model porcine DCD kidneys with the oxygenated pulsatile perfusion with blood-based perfusate at different temperatures (15 °C, 22 °C, and 37 °C) followed by 37 °C oxygenated whole blood reperfusion of all kidneys. The end points included multiple markers of injury and finally *ex vivo* function of kidneys at reperfusion.

METHODS

Animals and Blood Collection

Large (50–55 kg) white male Landrace adult pigs were used in all experiments. All procedures performed on animals were approved by the research ethics board of Western University, Canada. DCD conditions were simulated by cross-clamping the renal pedicle *in situ* for 30 minutes. Venous draw before clamping (blood used for perfusion) and direct cardiac puncture (blood used for reperfusion) was used to collect blood in sterile containers with 25,000 units of heparin. For perfusion, we obtained blood before renal clamping. This blood is devoid of any stressors such as cytokines or damage factors such as HMGB1 (i.e., nonstressed blood). To maintain ideal electrolyte balance and pH, and to compensate the amount of urine generated during 4 hours of perfusion, we diluted this blood with PlasmaLyte solution (1:2; Baxter Corporation, Deerfield, IL). For reperfusion at 37 °C, which mimics the posttransplant situation in clinic (i.e., reestablishment of blood supply), we used blood obtained after clamping (i.e., stressed blood).

Study Design

After procurement, DCD kidneys were briefly flushed with cold histidine-tryptophan-ketoglutarate solution. For control group representing SCS, organs were submerged in histidine-tryptophan-ketoglutarate solution and stored in a cooler with ice (2–4 °C) for 4 hours. These kidneys were used as controls. For blood perfusion groups, kidneys were assigned to storage in pulsatile perfusion with autologous oxygenated blood devoid of any vasodilators or exogenously added nutrients. We have adjusted pH time to time with added bicarbonate. Perfusion of kidneys with blood produces urine. We have replenished the amount of urine equivalent (volume) by adding PlasmaLyte solution. A mean pressure of 70 mm Hg was maintained during RM3 pump (Waters Instrument Inc., Rochester, MN) pulsatile perfusion for 4 hours. Perfusate oxygenation was set at 40% at 15 °C, 22 °C, and 37 °C ($n = 5$ in each group). After storage, all kidneys were reperfused with autologous blood obtained from the pigs after clamping for another 4 hours on the RM3 machine at 37 °C at a mean pressure of 70 mm Hg. Metabolic activity during reperfusion at 37 °C was assessed by hourly blood pO_2 analysis, pH, and osmolality. *Ex vivo* renal function was assessed by adding creatinine (10 mg/l) exogenously to the blood and measuring urinary creatinine hourly via the urine collected during the reperfusion stage.

Physiological Data Analysis

Urine output and blood flow rate was recorded hourly. Biochemical analysis of urine samples (total urinary protein, urine creatinine/hour, protein/creatinine ratio in total urine) were carried out in the hospital core facility and clinical medical laboratory.

Histology

At the end of the reperfusion, kidney tissue was fixed in 10% formalin in phosphate-buffered saline solution, embedded in paraffin, sectioned in 5 μm and stained with hematoxylin-eosin for ATN, terminal deoxynucleotidyl-transferase dUTP nick end-labeling for apoptosis, and Martius Scarlet Blue for blood (yellow), fibrin, and collagen (blue). In a blinded manner, scoring for cell death was determined by a pathologist by using a semiquantitative graded scale: 0, no change; 1, <10%; 2, 11% to 25%; 3, 26% to 45%; 4, 46% to 75%; and 5, 76% to 100%. ATN injury was assessed as percentage of tissue involved with histological changes. Average score of a minimum of 3 independent tissue sections from the similar treatment with 3 nonoverlapping fields for each kidney were used for statistical analysis.

Enzyme-Linked Immunosorbent Assay

Neutrophil gelatinase-associated lipocalin (NGAL) is a standard kidney injury marker. The amount of porcine urinary NGAL was determined by enzyme-linked immunosorbent assay (ELISA) as per supplied protocol. Briefly, total *ex vivo* urine was collected and centrifuged to remove particulate matter and diluted to 1:10 in the buffer supplied with the kits; 100 μl of standards and samples were added to the ELISA plate wells to facilitate binding with NGAL-specific precoated antibodies. Addition of biotinylated detection antibodies (100 μl) followed by Avidin–horseradish peroxidase conjugate and TMB substrate incubation was performed at room temperature until a suitable intensity of blue color appeared. The reaction was terminated by sulfuric acid solution and measured by a spectrophotometer at a wavelength of 450 ± 2 nm. Concentration of NGAL was determined from the standard curve. Interleukin (IL)-6 is a prominent marker of inflammation in IRI. Urine IL-6 ELISA was carried out following the manufacturer's protocol (Abcam, Cambridge, UK). Briefly, 100 μl urine during the first hour of reperfusion was incubated for 2.5 hours at room temperature in the plate coated with IL-6 capture antibody. After washing, the plate was incubated for 1 hour at room temperature with the biotinylated IL-6 detection antibody. The plate was then washed and incubated with horseradish peroxidase–Streptavidin for 45 minutes at room temperature, followed by adding TMB substrate. The stop solution was added after 30 minutes of color development. The plate was read at 450 nm by iMark microplate reader (Bio-Rad, Hercules, CA). IL-6 concentration was interpolated from the standard curve by GraphPad Prism 6 (La Jolla, CA).

