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Prolonged Normothermic Ex Vivo Kidney Perfusion Is Superior to Cold Nonoxygenated and Oxygenated Machine Perfusion for the Preservation of DCD Porcine Kidney Grafts

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Background. The increased usage of marginal grafts has triggered interest in perfused kidney preservation to minimize graft injury. We used a donation after circulatory death (DCD) porcine kidney autotransplantation model to compare 3 of the most frequently used ex vivo kidney perfusion techniques: nonoxygenated hypothermic machine perfusion (non-oxHMP), oxygenated hypothermic machine perfusion (oxHMP), and normothermic ex vivo kidney perfusion (NEVKP). **Methods.** Following 30 min of warm ischemia, grafts were retrieved and preserved with either 16 h of non-oxHMP, oxHMP, or NEVKP (n = 5 per group). After contralateral nephrectomy, grafts were autotransplanted and animals were followed for 8 d. Kidney function and injury markers were compared between groups. **Results.** NEVKP demonstrated a significant reduction in preservation injury compared with either cold preservation method. Grafts preserved by NEVKP showed superior function with lower peak serum creatinine (NEVKP versus non-oxHMP versus oxHMP: 3.66 ± 1.33 mg/dL, 8.82 ± 3.17 mg/dL, and 9.02 ± 5.5 mg/dL) and more rapid recovery. The NEVKP group demonstrated significantly increased creatinine clearance on postoperative day 3 compared with the cold perfused groups. Tubular injury scores on postoperative day 8 were similar in all groups. **Conclusions.** Addition of oxygen during HMP did not reduce preservation injury of DCD kidney grafts. Grafts preserved with prolonged NEVKP demonstrated superior initial graft function compared with grafts preserved with non-oxHMP or oxHMP in a model of pig DCD kidney transplantation.

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INTRODUCTION

Kidney transplantation improves survival and quality of life of patients with end-stage renal disease and has therefore become the preferred treatment for patients with end-stage renal disease.[1,2] Because of the severe organ shortage, marginal grafts have been increasingly used for transplantation.[3] Static cold storage (SCS) results in excellent outcomes for standard criteria grafts; however, grafts from extended criteria donors and donation after circulatory death (DCD) tolerate SCS only poorly, with a higher rate of postoperative delayed graft function, primary nonfunction and graft loss.[3-5] To reduce preservation injury and to assess graft functionality and viability before transplantation, the research community has explored several ex vivo machine perfusion techniques.

Hypothermic machine perfusion (HMP) without oxygen has been extensively used in a clinical setting and studies report decreased delayed graft function and improved 1- and 3-y graft survival in grafts preserved with HMP compared with SCS.[6-8] Oxygenation during HMP has also been explored, and animal studies show conflicting results regarding the benefits of this approach.[9-11] However, a recent clinical trial found that oxygenated hypothermic machine perfusion (oxHMP) results in increased 1-y graft survival compared with non-oxHMP.[12]

To reduce preservation injury and improve the outcome of marginal grafts, novel technologies, such as normothermic ex vivo kidney perfusion (NEVKP), have been increasingly explored in the past years. Preclinical results suggest that NEVKP is superior to non-oxHMP and SCS.[13-15] Clinical studies investigating the safety and benefits of NEVKP are scarce, but first results are promising.[16,17]

Studies comparing the postoperative function of DCD grafts stored with NEVKP and oxHMP are lacking. Also, previous studies have shown contradicting results regarding the effects of adding oxygenation during cold perfusion.[9,18] This study is the first to directly compare nonoxygenated and oxygenated HMP with NEVKP in a standardized porcine DCD kidney autotransplantation model. Kidney injury and graft function was assessed in vivo over 8 d follow-up.

MATERIALS AND METHODS

Animals and Study Groups

The study was approved by the Animal Care Committee of the University Health Network Research Institute, Ontario, Canada. Twelve-wk-old male Yorkshire pigs (~30 kg) were utilized.

The pigs were randomly assigned to 1 of the 3 groups. After 30 min of warm ischemia (WI), grafts were retrieved and preserved either by nonoxygenated HMP (non-oxHMP group), oxygenated HMP (oxHMP group), or NEVKP (NEVKP group) (Figure 1). After contralateral kidney resection and autotransplantation of the preserved grafts, animals were followed for 8 d. During the follow-up period, blood was collected daily and fluids and antibiotics were administered twice daily as previously reported.[19] At the end of the 8 d, animals were euthanized under anesthesia. All animals received humane care and all procedures were performed in accordance with the "Principles of Laboratory Animal Care" and the "Guide for the Care of Laboratory Animals" published by the National Society for Medical Research and by the National Institutes of Health, respectively. Some of the data of the NEVKP and non-oxHMP groups includes historical controls.[13]

Surgical Protocol

The anesthetic and surgical procedures were performed as previously described by our group.[19] After anesthesia induction and intubation, general anesthesia was maintained by administration of inhaled isoflurane. Next, a central venous catheter was placed into the internal jugular vein for blood collection and administration of fluids and medications. Following midline incision and dissection of the right kidney and its vessels, the renal artery and vein were clamped with vascular clamps for 30 min to induce WI, mimicking DCD conditions. Subsequently, grafts were retrieved, the renal vessels were cannulated and the grafts were flushed with 300 mL histidine-trytophan-ketoglutarate (HTK), containing 10 000 IU/L heparin. After flush, the grafts were connected to the cold or warm perfusion device for the preservation period. The abdomen was closed, and animals were recovered from surgery. Toward the end of the preservation time, animals were reanesthetized. Following reintubation, anesthesia was maintained by continuous intravenous propofol administration and inhaled isofluorane. The midline laparotomy was reopened, and the left kidney was resected. The stored kidney was then flushed with 300 mL heparinized HTK, and the renal anastomoses were sewed (renal vein end-to-side to vena cava, renal artery end-to-side to aorta, and donor ureter side-to-side to recipient ureter). The abdomen was closed, and the animals were recovered and followed for 8 d.

