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The Impact of Nutritional Supplementation on Donor Kidneys During Oxygenated Ex Vivo Subnormothermic Preservation

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Background. Evidence suggests that nutritional supplementation during normothermic ex vivo perfusion improves organ preservation. However, it is unclear whether the same benefit is observed during room temperature (subnormothermic) oxygenated perfusion. In this study, we tested the impact of providing complete nutrition during subnormothermic perfusion on kidney outcomes. **Methods.** Porcine kidneys were recovered after 30 min of cross clamping the renal artery in situ to simulate warm ischemic injury. After flushing with preservation solution, paired kidneys were cannulated and randomly assigned to perfusion with either (1) hemoglobin-carrier hemoglobin-based oxygen carrier or (2) hemoglobin-based oxygen carrier + total parenteral nutrition (TPN) for 12 h at 22 °C. To mimic reperfusion injury, all kidneys were reperfused with whole blood for an additional 4 h at 37 °C. Kidney function and damage were assessed. **Results.** Kidneys preserved with or without TPN performed equally well, showing similar renal function postreperfusion. Histological findings indicated similar levels of damage from apoptosis staining and acute tubular necrosis scores in both groups. Additionally, markers of renal damage (KIM-1) and inflammation (IL-6; high-mobility group box 1) were similar between the groups. **Conclusions.** Unlike other studies using normothermic oxygenated perfusion platforms, nutritional supplementation does not appear to provide any additional benefit during ex vivo kidney preservation over 12 h evaluated by whole blood-based reperfusion method at subnormothermic temperature. Further study should include a kidney autotransplant model to assess the role of TPN in vivo.

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INTRODUCTION

Kidney transplantation remains the best treatment option for patients suffering from end-stage renal disease. Access to transplantation is limited by a shortage of acceptable donor organs. In an effort to expand the donor pool, older and donation after circulatory death (DCD) kidneys are being transplanted more frequently.[1,2] However, these kidneys are more susceptible to damage from ischemia-reperfusion injury (IRI) during transplantation than organs from traditional donors, which can lead to an increased risk of delayed graft function and early graft loss.[3-5] Current clinical standards for organ storage include static cold storage and hypothermic machine perfusion at 4 °C to reduce metabolic activity and prevent associated cell death within the kidney. These methods fail to adequately protect the kidney from preservation-related damage from cold and prolonged anoxia which includes profound edema, capillary compression, and interstitial expansion giving way to tubular apoptosis and necrosis postreperfusion.[6]

Recent studies have shown that ex vivo preservation at normothermic temperatures (37 °C) can improve immediate graft function and reduce kidney injury compared with current clinical practice.[7-10] However, to address ongoing metabolic demands, organs undergoing normothermic ex vivo preservation are supplemented with insulin, glucose and occasionally,

amino acids to provide nutrition. As it was unclear whether complete nutritional supplementation is required during normothermic preservation, Buchko et al supplemented perfusion solution with total parenteral nutrition (TPN) for lung preservation. After perfusing the lungs for 24 h, they found that TPN improved early graft function with substantial increase in oxygenation.[11] However, TPN creates the need for additional monitoring, costs, and complexity of the ex vivo perfusion system, complicating its clinical use.

Although cold perfusion is currently the conventional method of organ preservation in clinical practice, it has severe downfalls. As an alternative, we have primarily focused on developing a pump circuit system that can perfuse blood or blood-like products. By utilizing 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 post-transplant outcomes. Our group has shown that subnormothermic (room temperature; 22 °C) ex vivo perfusion can mitigate IRI-damage and improve graft function compared with both normothermic and hypothermic storage strategies.[12] Furthermore, we showed that a synthetic hemoglobin-based oxygen carrier (HBOC) can replace blood products in providing cells with oxygen during kidney preservation.[13] In the subnormothermic environment, in which metabolic demands are reduced compared with normothermic storage, it is not clear whether nutritional supplementation is required at all during clinically relevant preservation times (12 h). Therefore, we embarked upon an "all or nothing" study in which 1 kidney from donor pairs was treated with full nutritional supplementation (TPN) and the other kidney was treated with no nutritional supplementation. The endpoints in this study were inflammation, reperfusion damage (apoptosis and necrosis), and early renal function postreperfusion.

MATERIALS AND METHODS

Animals

Large (50–55 kg) adult male Landrace pigs were used in all experiments. All procedures on animals were approved by Western University's research ethics board and conducted according to CCAC Guidelines for animal care. After kidney removal, blood for reperfusion was collected into sterile, heparinized (25 000 units) containers via direct cardiac puncture.