Quantitative Real-Time Polymerase Chain Reaction

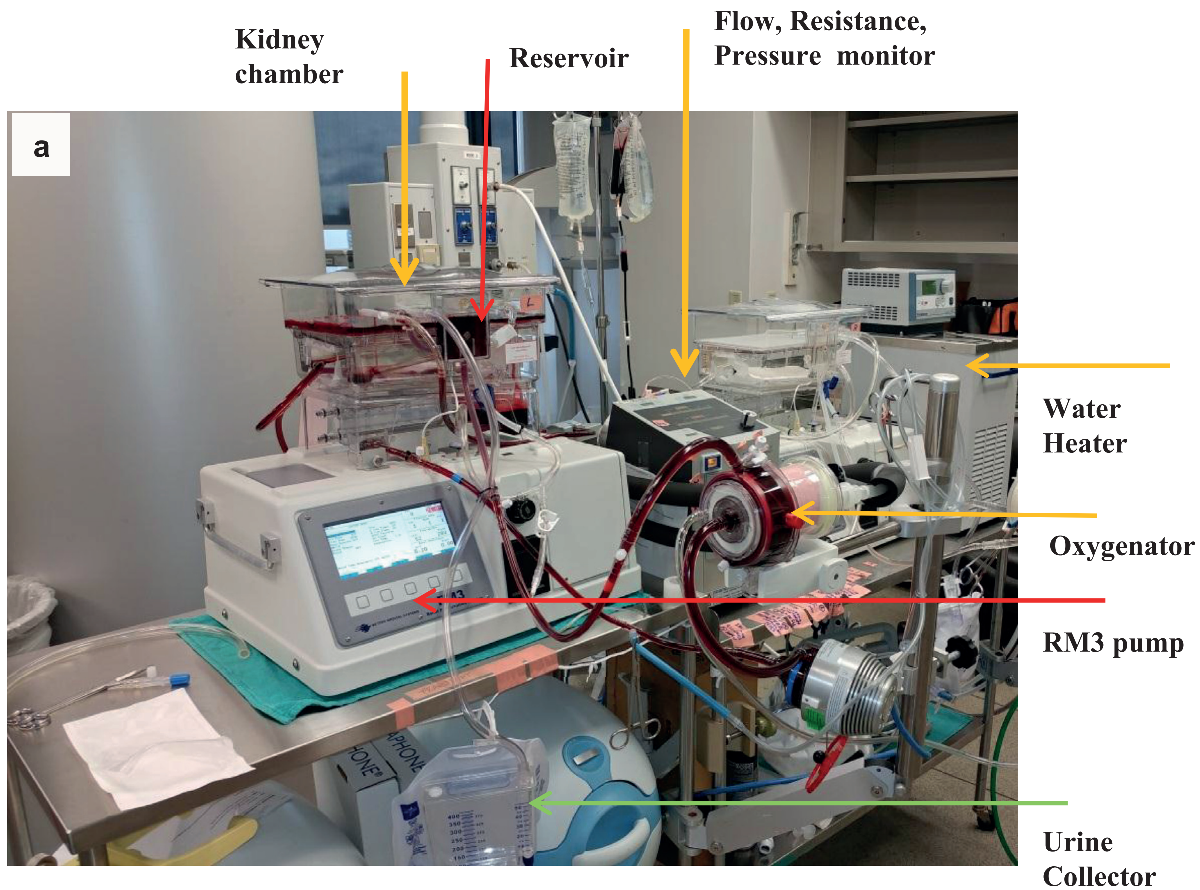
Total RNA was extracted from 40 mg of thoroughly homogenized fresh kidney tissues in lysis solution with PerfectPure RNA and Tissue kits (Applied Biosystems, Foster City, CA); 500 μl of whole tissue lysate was then passed through Preclear followed by washing with 500 μl wash 1 and wash 2 solutions. This eliminates proteins and DNA. Finally, the purified RNA was eluted with Diethyl pyrocarbonate–treated water and measured by a nanodrop spectrophotometer. Complementary DNA was synthesized with the First-strand synthesis System according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Real-time quantitative polymerase chain reaction was done in the presence of SYBR Green QPCR Master Mix kit (Invitrogen). Amplification of beta-actin mRNA was used as an internal control. The normalized delta threshold cycle value and relative expression levels ($2-\Delta\Delta$Ct) were calculated according to the manufacturer's protocol.

Ex vivo Oxygenated Perfusion in Modified RM3 Pump

All procedures relating DCD simulation *in situ* and running of *ex vivo* circuit during perfusion were technically flawless. Kidneys from 10 DCD donor animals were evaluated during *ex vivo* perfusion. Our circuit provided both inflow and outflow to the kidney in which deoxygenated blood leaving the reservoir was rerouted through the oxygenator in order to maintain a regulated amount of oxygen. Figure 1a shows the composition of the circuit including pulsatile peristaltic pump, disposable cassette, a membrane oxygenator, venous blood reservoir, and water-heating unit. A simplified design is shown in schematic (Figure 1b). Our study design was based on DCD kidneys subjected to 30 minutes of *in situ* warm ischemia followed by 4 hours of SCS (2–4 °C) versus subnormothermic (15 °C and 22 °C) and normothermic (37 °C) storage with pulsatile oxygenated blood (Figure 1c).

Statistical Analysis

Data were compared using Student *t*-test for unpaired values, 1-way analysis of variance for 1 factor and 2-way analysis of variance for 2 factors. A P value $\leq$0.05 was considered to be significant. Data are represented as means ± SD. All statistical analyses were performed with GraphPad Prism 6.