Nonoxygenated Hypothermic Machine Perfusion

HMP without oxygen was performed using a LifePort 1.0 device (Organ Recovery Systems, Itaska, IL). The circuit was prepared sterile according to the manufacturer instructions and primed with 1 L of Belzer's Machine Perfusion Solution (Bridge to Life Ltd, Columbia, SC). After cannulation of the renal

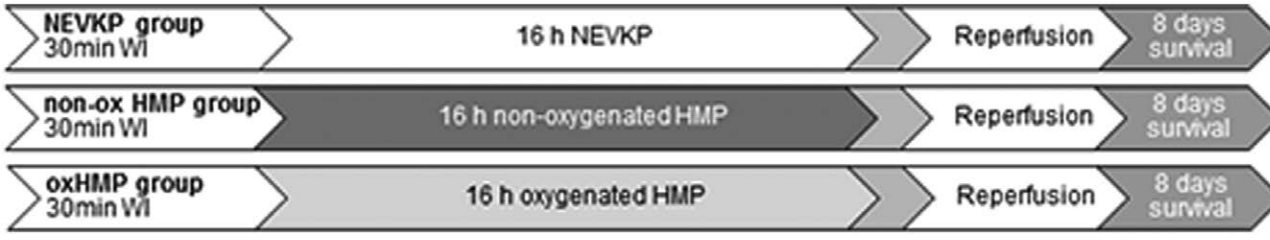

FIGURE 1. Study groups: Pigs were randomly assigned to 1 of the 3 groups (n = 5 in each group). After 30 min of warm ischemia, kidneys were retrieved and preserved with either 16 h of nonoxygenated HMP (non-oxHMP group), oxygenated HMP (oxHMP group), or normothermic ex vivo kidney perfusion (NEVKP group). Following perfusion and after contralateral nephrectomy, grafts were autotransplanted and animals were followed for 8 d. HMP, hypothermic machine perfusion; NEVKP, normothermic ex vivo kidney perfusion; non-oxHMP, nonoxygenated hypothermic machine perfusion; oxHMP, oxygenated hypothermic ex vivo machine perfusion.

artery, the graft was flushed, placed into the cassette, and perfused with a mean arterial pressure of 30 mm Hg. The temperature was maintained at 3–6°C throughout the preservation. No oxygen was provided during the perfusion, and because of the closed circuit, Po_2 levels decreased during perfusion from circa 250 mm Hg at baseline to 50 mm Hg at the end of perfusion.

Normothermic Ex Vivo Kidney Perfusion

Our NEVKP system has been previously described.[20] The S3 heart-lung machine and neonatal cardiopulmonary bypass equipment consists of a centrifugal pump, an oxygenator, a venous reservoir, an arterial bubble filter, and PVC tubing (Sorin Group Inc., Markham, Canada). Additionally, a heat exchanger and a customized double-walled heated organ chamber were built into the system. Perfusion circuit parameters (temperature, arterial and venous pressure, and arterial flow) were continuously recorded. The perfusate solution is made of Ringer's lactate, STEEN Solution (XVIVO Perfusion AB, Goteborg, Sweden), washed leukocyte-filtered erythrocytes, double reverse osmosis water, sodium bicarbonate, calcium gluconate, and heparin. Oxygen/carbon dioxide gas (95%/5%; 2 L/min) was provided continuously during perfusion, resulting in Po_2 levels around 650 mm Hg during the entire preservation time. Also, verapamil and an infusion of amino acids, glucose, and insulin were administered continuously during perfusion.[21] Arterial pressure was initially set at 75 mm Hg and maintained at 65 mm Hg by adjusting the rate of the centrifugal pump.

Oxygenated Hypothermic Machine Perfusion

Our pressure controlled, nonpulsatile NEVKP system, that was previously described[20] and briefly presented above, was modified to perform HMP. Instead of a heat exchanger, a heater-cooler device was attached to cool down the system. The system was primed with 1.5 L of Belzer's Machine Perfusion Solution. The kidney was placed in a chamber that was surrounded by ice. The temperature was maintained at 3–6°C throughout the perfusion. Oxygen (75 mL/min) was administered continuously during the perfusion using a membrane oxygenator, maintaining the Po_2 levels at around 650 mm Hg for the whole preservation time. Arterial pressure was set and maintained at 30 mm Hg, just as in the LifePort system. To mimic the conditions in the LifePort 1.0 device, the vein was not cannulated and the venous perfusate gathered around the kidney. While the kidney was continuously submerged in solution, a roller pump also recirculated this reservoir fluid to the perfusion circuit.

During all perfusions, perfusate samples were collected regularly and stored at −80°C for further investigation. Additionally, during NEVKP urine samples were collected regularly. At the end of each preservation method, the kidney was removed from the device, reflushed with 300 mL heparinized HTK, and stored on ice until transplantation.

Sample Collection

Blood gas analyses of the perfusate were performed hourly during graft perfusion. Additionally, blood gas analyses of the subject were taken before retrieval, before transplantation, and every day during postoperative care. Samples were also analyzed using a point-of-care comprehensive metabolic blood chemistry analyzer (Piccolo Xpress, Union City, Canada) and part of each sample was stored at −80°C for later analysis.

Using a customized metabolic cage, 24-h urine collection was done before transplantation and at postoperative day (POD) 2–3. During the 24-h urine collection in the metabolic cage, animals had to be housed separately, to allow individual urine collection. For creatinine clearance and lactate dehydrogenase (LDH) measurements, 24-h urine collection as well as serum samples were sent to the Toronto General Hospital Core Laboratory for analysis with the Abbott Architect Chemistry Analyzer using the manufacturer's reagents. For measurement of urinary neutrophil gelatinase-associated lipocalin (NGAL), porcine NGAL enzyme linked immunosorbent assays kit was used according to manufacturer's instructions.

Histology

At sacrifice on POD8, wedge biopsies were taken from the renal graft. All samples were placed in 10% neutral buffered formalin and transferred to 70% alcohol after 36 to 48 h. Following paraffin-embedding and sectioning (3-µm), periodic acid-Schiff (PAS) stained sections were used to score global tubular injury on a semiquantitative scale of 0 to 3 (0-no changes, 1-mild, 2-moderate, 3-severe changes) by a renal pathologist blinded to the experimental groups. The tubular injury score was based on the degree of brush border loss, tubular dilatation, epithelial vacuolation, thinning and sloughing, and luminal debris/casts. The changes were assessed over 20 high power fields and averaged.