Study Design

Kidneys were subjected to 30 min warm ischemia by cross clamping the renal pedicles in situ. Interruption of blood supply mimics DCD donation in humans by simulating DCD warm ischemic injury. Kidneys were then recovered and flushed with Histidine Tryptophan Ketoglutarate (Custodial, United States) solution before being placed on pump for oxygenated pulsatile perfusion at 22 °C using a modified RM3 pump (Figure 1A) as previously described.[12] Briefly, a simplified design is shown in Figure 1B and our study design is illustrated in Figure 1C. The medtronic centrifugal pump seen in Figure 1A is being used only to monitor flow and pressure because flow probe attached to the Waters pump was unable to get a reading perfectly.

The paired kidneys were randomly assigned to perfusion with (a) hemoglobin-carrier HBOC (Hbo_2 Therapeutics, Souderton, PA) or (b) HBOC with TPN constantly administered at a drip rate of 9 mL/h (HBOC + TPN). Oxygenation was provided by an oxygenator set to 40%. Insulin was added to the TPN group to promote uptake of glucose. To adjust pH to physiological levels, bicarbonate was added to the perfusion solutions. Fluid loss because of urine production was replenished by equivalent volumes of PlasmaLyte solution (Baxter Corporation, Opelika, AL). Pump pressure was maintained at ~70 mmHg throughout perfusion. After 12 h of storage perfusion, kidneys were reperfused with autologous stressed blood at 37 °C for another 4 h to mimic post-transplantation conditions. Pump pressure, oxygenation, and perfusate pH were maintained as per perfusion parameters. To evaluate kidney function during reperfusion, creatinine (10 mg/L) was added externally to the perfusion solution. Osmolality, pH, and partial pressure of oxygen of blood were monitored throughout reperfusion.

TPN Formulation

TPN solution was prepared by the hospital pharmacy with formulations suggested by a renal dietician (CF) to meet metabolic requirements. TPN contained: 1% Travasol, 5% Dextrose, 35 mmol/L sodium, 30 mmol/L potassium, 2.5 mmol/L magnesium, 5 mmol/L calcium, 15 mmol/L phosphate, 47 mmol/L chloride, 37 mmol/L acetate, and 5 mL of M.V.I-12. TPN was administered at a constant drip rate of 9 mL/h for 12 h.

Physiological Data Analysis

Urine and blood samples were collected, and urine output was recorded hourly during reperfusion. Samples were analyzed for creatinine and protein/creatinine ratio. Urine protein was quantified using a total protein assay (BioAssay Systems, Hayward, CA). Urine and serum creatinine were measured using a creatinine assay kit (BioAssay Systems, Hayward, CA) as per supplier's protocol. Creatinine clearance (Cr.Cl) was calculated using the equation: $Cr.Cl = U_{Cre} \times U_{output}/B_{Cre}$, where U_{Cre} is urine creatinine concentration, U_{output} represents total urine volume (mL) and B_{Cre} is blood creatinine concentration. Blood samples were also monitored hourly during reperfusion for glucose, lactate, and sodium levels using the iSTAT Handheld Blood Analyzer (Abbott Laboratories, Abbott Park, IL).

Histology

Following reperfusion, kidneys were fixed in 10% neutral buffered formalin. Tissue was embedded in paraffin and sectioned (5 μm). Kidney tissue was stained with hematoxylin and eosin for analysis of acute tubular necrosis (ATN) and terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) to detect apoptosis. Three randomly selected, nonoverlapping fields were analyzed from each section and at least 3 sections from each treatment were used to assess tissue injury. ATN and apoptosis were semiquantified in a anonymized manner using the following grading scale: 0, no change; 1, <10%; 2, 11%–25%; 3, 26%–45%; 4, 46%–75% and 5, 76%–100%.[14] Injuries were quantified as a percentage of tissue that sustained histological changes. The mean score from each experiment was utilized for statistical analysis.

ELISA

Kidney injury marker 1 in urine was determined by enzyme-linked immunoassay (ELISA) kit (MyBioSource, San Diego, CA) according to the supplier's protocols. Additionally, the presence of relevant proinflammatory molecules, interleukin-6 (IL-6; MyBioSource, San Diego, CA) and high-mobility group box 1 (HMGB-1; MyBioSource, San Diego, CA) in