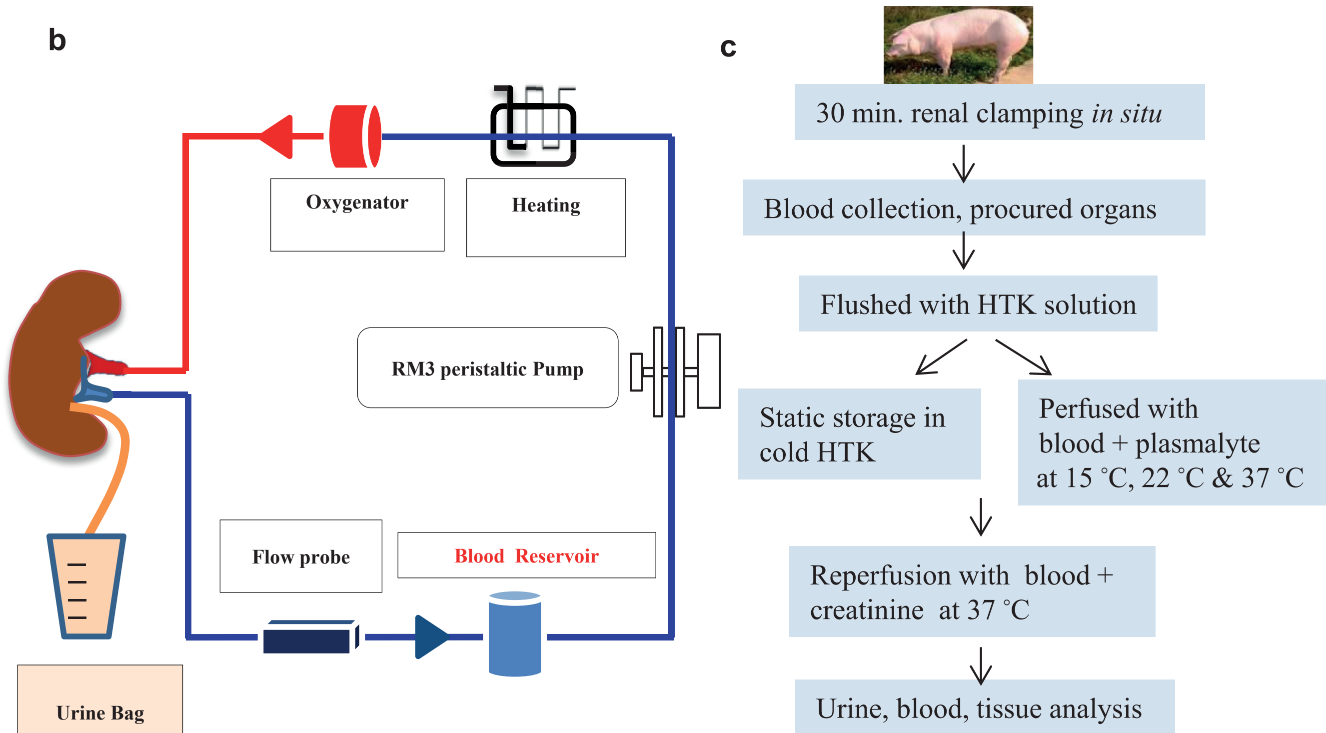


Figure 1. Modified RM3 pump (Water Instruments Inc., Rochester, MN) for blood perfusion/storage and reperfusion in the same circuit. (a) RM3 renal preservation pump was modified for whole blood perfusion and reperfusion in the same unit under different temperature settings. This system provides pulsatile perfusion and measures resistance and renal blood flow. For assessment of overall functional dynamics during *ex vivo* perfusion, several components, including the oxygenator, water heater exchanger, flow meter, and temperature probe, were attached. Kidneys were placed in a cassette reservoir as pictured with the cannulated artery, vein, and ureter as designed in the schematic (b). (c) Study design for *ex vivo* experiments at different temperature conditions. HTK, histidine-tryptophan-ketoglutarate.