High-sensitivity Flow Cytometry Analyses of Apoptotic Exosome-like Vesicles

All the analyses were performed on a BD Canto II Special Order Research Product (BD Biosciences) equipped with a small particle option, as described previously.[22,23] Perfusates (10 µL) were labeled in a total reaction volume of 50 µL at 32°C for 60 min with LWA 300 probe (300 nM). Cell tracker deep red (1 µM, ThermoFicher Scientific) was added for 30 min at 32°C and then 1 µL of Annexin V-BV421 (BD Biosciences) was added for 20 min at room temperature. Then, the sample was diluted by adding 250 µL of labeling buffer prior analysis by high-sensitivity flow cytometry.

Statistical Analysis

All statistical analyses were performed with RStudio software (version 1.1.463). Descriptive statistics were calculated (mean ± SD) and tests were conducted to compare variables between the study groups and subgroups. For continuous variables, when a normal distribution of data was identified, ANOVA test was used for comparing groups; in the case of a non-normal distribution, Kruskall-Wallis test was used for analysis. When significance was reached, t-tests, respectively, Wilcoxon tests were performed to determine which 2 groups were significantly different. A paired t-test was used to test significance of differences in normally distributed continuous parameters over time within the same group. Significance was defined as $P < 0.05$.

RESULTS

Animal Characteristics and Survival

Average animal weight was similar between groups (NEVKP: 30.2 ± 2.4 kg, non-oxHMP: 30.8 ± 1.2 kg, oxHMP: 29.1 ± 1 kg, $P = 0.47$). Pigs were randomly assigned to 1 of the 3 groups, and no animals were replaced in any of the groups.

All animals survived until day 8 in each group except 1 in the non-oxHMP group. This pig was euthanized on POD7 because of sudden respiratory decompensation of unclear etiology. Despite this, its renal function was comparable to other animals in this group, and the animal was not excluded from the analysis.

Perfusion Parameters

Hypothermic Machine Perfusion With and Without Oxygen

Kidney grafts that were subjected to non-oxHMP demonstrated improvement in flow rates and resistance index during preservation, with a significant difference between values at baseline (values measured at 5 min after the perfusion was started) compared with the end of the perfusion ($P < 0.001$). Similar perfusion dynamics were observed in kidney grafts that were subjected to oxHMP: flow rates improved over the course of perfusion ($P = 0.5$), and the resistance index was significantly lower at the end of the perfusion compared with baseline ($P = 0.02$) (Figure 2A and B). A significant increase in perfusate lactate concentration (baseline: 0.28 ± 0.07, 15 h: 0.88 ± 0.13 mmol/l, $P = 0.0033$) developed during non-oxHMP, along with a minimal decrease in perfusate glucose concentration (baseline: 10.2 ± 0.4, 15 h: 9.3 ± 0.5 mmol/L, $P = 0.053$). During oxHMP, both lactate concentration (1 h: 0.28 ± 0.19, 15 h: 0.53 ± 0.34 mmol/L, $P > 0.05$) and glucose concentration (1 h: 9.72 ± 0.21, 15 h: 10.24 ± 0.25 mmol/L, $P = 0.008$) increased (Figure 3A and C). During both non-oxHMP and oxHMP, injury marker LDH increased during perfusion, but overall LDH values remained low (non-oxHMP: 1 h: 10 ± 4.6, 15 h: 35.4 ± 10.6 U/L, $P = 0.003$; oxHMP: 1 h: 8.6 ± 5, 15 h: 58.8 ± 27.6 U/L, $P = 0.01$) (Figure 4A).

Normothermic Ex Vivo Kidney Perfusion

Renal blood flow progressively increased during the perfusion ($P < 0.01$), whereas the intrarenal vascular resistance significantly decreased ($P = 0.004$) (Figure 2A and B). The acid-base parameters (pH–Figure 3D, bicarbonate, base excess) and electrolyte concentrations (serum sodium, potassium, calcium, and chloride) were stable within physiologic range during the entire time of NEVKP. Lactate significantly decreased throughout the perfusion (baseline: 9.6 ± 0.6 versus 16 h: 1.6 ± 0.6 mmol/L, $P < 0.001$) (Figure 3B), whereas glucose significantly increased (baseline: 5.2 ± 0.9 versus 16 h: 9.8 ± 0.3 mmol/L, $P = 0.001$) (Figure 3C). LDH, a marker of injury, showed an increase during perfusion, but overall values remained low (1 h: 20 ± 5.5 U/L, 15 h: 57 ± 27.7 U/L, $P = 0.03$) (Figure 4A). All kidneys produced urine during the perfusion. Cumulative urine production was 161 ± 103 mL during NEVKP.

Posttransplant Graft Function and Injury

Kidneys preserved by NEVKP demonstrated improved graft function with lower mean peak serum creatinine (SCr) and faster recovery that occurred earlier compared with non-oxHMP and oxHMP (Figure 5A). The differences between daily serum creatinine levels reached significance between NEVKP and both non-oxHMP and oxHMP on POD1, 2, and 3 (all $P < 0.05$) and, in addition, between NEVKP and oxHMP on POD7 and 8 ($P < 0.05$) (Figure 5A). There was no difference in posttransplant graft function between the 2 cold perfused groups. The area under the curve analysis of the SCr from POD1 to POD8 did not show any significant difference between the 3 groups (all $P > 0.05$). Blood urea nitrogen showed a similar trend to SCr, with the NEVKP group having lower levels compared with the other 2 groups.

All 3 groups showed similar creatinine clearance at baseline (Figure 5B). In contrast, creatinine clearance on POD3 was significantly increased in the warm perfused group compared with the cold perfused groups (NEVKP: 63.6 ± 19.0 versus non-oxHMP: 11.4 ± 10.1 and oxHMP: 10.1 ± 12.5, $P = 0.001$).