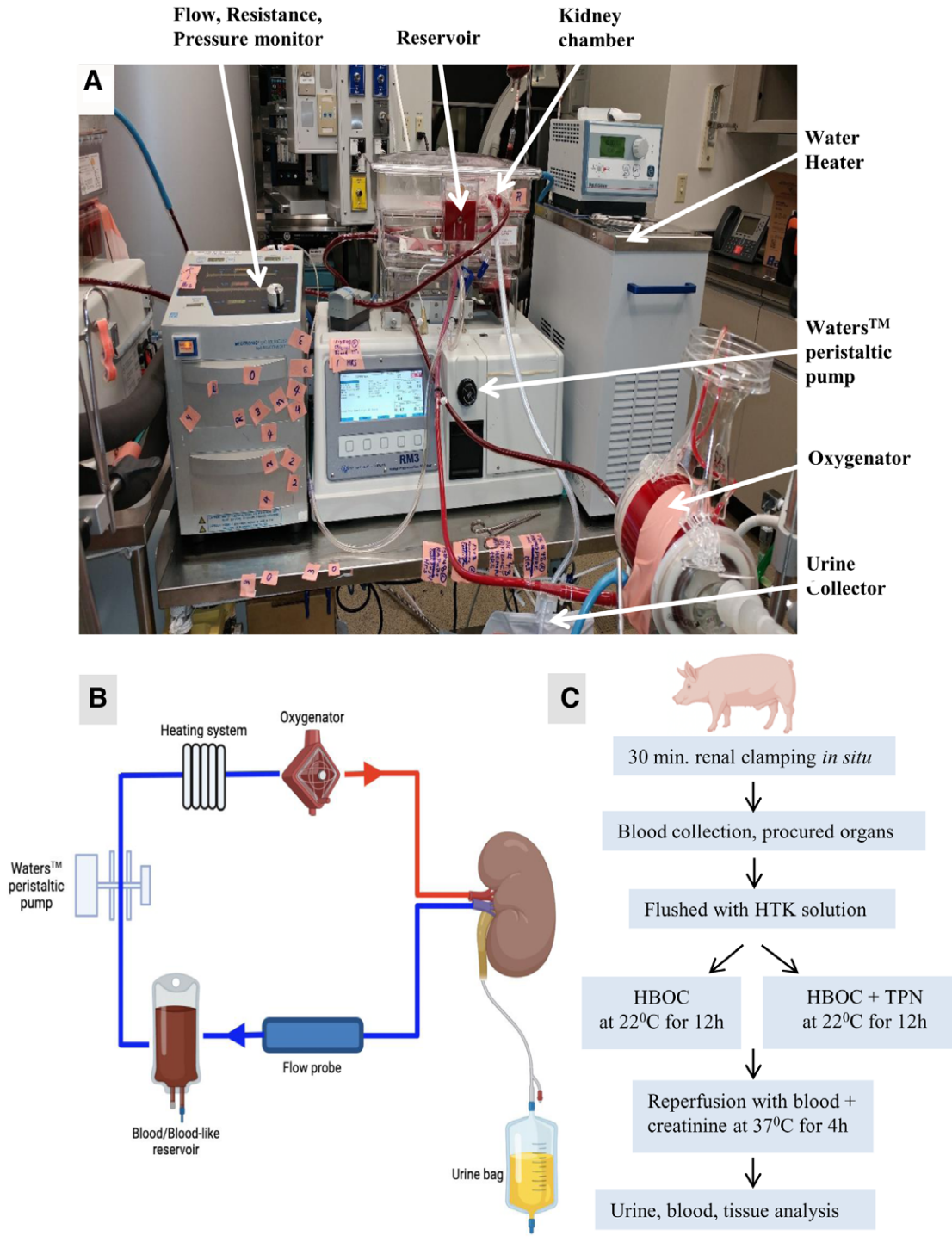

FIGURE 1. Modified Waters pump for blood perfusion/storage and reperfusion in the same circuit. (A) The Waters renal preservation pump was modified in a way that whole blood perfusion and reperfusion can be done in the same unit. This system provides pulsatile perfusion at varying temperatures and measures resistance and renal blood flow. For assessment of overall functional dynamics during ex vivo perfusion, several components including an oxygenator, water heater exchanger, flow meter and temperature probe were attached. As pictured in the designed schematic (B), kidneys were placed in a cassette reservoir with the cannulated artery, vein, and ureter. (C) Study design for ex vivo experiments for TPN treatment. HBOC, hemoglobin-based oxygen carriers; TPN, total parenteral nutrition.

urine samples were determined as per supplier's protocol. Briefly, debris was removed from urine samples by centrifuging for 5 min at 3000 rpm. Subsequently, 100 µL of standards and urine samples were incubated in the precoated ELISA plate for 1 h. After washing, 100 µL of biotinylated detection antibody was added to the plates and incubated for 1 h. Plates were washed and 100 µL of avidin-HRP working solution was added for 1 h. Following a final wash, substrate reagent was added to the plate and color was developed. Plates were read at 450 nm using an iMark microplate reader (BioRad, Hercules, CA). Concentrations of the target proteins in the samples were determined from the standard curve.

Statistical Analysis

Data were analyzed using Student's *t*-test for paired values with GraphPad Prism 9. Differences between groups were considered statistically significant at $P \leq 0.05$. All data are expressed as mean ± SEM.