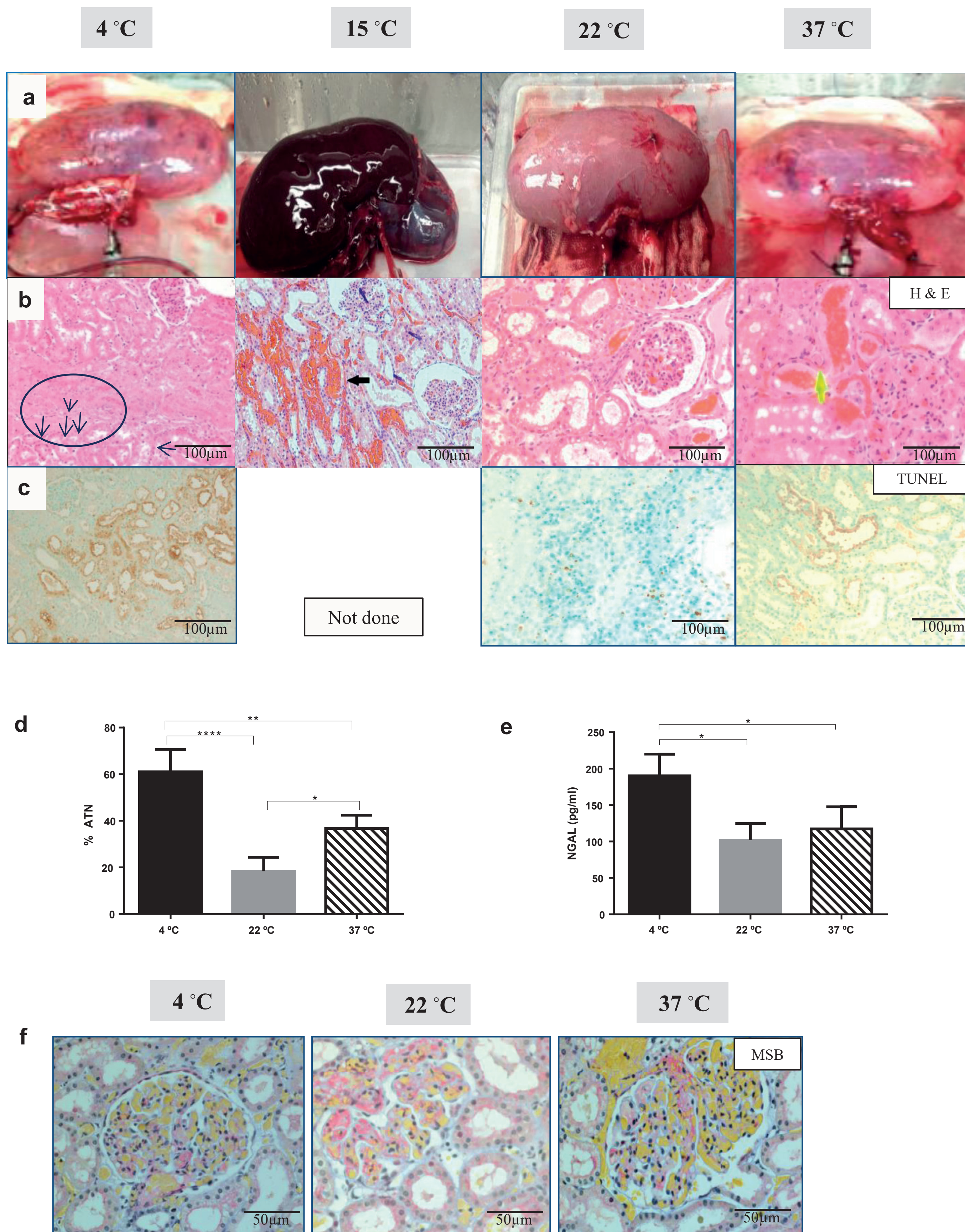


Figure 2. Oxygenated blood perfusion at 22 °C reduces ischemia-reperfusion injury and interstitial hemorrhage in donation after cardiac death model kidneys. (a) Kidneys subjected to 30 minutes of warm ischemia *in situ* were perfused/stored at different temperatures as indicated at the top of the figure. After 8 hours of blood perfusion-reperfusion, gross kidney (a) sections were stained with hematoxylin-eosin (H&E) (continued)

RESULTS

Validation of a New Perfusion Device for Comparison of Different Preservation Modalities

To provide oxygenated perfusion using physiological conditions such as pressure, temperature, and perfusate, we have reengineered an existing clinical pulsatile pump system (RM3; Water Instruments Inc., Rochester, MN). The RM3 pump has been approved by the Food and Drug Administration for hypothermic nonoxygenated pulsatile preservation (Figure 1a). A simplified design is shown in Figure 1b and our study design is illustrated in Figure 1c. Modification of this system allows this circuit to perfuse blood or blood-like perfusates by adding an oxygenator and creating a double-luer connector to provide both inflow and outflow to the organ. Deoxygenated blood leaving the renal vein is rerouted through the oxygenator to be reoxygenated and resupplied to the kidney via the renal artery. The Medtronic (Dublin, Ireland) centrifugal pump seen in Figure 1a is being used only to monitor flow and pressure because the flow probe attached to the RM3 pump was unable to read them perfectly.

Views of the DCD kidneys placed in the chamber at perfusion stage were photographed (Figure 2a). A conduit was routed from the ureter to a urinary bag to collect urine for analysis.

Ex Vivo Oxygenated Perfusion at 22 °C Led to Minimal Injury

In the current study, DCD kidneys stored in a cooler box at 2 to 4 °C (Figure 2a, left) were used as controls representing current standard of care for organ preservation. No visible differences in gross morphology (e.g., edema, and thrombosis) were found in kidneys treated either in 4 °C (SCS), 22 °C and 37 °C storage conditions. At 15 °C, however, DCD kidneys became fully thrombosed within 2 hours of perfusion. Severe hemorrhage was observed in hematoxylin-eosin staining. We assume that viscosity and clumping at lower temperatures are 2 main factors responsible for clotted kidneys at 15 °C. At set pressures, flow was poor and intrarenal resistances were exceptionally high in this group. All other groups sustained different degrees of tubular damage, as demonstrated by histology after 4-hour reperfusion. However, as shown in Figure 2b to 2d, ATN score was significantly lower for 22 °C kidneys compared with kidneys preserved in 4 °C (****$P < 0.0001$). Although *ex vivo* perfusion at 37 °C demonstrated reduced ATN score compared with SCS (**$P = 0.0026$; Figure 2d), oxygenated blood perfused kidneys at 22 °C had a significantly lower level of ATN injury compared with kidneys stored at 37 °C (*$P = 0.0137$). Presence of apoptotic death in terminal deoxynucleotidyl-transferase dUTP nick end-labeling staining was minimal at 22 °C compared with all other groups (Figure 2c). To measure overall or total damage during the entire procedure, we performed NGAL ELISA on total urine produced at the end of reperfusion. Consistent with ATN score, porcine urinary kidney injury marker NGAL was significantly lower in the urine produced by kidneys preserved in 22 °C–treated versus SCS (*$P = 0.0191$). Although a modest amelioration of injury was reported at 37 °C, intrarenal hemorrhage was evident in all experiments during this physiological temperature as seen by Martius Scarlet Blue staining (yellow for blood) (Figure 2f).

Subnormothermic Oxygenated Perfusion at 22 °C Resulted in Improved *Ex Vivo* DCD Kidney Function at Reperfusion

Ex vivo functional characteristics during pulsatile perfusion were also measured. Compared with SCS kidneys, renal blood flow was significantly higher in the subnormothermic group at 22 °C during the reperfusion phase (Figure 3a). Flow rate was not significantly different between perfusion at 37 °C and SCS at 4 °C ($P = 0.0625$) but comparatively higher at 22 °C compared with 4 °C groups (*$P = 0.0239$). Interestingly, on reperfusion, kidneys perfused at 22 °C produced significantly more urine than normothermic and SCS groups (308 ± 171 ml vs. 85 ± 34 ml; *$P = 0.0238$) (Figure 3b). In addition, urine produced during 22 °C perfusion had lower hourly protein/creatinine ratios during 4 hours of reperfusion (****$P < 0.0001$, Figure 3c) as well as total protein/creatinine ratios versus all other groups (Figure 3d; **$P = 0.0034$). Creatinine clearance was higher in the 22 °C and 37 °C groups