Postoperative urine NGAL was measured in a random sample from the 24-h urine collection. Urine NGAL measurements were normalized to urinary creatinine concentration. Grafts perfused with NEVKP showed significantly lower normalized NGAL values compared with the other 2 groups on POD3 (NEVKP: 0.5 ± 0.2 versus non-oxHMP: 1.8 ± 0.8 and oxHMP: 1.4 ± 0.3, $P = 0.01$) (Figure 6). There were no differences between the 2 groups that received cold perfusion on POD3.

Injury marker LDH decreased daily in all groups (Figure 4B). No significant differences were present on POD1, but on POD3 LDH was slightly lower in the oxHMP group compared with the non-oxHMP and NEVKP groups ($P = 0.01$).

Renal tissue samples were collected for histology and PAS stained slides were assessed by a blinded renal pathologist. Tubular injury scores were similar in all groups ($P = 0.17$) (Figure 7A and B).

As marker of endothelial injury, the levels of apoptotic exosome (ApoExo)-like vesicles were measured in the perfusate at 1 h of perfusion. The NEVKP group had lower levels of ApoExo compared with the cold perfused groups. However, significance between groups was reached only between the NEVKP and the oxHMP group ($P = 0.02$) (Figure 8).

DISCUSSION

This study is the first to compare, head-to-head, the outcomes of the 3 clinically utilized ex vivo machine perfusion techniques used for prolonged DCD graft preservation. Here, we demonstrated that grafts preserved with NEVKP from procurement until transplantation show initial superior graft function compared with non-oxHMP and oxHMP preserved grafts. No difference was found between the 2 cold preservation methods.

Grafts preserved with NEVKP demonstrated improved preservation injury, lower peak SCr, improved creatinine clearance at POD3, and lower urine NGAL as marker of kidney injury at POD3. Histology at the end of follow-up on POD8 showed no differences between groups regarding tubular injury and inflammation. This is in alignment with kidney function, which had nearly normalized in all groups by POD8. Animals were observed for 8 d to assess the time that the graft needed to return to a normal function. Because the animals cannot be biopsied during the follow-up period, histology could only be procured at the time of sacrifice. Histological difference would be expected at earlier timepoints. No differences were found between the cold perfused group with oxygen and without oxygen in terms of graft function and injury. Differences in lactate values over the course of perfusion between the cold perfused groups and the NEVKP group are likely because of the differences in perfusate. Of note, the perfusate of the NEVKP group contained Ringers Lactate, whereas the cold perfusion perfusates did not.

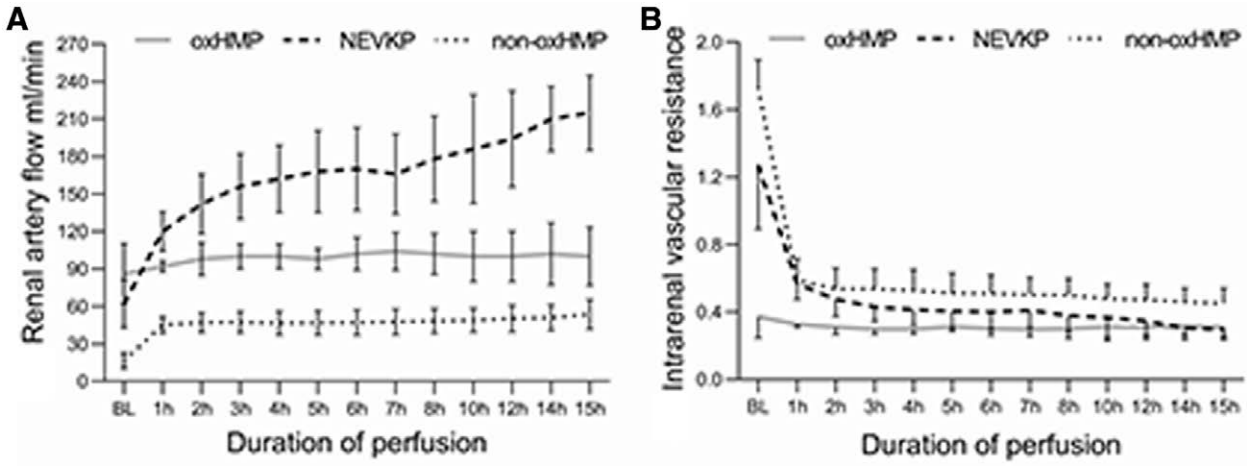

FIGURE 2. Pump parameters. All values are presented as mean ± SD (n = 5 in each group). (A) Renal arterial flow. (B) Intrarenal vascular resistance. In all 3 groups, there was an improvement in the renal arterial flow ($P < 0.01$ for the non-oxHMP and NEVKP groups, $P = 0.5$ for the oxHMP) and a significant decrease in the intrarenal vascular resistance (all $P < 0.05$) over the 16 h of perfusion. NEVKP, normothermic ex vivo kidney perfusion; non-oxHMP, nonoxygenated hypothermic machine perfusion; oxHMP, oxygenated hypothermic ex vivo machine perfusion.