RESULTS

New Perfusion Device for Comparison of Nutrional Supplementation

To achieve perfusion-reperfusion in relatively warm conditions, our group has reengineered a clinically utilized pulsatile perfusion system originally developed for hypothermic machine perfusion (RM3 Renal Preservation System, Waters Medical System, Rochester, MN). This reengineered ex vivo apparatus Figure 1A and simplified design schematics (Figure 1B) can mimic in vivo function by maintaining similar temperature conditions, degrees of oxygenation, fluid dynamics, pressure, resistance, and urine producing capacity. Modification of this system allows this circuit to perfuse blood or blood-like perfusates by adding an oxygenator, and a double-luer connector allows for inflow and outflow to the organ. Like the human body, the oxygenator oxygenates blood to supply to the kidney via the renal artery, after which the deoxygenated blood leaving the renal vein will return to the oxygenator. The current study approach utilizing this apparatus is described in Figure 1C. Views of the DCD kidneys placed in the chamber at perfusion stage were photographed (Figure 1A). A conduit is routed from the ureter to a urinary bag to collect urine for analysis.

Assessment of Renal Function Postreperfusion

To assess whether nutritional supplementation improved immediate renal function, kidneys were reperfused with oxygenated autologous blood at 37 °C for 4 h. The reperfusion phase simulates return of circulation post-transplantation, allowing us to evaluate post-transplant function of the organ. Standard metrics of kidney function, urine output and creatinine clearance, were determined for both groups. Urine output after preservation did not differ between kidneys provided with nutritional supplement and those without (Table 1; Figure 2A; 492 ± 124 mL versus 392 ± 62 mL; $P = 0.199$).

TABLE 1.

Semiquantitative analysis of renal tissue samples postreperfusion

	Acute tubular necrosis %		Apoptosis %	
Sample	HBOC	HBOC + TPN	HBOC	HBOC + TPN
1	75	11	45	0
2	11	45	11	45
3	75	45	45	24
4	24	24	24	24
5	24	24	24	0
6	45	24	45	24

Percentage area of acute tubular necrosis and apoptosis were quantified by a pathologist in a anonymized manner.
HBOC, hemoglobin-based oxygen carriers; TPN, total parenteral nutrition.

Furthermore, there was no difference in postreperfusion creatinine clearance between the groups (Figure 2B; $P = 0.525$).

Additionally, blood levels of sodium, lactate, and glucose were monitored during reperfusion. Both sodium (Figure 3A; $P = 0.621$) and lactate (Figure 3B; $P = 0.591$) levels rose slightly over the reperfusion period, but there was no significant difference between the groups. However, glucose levels were higher in blood samples from kidneys that were preserved with TPN than those without (Figure 3C, $P = 0.0034$).

Histologic Analysis of Kidneys Postreperfusion

No gross pathological differences in regard to color or edema were observed between the 2 treatments. Histopathologic analysis identified no significant difference in tissue damage sustained during preservation and reperfusion in the 2 groups (Figure 4A). Upon scoring, the percentage of tubules that displayed ATN were similar between kidneys preserved with HBOC alone and with nutritional supplementation (Figure 4B; 42.33% ± 11.25% versus 28.33 ± 5.51; $P = 0.199$), suggesting both prevent IRI to a similar degree.

Additionally, moderate positive staining for apoptotic tubular cells was observed upon microscopic examination of tissues from both groups (Figure 4C). Quantification showed no significant difference in tubular apoptosis between kidneys preserved without or with nutritional supplementation

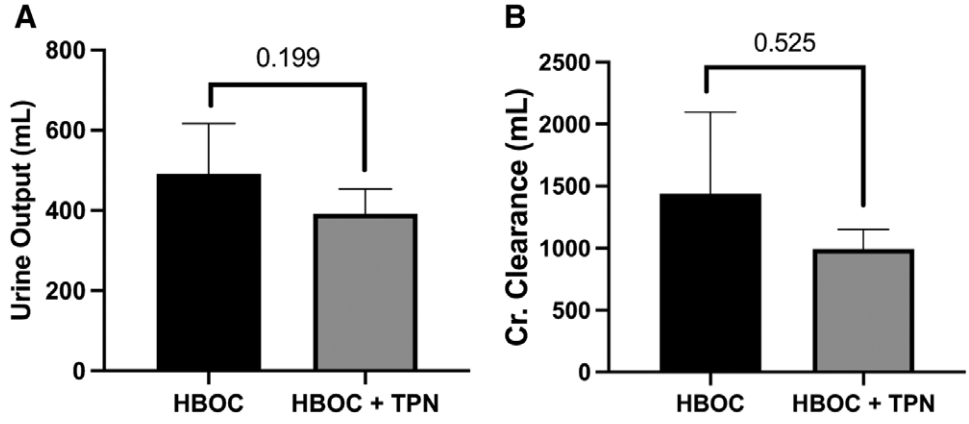

FIGURE 2. Early DCD kidney function is achieved by preservation with and without TPN. (A) Urine output during reperfusion was similar in kidneys preserved with HBOC alone and those preserved with the addition of TPN (HBOC + TPN); $P = 0.199$. (B) creatinine clearance ($P = 0.525$), during reperfusion was similar between kidneys preserved with HBOC alone or HBOC with nutritional supplementation with TPN. Results are shown as mean ± SEM; Student's *t*-test; n = 6. DCD, donation after circulatory death; HBOC, hemoglobin-based oxygen carriers; TPN, total parenteral nutrition.