Figure 2. (continued) (b), and terminal deoxynucleotidyl-transferase dUTP nick end-labeling (TUNEL) (c) to assess edema, acute tubular damage, and apoptosis, respectively. Kidneys perfused at 15 °C suffered a blood clot and succumbed to complete damage beyond evaluation compared with static cold storage (SCS). Arrow indicates extensive hemorrhage (b). Blue circle and arrowheads indicate the massive necrotic area at 4 °C (SCS), and the yellow arrowhead is showing thrombi formation (at 37 °C). Percentage of acute tubular necrosis (ATN) from H&E sections (original magnification ×40) was determined using representative images from 3 independent experiments by a qualified pathologist. The 22 °C perfusion showed the most significant reduction in ATN compared with 4 °C (SCS; 18.3% ± 6.0% vs. 61.0% ± 9.6%, ****$P < 0.0001$) and 37 °C (18.3% ± 6.0% vs. 36.7% ± 5.7%, **$P = 0.0026$) (d). In addition, it was associated with reduced kidney injury marker neutrophil gelatinase-associated lipocalin (NGAL) compared with 4 °C (102 ± 22 vs. 190 ± 30, *$P = 0.0191$ (e). (f) Intrarenal hemorrhage was indicated by staining the tissue section with Martius Scarlet Blue (MSB). Yellow staining in MSB represented blood.

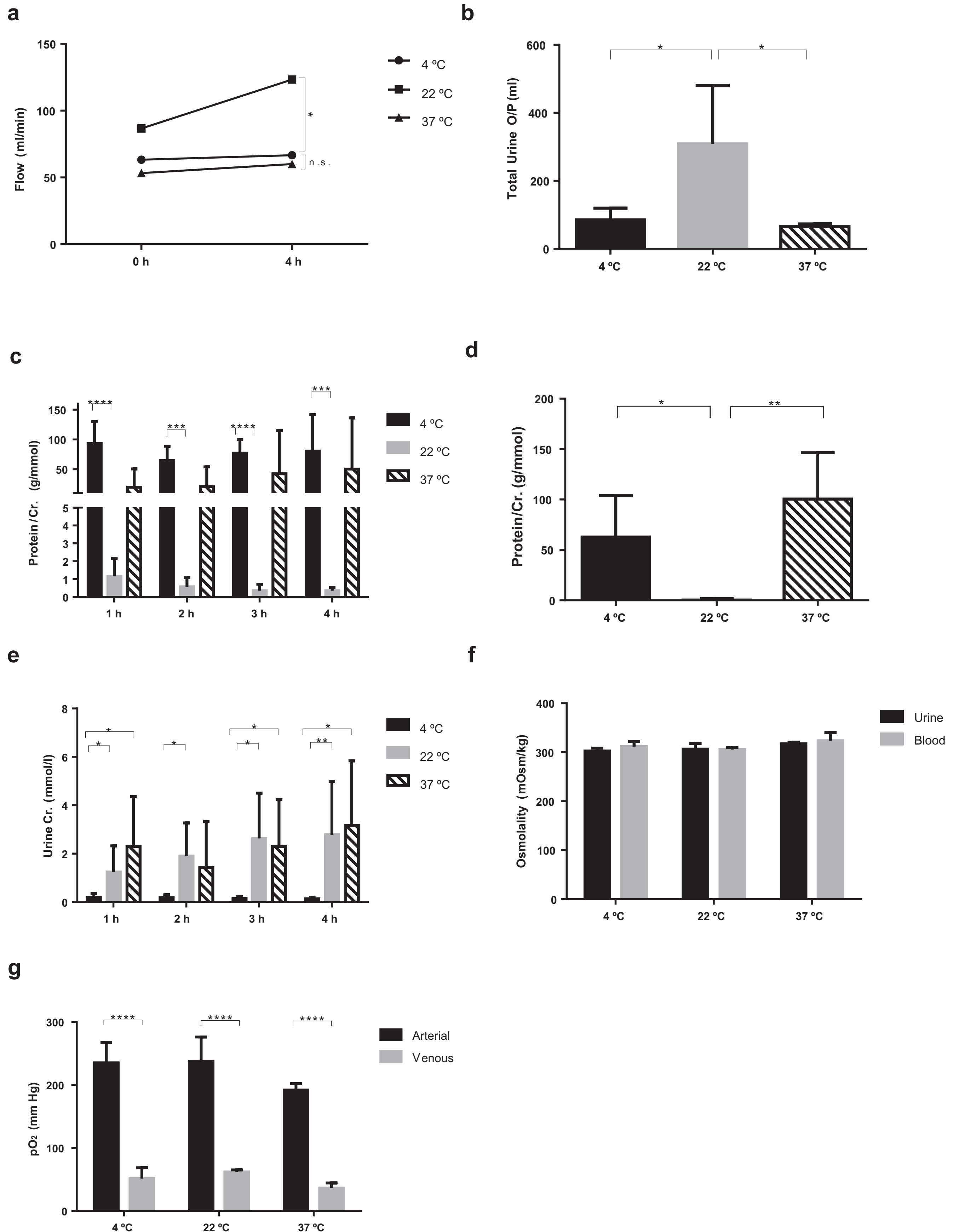


Figure 3. Oxygenated blood perfusion at 22 °C confers optimal kidney function *ex vivo.* Donation after cardiac death (DCD) kidneys subjected to 4 different temperature conditions were monitored at reperfusion. An improved *ex vivo* function of DCD kidneys was observed at 22 °C evaluated by (a) renal blood flow rate (ml/min) (112 vs. 54 ml/min, $*P = 0.024$). (b) Higher total urine output (308 $\pm$ 171 vs. 85 $\pm$ 34 ml [continued]

versus hypothermic group (1 hour, $^{*}P = 0.042$; 2 hours, $^{*}P = 0.012$; 3 hours, $^{*}P = 0.009$; 4 hours, $^{**}P = 0.015$; Figure 3e). Both paired urine and blood osmolality values show no difference between temperature groups (ranges between 302 ± 6 to 323 ± 16 mOsm/kg perfusate) (Figure 3g). Therefore, lower protein/creatinine levels, lower urinary NGAL, and higher urine output associated with perfusion at 22 °C are not due to diluted urine and lower osmolality. Significantly higher arterial pO_2 values during normothermic reperfusion stage in all groups ($^{****}P = 0.0001$) demonstrate higher delivery of oxygen and reduced venous pO_2 represents oxygen consumption by the kidneys during normothermic reperfusion. Acid base balance such as pH was maintained within the physiological range (7.48–7.56) by adding HCO_3^- during normothermic reperfusion. In conclusion, higher flow rates, increased urine output, and reduced urinary protein-creatinine ratio observed in kidneys perfused at subnormothermic temperatures (22 °C) are related to superior early graft function versus other groups.