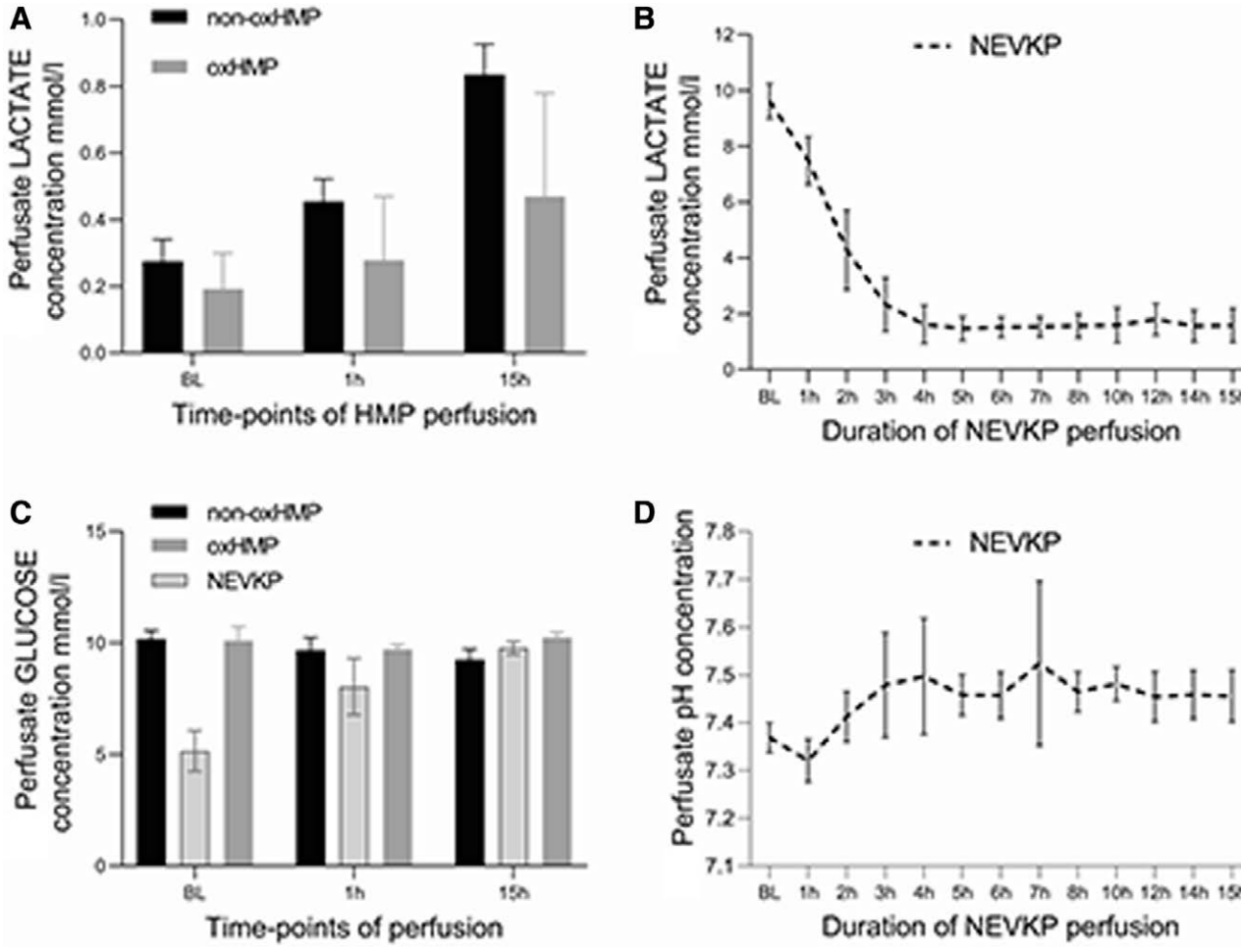

FIGURE 3. Pump metabolites. All values are presented as mean ± SD (n = 5 in each group). (A) Perfusate lactate during HMP with and without oxygen. Lactate increased both during non-oxHMP ($P = 0.0033$) and oxHMP ($P > 0.05$) over the course of perfusion. (B) Perfusate lactate during NEVKP. Lactate significantly decreased during NEVKP over the 16 h of perfusion ($P < 0.001$). (C) Perfusate glucose during HMP with and without oxygen and NEVKP. Glucose concentration slightly decreased during non-oxHMP ($P = 0.053$) and significantly increased during oxHMP and NEVKP (both $P < 0.01$). (D) Perfusate pH during NEVKP. Perfusate pH was stable and within physiologic range during the NEVKP perfusion. NEVKP, normothermic ex vivo kidney perfusion; non-oxHMP, nonoxygenated hypothermic machine perfusion; oxHMP, oxygenated hypothermic ex vivo machine perfusion.

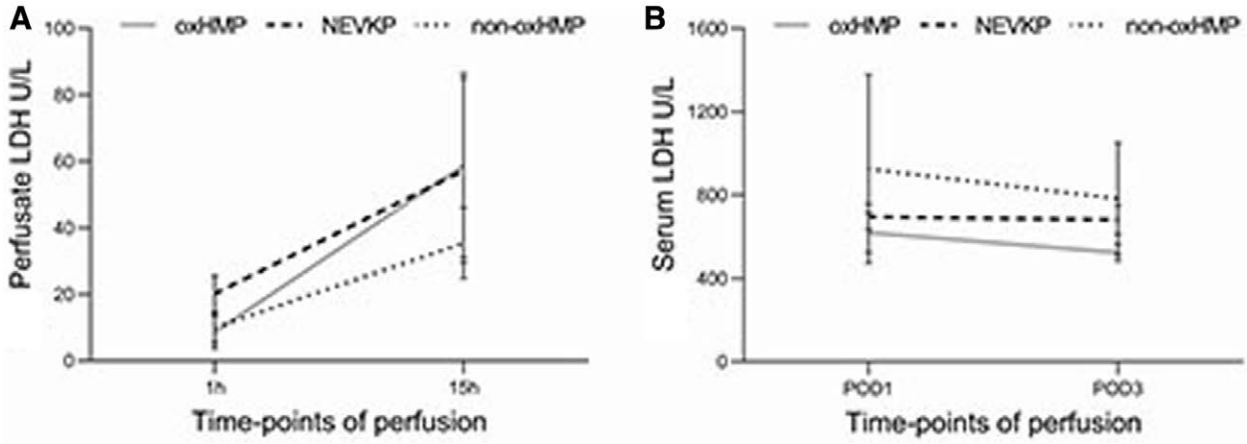

FIGURE 4. Injury marker LDH. All values are presented as mean ± SD (n = 5 in each group). (A) Perfusate LDH. Perfusate LDH increased during perfusion in all 3 groups; however, values remained low (all $P < 0.05$). (B) Serum LDH posttransplantation. Serum LDH decreased daily in all 3 groups; on POD3, LDH levels were lower in the oxHMP group compared with the other 2 groups ($P = 0.01$). LDH, lactate dehydrogenase; NEVKP, normothermic ex vivo kidney perfusion; non-oxHMP, nonoxygenated hypothermic machine perfusion; oxHMP, oxygenated hypothermic ex vivo machine perfusion; POD, postoperative day.