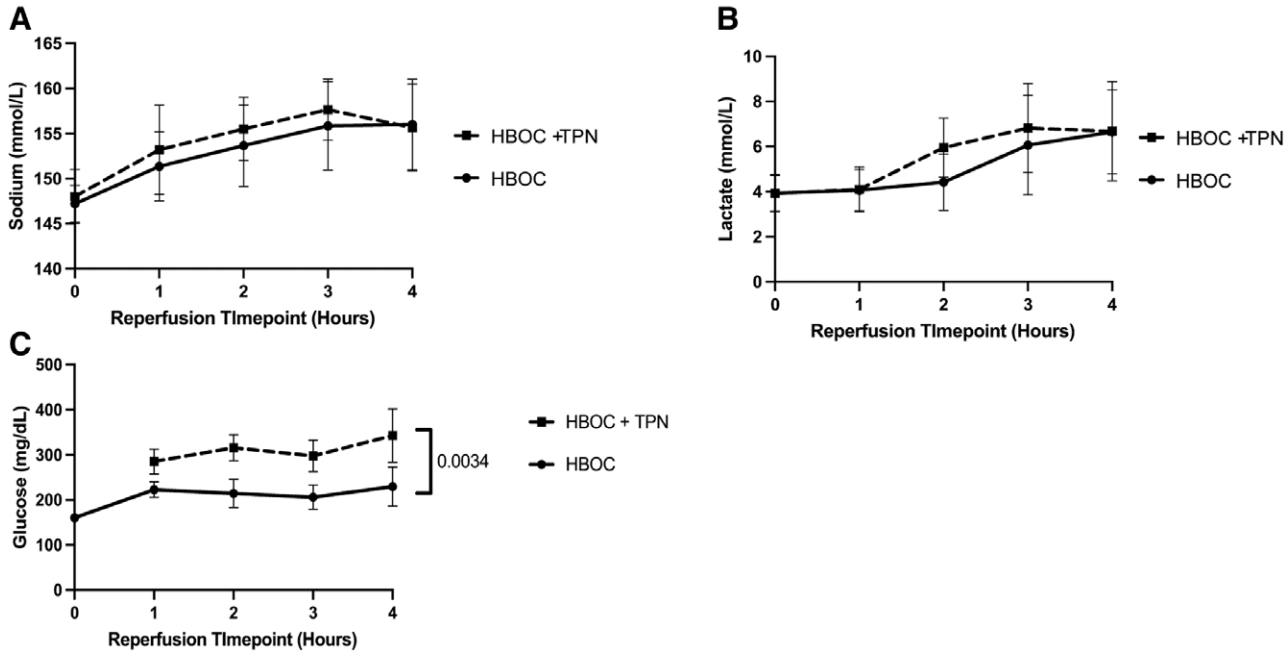

FIGURE 3. Perfusate parameters during reperfusion. (A) Blood levels of sodium (mmol/L) were monitored throughout reperfusion in kidneys preserved with HBOC alone or with the addition of TPN (HBOC + TPN) and showed similar trends between the groups; $P = 0.621$. (B) Blood lactate levels also rose slightly but showed no difference between the groups during reperfusion; $P = 0.591$. (C) Blood glucose levels were significantly higher during reperfusion in the HBOC + TPN preservation group compared with HBOC alone; $P = 0.0034$. Results are displayed as mean ± SEM; Student's *t*-test; n = 6. HBOC, hemoglobin-based oxygen carriers; TPN, total parenteral nutrition.

(Figure 4D; 32.33% ± 5.99% versus 19.50 ± 7.00; $P = 0.297$), indicating that prevention of cell death is not reduced by the addition of TPN.

Evaluation of Urinary Markers for Renal Damage and Inflammation

Urine produced during the reperfusion phase was collected for analysis of renal damage and inflammation. Consistent with the other parameters, urine quality, as measured by protein/creatinine ratio, was not significantly different between kidneys preserved with HBOC alone or with the addition of TPN (Figure 5A; 68.80 ± 31.84 versus 100.2 ± 46.05; $P = 0.114$). Concentrations of renal injury marker, KIM-1, were also not significantly different between preservation without or with nutritional supplementation (Figure 5B; 1.16 ± 0.41 ng/mL versus 0.87 ± 0.12 ng/mL; $P = 0.472$). Comparably, markers of inflammation, IL-6 (Figure 6A; 115.7 ± 41.25 pg/mL versus 86.91 ± 29.33 pg/mL; $P = 0.470$) and HMGB-1 (Figure 6B; 86.48 ± 23.42 pg/mL versus 102.8 ± 24.54 pg/mL; $P = 0.599$), had similar concentrations in urine from kidneys preserved without and with TPN.