Subnormothermia Suppresses the Expression of Key TLR Signaling Molecules

Innate immune activation commonly observed in DCD organ preservation is predominantly due to the responses from TLR-induced proinflammatory reactions.[10] We anticipate that the subnormothermic temperature may attenuate the expression of key molecules commonly shared by almost all the TLR signaling pathways. Therefore, we assessed the expression of MyD88 (a common TLR adaptor protein), NF-κB (key transcription factor responsible for the production of multiple cytokines, chemokines, and interleukins) and HMGB1 (the most prevalent DAMPs ligand for TLR in IRI) in DCD kidneys preserved in different temperature conditions. We found that HMGB1 was highly expressed in kidneys preserved in cold static condition followed by normothermic blood reperfusion. Although perfusion at 37 °C resulted in reduced HMGB1, only a significant reduction was observed in subnormothermic 22 °C perfusion compared with SCS ($P = 0.0172$; Figure 4a). Similarly, the same pattern of expression of TLR0 signaling molecules, for example, MyD88 ($P = 0.0186$) (Figure 4b), and NF-κB ($P < 0.0001$; Figure 4c) fluctuated with temperature as assessed by quantitative reverse-transcription polymerase chain reaction. We anticipated that the proinflammatory reactions that occurred during 4 hours of perfusion would be reflected substantially in their excretion into urine generated during the first hour of reperfusion. Therefore, first-hour urine was assessed for IL-6 concentration by a porcine IL-6–specific ELISA kit. Compared with 22 °C and 37 °C perfusion, kidneys stored at 4 °C excreted a significantly higher amount of IL-6. In contrast, 22 °C and 37 °C perfusion minimized IL-6 production (Figure 4d; $P = 0.0004$, $P = 0.0002$).

DISCUSSION

It has long been known to the transplant community that the current clinical standard of organ preservation using cold solution cannot fully protect organs from storage injury. Popular belief is that hypothermic storage can decrease oxidative metabolism and may prevent kidney injury secondary to IRI. On the contrary, the lack of oxygen and metabolic inactivity in cold preservation trigger a cascade of inflammatory reactions and cause necrotic-apoptotic cell death in donor kidneys. One approach is to preserve these kidneys through pulsatile perfusion in a pump circuit with physiologic nutrients, pressure, and temperature. In this new setting, the need for oxygenation in the perfusate using clinical devices or delivering oxygen to the isolated ischemic kidneys by an extracorporeal membrane oxygenator has also been considered. At this low level of metabolism, delivering oxygen with crystalloid preservation solution (e.g., University of Wisconsin, Steen [XVivo Solution, Lund, Sweden]) is not a viable option. To find out the importance of oxygenation in cold perfusate, Thuillier *et al.*[11] used a porcine model of transplantation similar to what we studied in this article. This group found that transplanted kidneys perfused with oxygen using a hypothermic machine perfusion in absence of an effective oxygen carrier can function better on transplantation. However, posttransplant evaluation did not include parameters of ischemic reperfusion injury, particularly activation of any proinflammatory markers. Moreover, during the hypothermic preservation stage, there was no statistical difference in terms of flow and resistance between groups.

At present, clinical data are heterogeneous about the use of warm perfusate for *ex situ* machine perfusion to

Figure 3. (continued) in cold, $^{*}P = 0.0239$) between groups and (c) reduced urinary protein-creatinine ratio at 22 °C was found in both hourly samples ($^{***}P = 0.0005$ and $^{****}P < 0.0001$) as well as in total urine (d) compared with hypothermic static cold storage 4 °C (1 ± 0.45 g/mmol vs. 62 ± 41 g/mmol, $^{*}P = 0.04$) and normothermic perfusion 37 °C (1 ± 0.45 g/mmol vs. 100 ± 45 g/mmol, $^{*}P = 0.003$). $^{**}P = 0.0034$. (e) Hourly urine creatinine levels indicate both 22 °C and 37 °C perfused kidneys' superior functionality as compared to 4 °C ($^{*}P < 0.05$ and $^{**}P = 0.0015$). (f) Both urine and blood osmolality values showed no difference between groups (ranges between 302 ± 6 mOsm/kg and 323 ± 16 mOsm/kg) and (g) showed significantly higher arterial pO_2 versus venous values during normothermic reperfusion stage in all groups ($^{****}P = 0.0001$). Each line or bar represents the mean ± SD. Cr, creatinine.