To date, the effects of oxygenation during HMP on preservation injury and kidney function have been contradicting. Venema et al found that the addition of oxygen during HMP did not improve kidney function compared with non-oxHMP.[18] In this study, kidneys subjected to 30 min of WI were preserved for 24 h with SCS, HMP with 21% oxygen or HMP with 100% oxygen and then reperfused ex vivo for 4 h to mimic transplantation. HMP was superior to SCS; however, the oxygen levels did not influence graft function. The authors reported reduced oxidative stress and energy status in grafts perfused with 100% oxygen. However, using a reperfusion model instead of a transplant model has its limitations. Duration of follow-up is only limited to a few hours and important cellular components and mediators of injury and regeneration are not present in the perfusate. In addition, urine production, as an endpoint of function, requires hormonal support, which is absent during ex vivo reperfusion.

Our results are in contrast with a study presented by Darius et al, who found that oxHMP is superior to non-oxHMP.[9] After 30 min of WI and either 22 h of HMP with or without oxygen or 20 h of non-oxHMP followed by 2 h of warm perfusion, grafts were transplanted, and animals were followed for 13 d. Peak SCr was lowest in the oxHMP group, though without reaching significance compared with the other 2 groups. The area under the curve analysis of SCr showed a significantly lower creatinine for the oxHMP group compared to the non-oxHMP group, and the HMP+NEVKP group. When normalizing the SCr to pig body weight, there was a significant difference on POD2 and POD3 between the oxygenated and nonoxygenated HMP groups. The authors argued that this demonstrates the superiority of oxHMP over non-oxHMP and HMP+NEVKP. The variation in cold ischemia times between the studies might contribute to the different findings.[24]

A recent clinical trial investigated the benefits of continuous oxygenated versus nonoxygenated cold perfusion in DCD

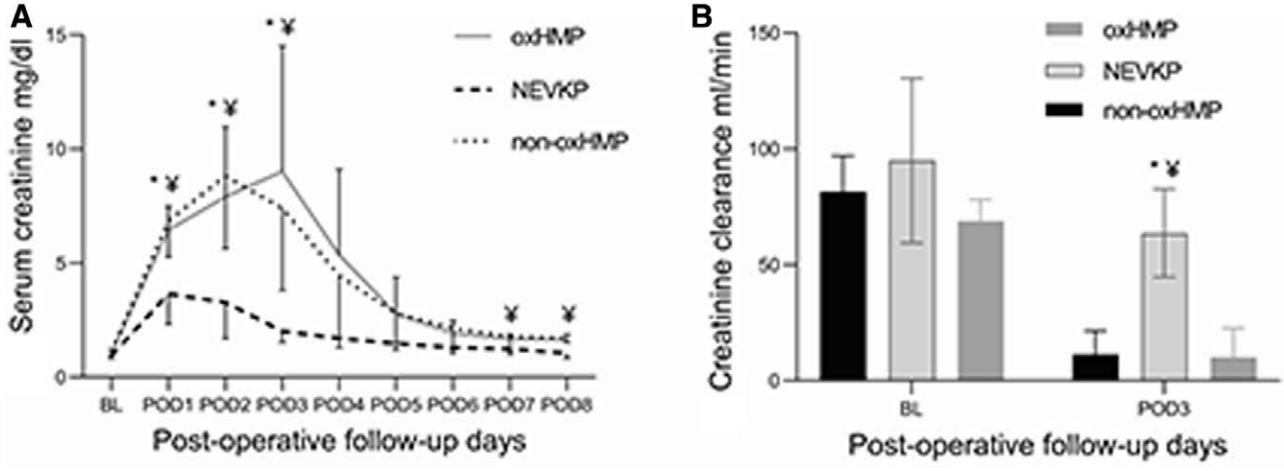

FIGURE 5. Posttransplant graft function. All values are presented as mean ± SD (n = 5 in each group). (A) Serum creatinine of transplanted animals during the 8 d follow-up. Overall, there was a significantly lower serum creatinine in the NEVKP group compared with the other 2 groups (all $P < 0.05$). There were no significant differences between the 2 cold perfused groups. (B) Creatinine clearance at baseline and POD3. Creatinine clearance on POD3 was significantly higher in the NEVKP group vs the other 2 groups ($P = 0.001$). No differences were found between the 2 cold perfused graousp. * indicates significance between NEVKP and non-oxHMP; ¥ indicates significance between NEVKP and oxHMP. non-oxHMP. NEVKP, normothermic ex vivo kidney perfusion; non-oxHMP, nonoxygenated hypothermic machine perfusion; oxHMP, oxygenated hypothermic ex vivo machine perfusion; POD, postoperative day.

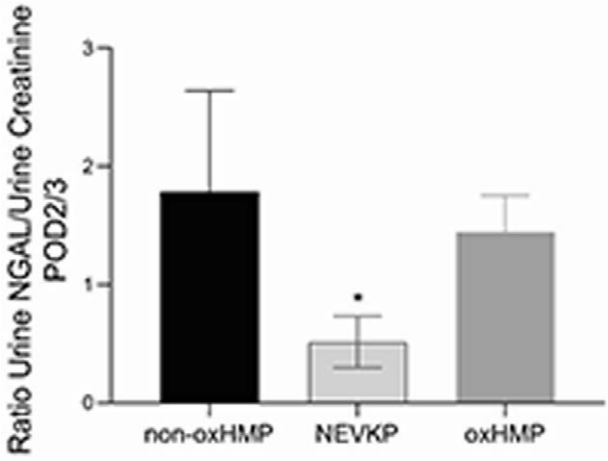

FIGURE 6. Urinary NGAL. NGAL measured in the urine was normalized to urinary creatinine concentration. Urinary NGAL was significantly lower in the NEVKP group vs the cold perfused groups ($P=0.01$). * indicates significance between NEVKP and the other 2 groups (n = 5 in each group). NEVKP, normothermic ex vivo kidney perfusion; NGAL, neutrophil gelatinase-associated lipocalin; non-oxHMP, nonoxygenated hypothermic machine perfusion; oxHMP, oxygenated hypothermic ex vivo machine perfusion.

grafts.[12] This European trial found no differences in the estimated glomerular filtration rate at 1-y but a decreased graft loss in the oxygenated group. Also, the authors report less acute rejection episodes in the oxygenated group. Another clinical trial investigated the benefits of end-ischemic oxygenated HMP after SCS in extended criteria brain dead donors.[25] The group that received short-term oxHMP before transplantation showed no improvement in graft function and survival compared with the group that received SCS alone. Our findings are in accordance with these studies, highlighting that oxygenation during HMP did not result in superior graft function compared with HMP without oxygen. The benefits of oxygenation during HMP remain inconclusive. However, our study indicates that the mere addition of oxygen during HMP is not enough to improve graft function after transplantation in moderately damaged kidney grafts. Further studies need to determine whether oxygenated HMP could have advantages in more severely injured grafts.