DISCUSSION

Normothermic and subnormothermic oxygenated ex vivo perfusion have been shown to be capable of better preservation of kidneys than the current clinical standard and provide the opportunity to repair organs that may otherwise not be suitable for transplant.[7,8,12] However, increasing storage temperatures introduces the complexity of addressing increased metabolic demands. Currently, the only clinical normothermic ex vivo perfusion protocols for kidney preservation requires nutritional supplementation.[9] Additionally, TPN, a complete form of nutrition containing carbohydrates, amino acids, and essential vitamins and minerals, has been shown to be effective at reducing inflammation and improving immediate organ function when administered as metabolic support during normothermic ex vivo perfusion in lungs.[11]

Although the advantages of nutritional supplementation in normothermic perfusion are clear, whether supplementation is also required during subnormothermic temperatures which potentially confer less metabolic stress was the focus of our study. We compared subnormothermic preservation of DCD porcine kidneys with no or complete nutritional supplementation with TPN. Interestingly, we found that even over 12 h of perfusion there were few benefits of nutritional supplementation. Contrary to normothermic perfusion studies, kidneys post-subnormothermic preservation performed equally well regardless of nutritional supplementation in terms of urine production and creatinine clearance. Additionally, we identified no difference in inflammation or tissue damage in kidneys that were perfused with TPN and those without, as assessed by urinary markers (IL-6, KIM-1) and histological analysis.

Although our results do not negate the requirement for nutritional supplementation at normothermic temperatures, it is possible that metabolic demand is not as exacerbated during preservation at subnormothermic temperatures in kidneys. It is also possible that TPN may not necessary to have a significant impact on preservation conditions during this period of assessment (12 h). However, some of the nutritional components may indeed require for prolonged period of storage such as beyond 20 h. Our experimental approach is limited by only 4 h of postreperfusion evaluation of organ function which truly does not reflect a post-transplant scenario. Interestingly, our previous studies indicate that 12 h of oxygenated subnormothermic storage was sufficient to neutralize the detrimental effects of innate immune hyperactivation seen in static cold storage for the same period of time. Therefore, we are adding new information here that additional support through nutrients supplementation is not helpful to further