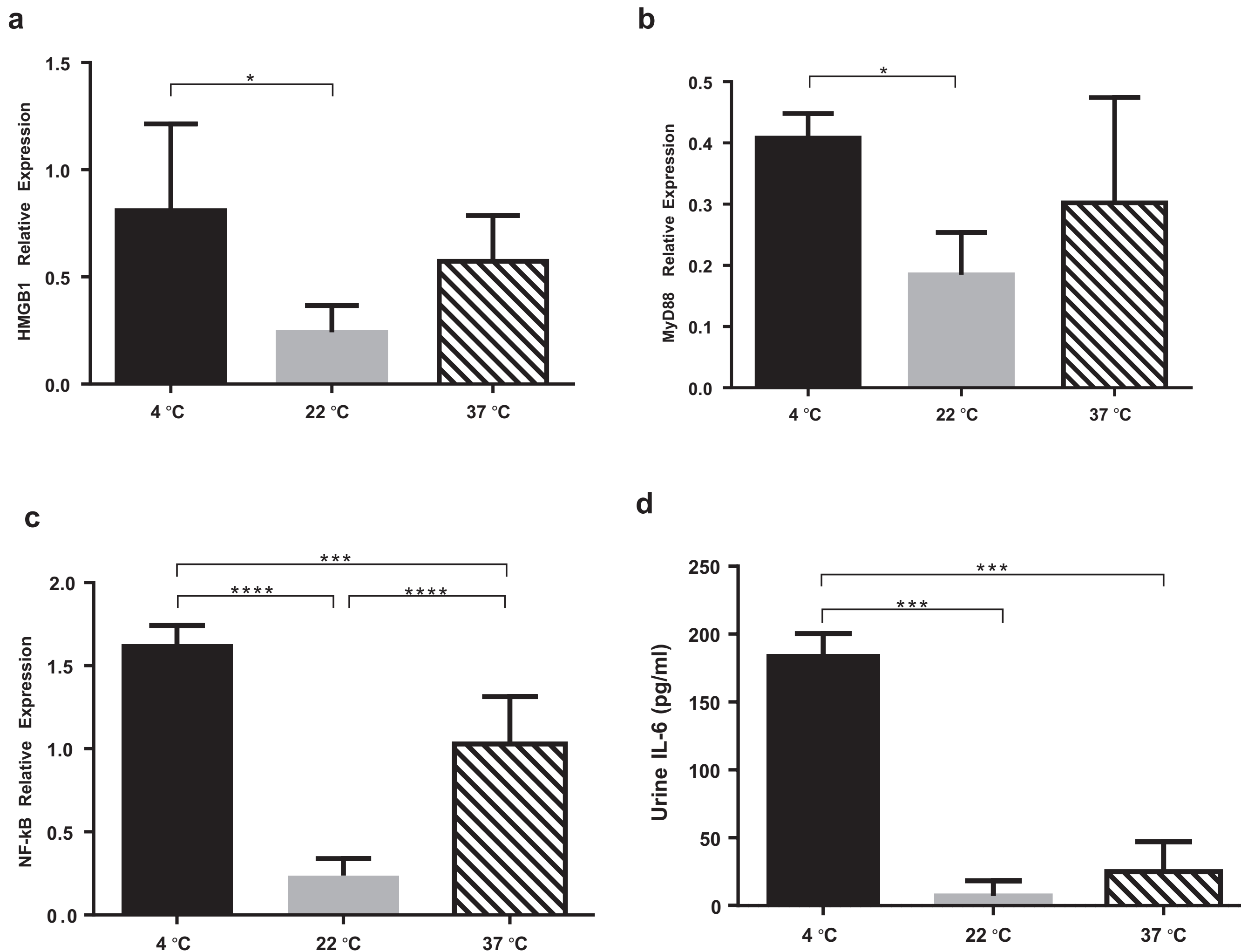


Figure 4. Toll-like receptor (TLR) signaling molecules and urinary interleukin (IL)-6 production are ameliorated at 22 °C. Quantitative reverse-transcription polymerase chain reaction of (a) a damage-associated molecule high-mobility group box 1 (HMGB1) (*$P = 0.0172$) released after necrotic tissue injury, (b) the selective common adaptor protein MyD88 (*$P = 0.0186$), and (c) a key transcription factor of TLR signaling pathway NF-κB (****$P < 0.0001$, ***$P = 0.0007$) were evaluated from the RNA isolated from the pig kidney tissues perfused at different temperatures. Relative changes of the genes are shown after normalizing it with a housekeeping gene. Urinary porcine IL-6 enzyme-linked immunosorbent assay (ELISA) was used to measure IL-6 obtained from the first-hour urine at the reperfusion stage. Reduced IL-6 production was observed at 22 °C and 37 °C perfusion (***$P = 0.0004$, ***$P = 0.0002$; d).

protect and preserve renal grafts. At the same time, composition of an ideal perfusate suitable for prolonged *ex vivo* perfusion in warm temperature has not been studied thoroughly. Several studies, including ours, that attempt to see the effects of normothermic perfusion in liver and kidneys, showed a reduction of tissue injury compared with the current cold preservation.

With the use of greater proportions of suboptimal allografts from DCD and expanded-criteria donors, there has been a movement away from SCS to cold anoxic pulsatile perfusion to reduce delayed graft function rates.[12] Lack of efficacy in cold storage necessitates the development of an alternative method that is more physiological in nature, such as delivering oxygen to ischemic kidneys at higher temperature. More recently, Nicholson and Hosgood[5] showed that delayed graft function rates can be further reduced by using normothermic *ex vivo* perfusion of renal allografts with oxygenated blood during the storage phase. This strategy may reduce graft damage by minimizing cold injury or by reducing inflammation as a result of ischemic reperfusion injury. However, the ideal temperature for *ex vivo* perfusion is unknown, and with clinical evidence that therapeutic whole-body hypothermia (34 °C–35 °C) may be beneficial in preserving kidney function in renal donors,[6] we wanted to compare the impact of both normothermic and subnormothermic *ex vivo* perfusion of blood on kidney preservation compared with SCS in a standard storage solution (histidine-tryptophan-ketoglutarate). This study is limited to 4 hours of perfusion and 4 hours of reperfusion, which is relatively a short run compared with the clinical standard of somewhere

between 16 and 24 hours of preservation time. Although achieving that goal is our final objective, an optimal perfusate to carry out such prolonged perfusion under normothermic and subnormothermic conditions does not exist to date. This study is a work in progress in which oxygenated perfusate without any additives. such as insulin or glucose, confers optimal storage condition for donor kidneys up to 8 hours *ex vivo* in a close to room temperature environment. Importantly, as indicated by urinary NGAL, inflammation, and ATN score, 4 hours of reperfusion with blood has been able to show significant differences between perfusion groups. We anticipate that this new information can be replicated in future protocol design, which would include acellular oxygen-carrying solution use to perfuse isolated kidneys at room temperature. This would also open a new door for therapeutic intervention to reduce the debilitating effects of IRI in donor organ preservation.