The benefits of NEVKP have also been previously investigated by other groups. Blum et al compared non-oxHMP with NEVKP for the preservation of DCD porcine kidney grafts.[26] After 45 min of WI and 5 h of SCS, grafts were perfused for 8 h, and then graft function was assessed using a reperfusion model. Oxygen consumption, urine production, creatinine clearance, fractional excretion of sodium, proteinuria, and LDH and aspartate aminotransferase release into the perfusate did not differ between groups. The differences in kidney function between groups might have not become apparent during reperfusion because of the limited time of assessment. In our study, because of the transplantation model, we were able to observe the function for several days and this might have made the differences between groups more obvious.

The Nicholson group has also compared NEVKP and HMP for the preservation of kidney grafts. In one study, after 8 min of warm ischemia and 2 h of SCS, grafts were perfused for 16 h with either NEVKP or non-oxHMP and then an ex vivo reperfusion model was used for graft assessment.[27] Renal function was similar in the 2 groups, but NEVKP preserved kidneys could concentrate creatinine and conserve sodium better. Graft assessment was limited to a few hours of reperfusion, which makes function assessment more difficult. These results support our findings regarding the improved kidney function in NEVKP perfused grafts compared with HMP perfused grafts.

To our knowledge, only 1 other study compared NEVKP and HMP with or without oxygen in an in vivo model.[28] Using a rodent model, grafts were exposed to 30 min of WI, 4 of SCS, 1 h of either end-ischemic NEVKP or oxHMP or nitrogenated HMP and then transplanted. Grafts perfused with end-ischemic oxHMP performed better than the other groups with better survival rates, lower peak SCr, and less reactive oxygen species release. These results are different to our findings; however, following several hours of SCS, the limited perfusion of 1 h might have not been enough to unfold the benefits of NEVKP.

We also found that during NEVKP, less ApoExo vesicles are present in the perfusate, compared with the cold perfused groups. Recent findings have suggested that in injured endothelial cells, caspase-3 activation promotes the release of extracellular vesicles, including ApoExo, which enhance the recruitment of inflammatory cells and favor the production of antibodies, which aggravate vascular inflammation.[29,30] Also, using a model of acute kidney injury in mouse, Dieude et al found that vascular injury increases proteasome caspase-like activity in exosome-like vesicles preparations.[30] Reduced levels of ApoExo vesicles during NEVKP are in line with our other findings and these results support our conclusion that NEVKP preservation is more protective compared with the cold preservation techniques.

NEVKP maintains metabolism active instead of slowing it down by cooling and allows for graft assessment and possibly graft modification and repair. However, warm perfusion is technically and logistically challenging, involving higher costs and more trained personnel. One main advantage of HMP is its simplicity and the fact that it can be performed during transportation. Because of the low temperature, both graft assessment and treatment are limited.

Our model involved healthy young pigs without any underlying kidney disease and a moderate kidney injury (30 min WI). It is possible that non-oxHMP and oxHMP offer benefits in older grafts or kidneys with underlying changes, for example due to hypertension or diabetes. Also, compared with SCS, HMP with and without oxygen might improve preservation injury.

One of the main strengths of our study is that kidney injury and function were observed in an in vivo model. Graft injury occurs not only during the ischemia period but is a complex process that also continues after reperfusion. Therefore, a transplantation survival model is best suited to fully apprehend the complexity of this process.

We acknowledge that our study has several limitations. The small number of animals per group might not allow for significant results to become obvious. Also, the allotransplantation model does not allow the possible immunological effects of the ex vivo preservation methods. Animals were only followed for 8 d, which does not allow us to assess long-term graft function. The lack of histologic differences may be because of the late time-point at which the biopsy specimens were obtained, considering that by day 8 the kidney function was almost normal in all groups. It is possible that differences between the groups may exist at earlier time points, and these could potentially have long-term effects. Also, the lack of prepreservation