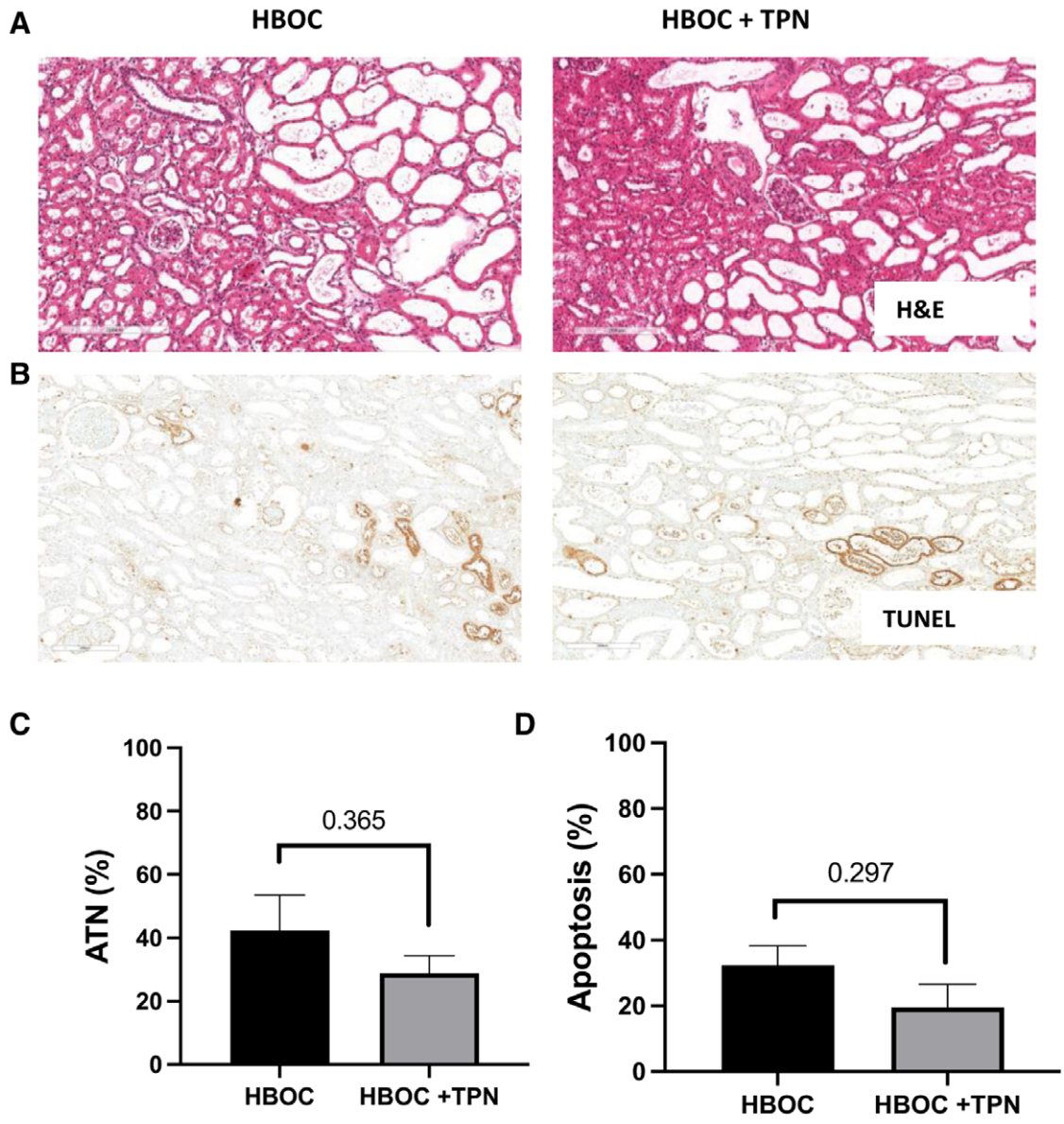

FIGURE 4. Ex vivo perfusion with HBOC alone or with the addition of TPN (HBOC + TPN) show similar levels of ATN and apoptosis in porcine kidneys. (A) Hematoxylin and eosin staining show renal tubules sustained similar loss of tubule cells in HBOC and HBOC + TPN conditions. Scale bar = 200 μm. (B) No significant difference in acute tubular necrosis scoring was observed between the HBOC alone and HBOC + TPN groups; $P = 0.365$. (C) Terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-TUNEL staining revealed similar levels of apoptosis (brown staining) in tubules of HBOC and HBOC+ TPN perfused kidneys. (D) Apoptosis was scored by a qualified pathologist and is shown as percent of apoptotic tubule cells; $P = 0.297$. Results are displayed as mean ± SEM; Student's *t*-test; n = 6. ATN, acute tubular necrosis; HBOC, hemoglobin-based oxygen carriers; TPN, total parenteral nutrition; TUNEL, triphosphate nick end labeling.

improve proinflammatory reactions. Studies in liver showed a significant decrease in metabolic demand between normothermic and subnormothermic ex vivo perfusion.[15] Reduced metabolic activity at this temperature may render the addition of nutritional supplementation redundant. At this stage, we are optimizing several parameters of optimal organ preservation which are physiologically relevant to validate this new method for future clinical implication and replace existing organ storage at cold. Once established, a limited number of kidneys will be auto-transplanted in porcine model for further assessment for extended period of time.

A potential limitation of our study includes testing only 1 formulation of TPN. Although we believe this to be an accurate representation of the nutritional needs of a kidney, as specified by a renal dietician, it is possible other formulations or other forms of nutritional supplementation may have improved preservation. Also, we did not directly compare the use of TPN during subnormothermic and normothermic preservation, as it is well known that normothermic perfusion requires metabolic support.[16] Additionally, our reperfusion period was relatively short at only 4 h; however, we have shown in our previous studies that this is a sufficient length of time to assess damage and early renal function.[12-14]

We have previously shown subnormothermic preservation at 22 °C via pulsatile perfusion to be superior to static cold storage in anoxic conditions.[12] This study demonstrates an additional advantage to subnormothermic preservation as the reduced metabolic activity at this temperature allows for effective preservation of kidneys without the need for nutritional supplementation. The results of this study add to the