Consistent with other groups, by using our newly designed renal pump circuit system, we showed that normothermic *ex vivo* perfusion of kidneys with blood can better protect the organ than SCS with regard to IRI (both necrosis and apoptosis), early organ function, and injury; however, intrarenal hemorrhage and blood clot formation cannot be prevented at normothermia. By adding an anti-inflammatory agent called carbon monoxide releasing molecule (CORM401) in the perfusate, we were able to partially reduce blood clots/hemorrhage at normothermia.[13] To our surprise, when blood perfusate temperature was lowered down to room temperature (22 °C) during storage, all parameters of renal and vascular resistance, renal blood flow, urine output blood clots, and hemorrhage were further improved after. The reduced level of kidney damage marker (NGAL) and IL-6 in the urine along with reduced apoptosis (terminal deoxynucleotidyltransferase dUTP nick end-labeling) and necrosis (ATN) scores also suggested lower initial burden of tubular damage at 22 °C. This may result in higher volumes of urine production with improved creatinine clearance during the reperfusion stage of the experiments in both normothermic and subnormothermic (22 °C) groups. Interestingly, reduction of temperature down to 15 °C reduced renal blood flow with detrimentally high resistance, urine output was virtually absent, and total thrombosis of the grafts occurred.

Proteinuria occurs secondary to tubular damage. Although 37 °C perfusion decreases ATN (Figure 2d) and NGAL (Figure 2e) compared with the cold storage, poor blood flow (Figure 3a) and formation of extensive blood clots (Figure 2f) were evident during 37 °C perfusion. By decreasing perfusion temperature to 22 °C, we were able to eliminate blood clots and further improve ATN and NGAL, which may subsequently cause better proteinuria profile. Although 37 °C is a physiological temperature, in *ex vivo* perfusion settings, it may not be an optimal temperature to preserve the organs.

Recently Niemann *et al.*[6] demonstrated that donor kidneys were protected when the deceased organ donor was cooled to 34 °C before organ procurement. By using a red blood cell–based perfusate containing Steen solution and insulin, it has also been shown that longer periods of normothermic perfusion following SCS can reduce the damage associated with cold storage of renal grafts.[14] However, it had yet to have been shown that subnormothermic *ex vivo* perfusion of kidneys with blood could protect kidneys better than the normothermic approach. In fact, Adams *et al.*[15] recently showed that normothermic perfusion of kidneys for 1 hour was superior to subnormothermic 32 °C perfusion in a porcine renal preservation model with regard to early functional parameters. However, in their experiment, a centrifugal pump system was used, blood/perfusate conditions were different from those used in our experiments, and the *ex vivo* perfusion lasted only 1 hour, in addition to 24-hour cold static storage. We have reliable results using this novel perfusion system with blood and significantly improved preservation timing *ex vivo* for total of 8 hours (4 hours perfusion and 4 hours of reperfusion with blood).

Although kidneys treated with 1 hour of normothermia were superior with regard to early functional results, comparison of renal damage with histopathology was limited in their study and superiority was not seen with regard to the tubular damage. Hence, although we tried to preserve the kidneys using *ex vivo* perfusion, their group tried to engage normothermic activity to transiently engage oxidative metabolism. Importantly, if a subnormothermic condition can significantly reduce cell death, and prevent IRI and blood clot as we have seen in this study, this close to room temperature condition will bring convenience in transporting kidneys from distant sources at ease without an expensive and complicated heating-cooling device attached to the pump circuit. Considering the clinical and rationale aspect of the study, we have concluded that 22 °C pulsatile perfusion could be the best way to preserve kidneys in the presence of oxygen. With new bloodless oxygen-carrying solutions that cannot clot,[16,17] it is also possible that *ex vivo* perfusion of kidneys at or below 15 °C may be feasible. Hence, the optimal temperature for organ storage may vary depending on the *ex vivo* conditions of organ perfusion.

We observed similar effects on TLR signaling pathways specifically key molecules such as NF-κB and

MyD88. Transcription factor NF-κB is central to the TLR-mediated production of inflammatory cytokines and chemokines. Finally, the expressions of HMGB1 and IL-6 were significantly reduced in subnormothermic preservation. We found that cold ischemia was not able to prevent cellular injury in donor kidneys. When normothermic blood reperfusion was applied to kidneys previously preserved in an anoxic static cold histidine-tryptophan-ketoglutarate solution for as little as 4 hours, they suffered higher degrees of injury and inflammation compared with kidneys stored at 22 °C with oxygenated pulsatile perfusion. In fact, we found that hypothermia-based anoxic storage may exacerbate reperfusion injury through upregulating proinflammatory response in transplanted donor organs with >10-fold overexpression of IL-6 in the control SCS group compared with the 22 °C group.

We conclude that under our conditions of oxygenated *ex vivo* perfusion for kidney storage, 22 °C subnormothermia provides the best temperature to preserve kidneys with regard to renal blood flow, early renal function, and organ damage on reperfusion. After concluding beneficial effects of subnormothermic (22 °C) blood perfusion on the preservation of model porcine DCD kidney, we are assessing the impact of an acellular hemoglobin-based oxygen carrier in these model kidneys. We will continue to explore other aspects of the *ex vivo* perfusion of kidneys, including the use of endogenous gasotransmitters and drug repositioning to protect kidneys against IRI and to achieve the best condition for kidney preservation.

DISCLOSURE

All the authors declared no competing interests.

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