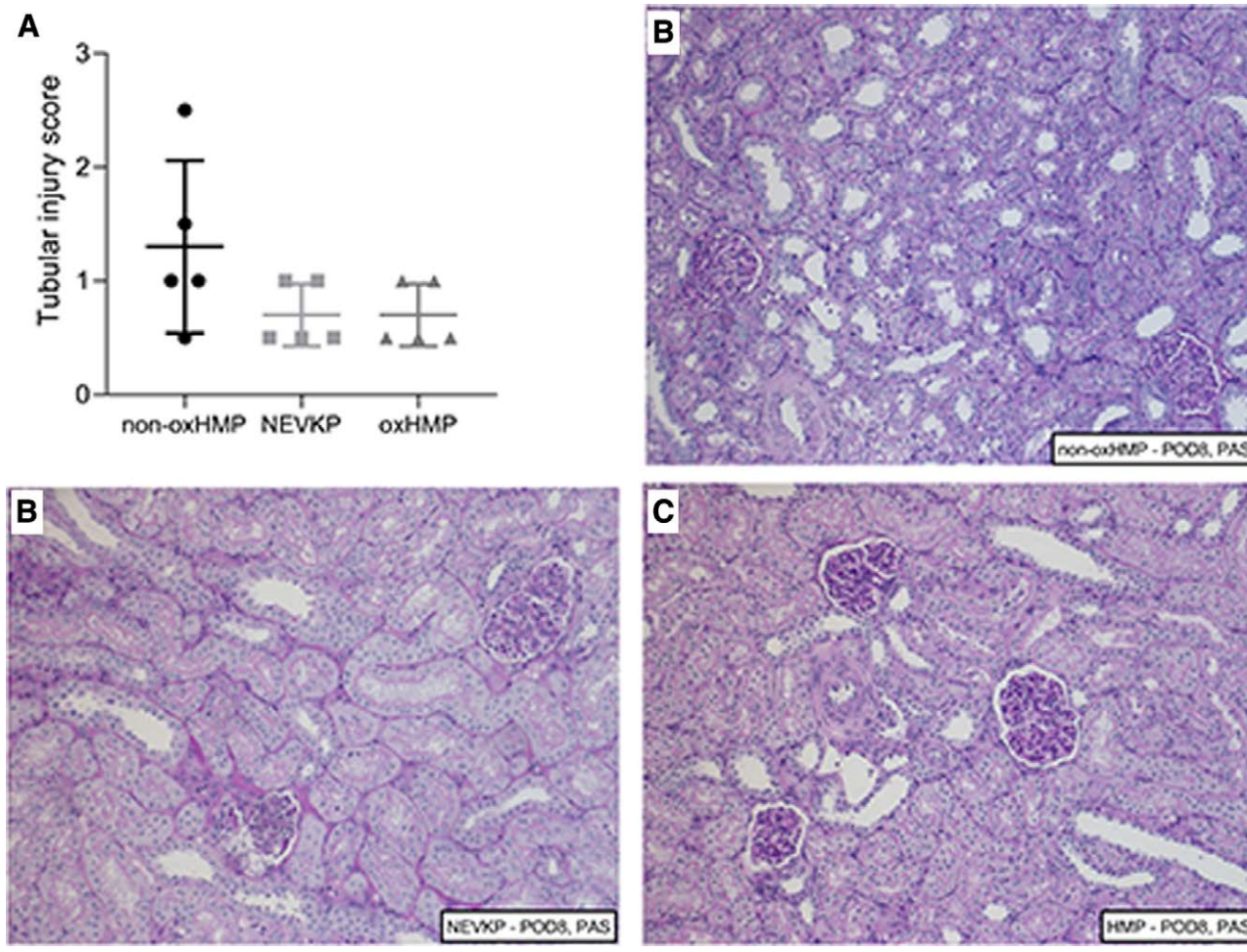

FIGURE 7. Histopathologic changes among the 3 experimental groups. (A) Graphical representation of tubular injury score comparing NEVKP, non-oxHMP, and oxHMP groups, respectively, showing slightly greater injury (although not statistically significant) in the non-oxHMP group (n = 5 in each group). (B) PAS stained representative histology images from NEVKP, non-oxHMP, and oxHMP groups. NEVKP, normothermic ex vivo kidney perfusion; non-oxHMP, nonoxygenated hypothermic machine perfusion; oxHMP, oxygenated hypothermic ex vivo machine perfusion; PAS, periodic acid-Schiff.

graft injury and the good condition of the recipient might have resulted in a much sooner recovery of graft function, overstating the benefits of the ex vivo preservation techniques. Young pigs have the capacity to regenerate well after ischemic injury. The presence of only 1 kidney at termination of the experiment might have resulted in a higher glomerular filtration rate of the transplanted kidney when compared with baseline. It is possible that the reported kidney function 8 d after transplantation overestimates the actual kidney function.

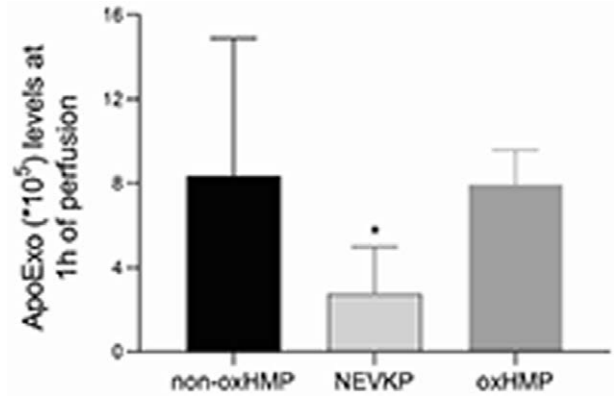

FIGURE 8. ApoExo perfusate levels. Levels of ApoExo in the perfusate at 1 h of perfusion (n = 5 in each group). Apoexo levels were lower in the NEVKP group vs the cold perfused groups. Significance was reached only between the NEVKP and oxHMP group ($P = 0.02$). * indicates significance between NEVKP and the oxHMP group. ApoExo, apoptotic exosome; NEVKP, normothermic ex vivo kidney perfusion; oxHMP, oxygenated hypothermic ex vivo machine perfusion; non-oxHMP, nonoxygenated hypothermic machine perfusion.

Our study used 2 different devices for the preservation of the grafts. Because non-oxHMP is already well established in clinical trials, we choose to use a commercially available device for this group. Because in our country, there is no commercial device available for oxHMP, we adapted our NEVKP system to perform oxHMP. To validate our oxHMP system, we initially performed several experiments comparing graft function in kidneys perfused with non-oxHMP with the LifePort System and with our device. The results were similar in both groups; however, because of space limitations, these data were not included in the current article.

Furthermore, the technical challenges of warm perfusion in the human setting should not be underestimated. Currently, there is no portable perfusion device available for NEVKP. Therefore, warm perfusion for the entire preservation period, as used in this study, is currently impractical in the human setting. It is possible that the optimal future preservation technique will include a cold perfused technique, with or without

oxygen, followed by warm perfusion after arrival of the graft at the transplant center.

In summary, this study demonstrated superior initial graft function with prolonged NEVKP when compared with HMP with or without oxygen in a pig kidney DCD transplant model. No differences were found between HMP with and without oxygen. Faster recovery with NEVKP could possibly result in better and higher long-term graft survival. Moreover, during NEVKP graft viability could be assessed in real-time. Using NEVKP in a clinical setting could result in increased graft utilization. Future studies should aim to confirm our results and augment the usage of NEVKP in a clinical setting.

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