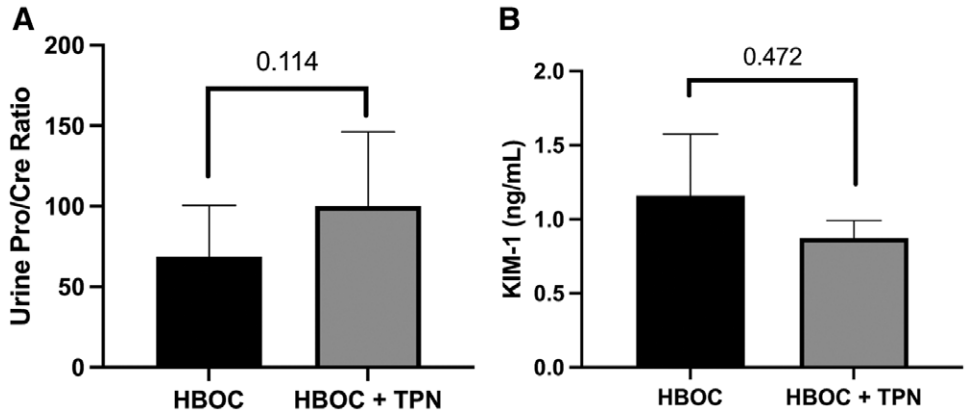

FIGURE 5. Subnormothermic oxygenated perfusion with a HBOC alone or with the addition of TPN (HBOC + TPN) equally protect kidneys from damage. (A) Urine quality, as measured by urine protein/creatinine ratio, was not significantly different between HBOC and HBOC + TPN perfused kidneys; $P = 0.114$. (B) Levels of kidney injury marker 1 (KIM-1), measured in urine samples during reperfusion by ELISA were similar between kidneys perfused with HBOC alone and those which received TPN; $P = 0.472$. Results are shown as mean ± SEM; Student's *t*-test; n = 6. ELISA, enzyme-linked immunoassay; HBOC, hemoglobin-based oxygen carriers; KIM-1, kidney injury marker 1; TPN, total parenteral nutrition.

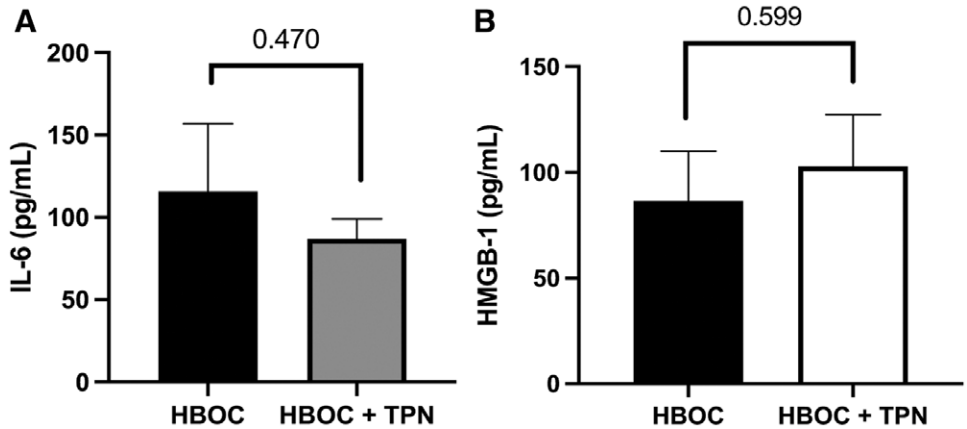

FIGURE 6. Expression of inflammatory molecules during reperfusion after ex vivo perfusion with HBOC alone or with nutritional supplementation (HBOC + TPN). (A) Kidney inflammation marker, IL-6 in urine measured by ELISA was not significantly different in kidneys perfused with HBOC or HBOC + TPN; $P = 0.470$. (B) Urine levels of inflammatory molecule HMGB-1 measured by ELISA were similar between the HBOC and HBOC + TPN perfusion groups; $P = 0.599$. Results are reported as mean ± SEM; Student's t-test; n=6. HBOC, hemoglobin-based oxygen carriers; HMGB-1, high-mobility group box 1; IL-6, interleukin-6; TPN, total parenteral nutrition.

clinical relevance of our system as subnormothermic preservation allows for a less complex setup with less monitoring than normothermic counterparts.

Our previous studies have optimized pump parameters, temperature, and oxygen requirements for subnormothermic ex vivo perfusion of kidneys.[12,13,17] Compared to normothermic preservation pumps, our system does not require maintenance of specific temperatures and can be used with a bloodless oxygen carrier (HBOC), which reduces the complexity and cost of utilizing our system in the clinical setting. In this study, we expanded upon our previous work to optimize perfusate composition. Our finding that nutritional supplementation is not required for optimal preservation further adds to the simplicity of our design and its potential for easy clinical translation.

Future studies could assess the use of our pump system for preservation up to 24 h, which if successful, could allow for more flexible scheduling of transplant surgeries. Additionally, a major advantage of our pump is the ability to incorporate additives, such as drugs to reduce IRI or prevent infection, that could be used to recondition organs that may be deemed unsuitable for transplantation.

In conclusion, our findings that nutritional supplementation is not required for optimal kidney preservation during subnormothermic ex vivo perfusion further contribute to the field of organ preservation. Additionally, our findings indicates that *ex vivo* perfusion may be more clinically feasible and cost-effective than previously thought if it is able to be carried out without the need for metabolic support.

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