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Continuous Normothermic *Ex Vivo* Kidney Perfusion Is Superior to Brief Normothermic Perfusion Following Static Cold Storage in Donation After Circulatory Death Pig Kidney Transplantation

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Hypothermic preservation is known to cause renal graft injury, especially in donation after circulatory death (DCD) kidney transplantation. We investigated the impact of cold storage (SCS) versus short periods of normothermic *ex vivo* kidney perfusion (NEVKP) after SCS versus prolonged, continuous NEVKP with near avoidance of SCS on kidney function after transplantation. Following 30 min of warm ischemia, kidneys were removed from 30-kg Yorkshire pigs and preserved for 16 h with (A) 16 h SCS, (B) 15 h SCS + 1 h NEVKP, (C) 8 h SCS + 8 h NEVKP, and (D) 16 h NEVKP. After contralateral kidney resection, grafts were autotransplanted and pigs followed up for 8 days. Perfusate injury markers such as aspartate aminotransferase and lactate dehydrogenase remained low; lactate decreased significantly until end of perfusion in groups C and D ($p < 0.001$ and $p = 0.002$). Grafts in group D demonstrated significantly lower serum creatinine peak when compared to all other groups ($p < 0.001$) and 24-h creatinine clearance at day 3 after surgery was significantly higher (63.4 ± 19.0 mL/min) versus all other groups ($p < 0.001$). Histological assessment on day 8 demonstrated fewer apoptotic cells in group D ($p = 0.008$). In conclusion, prolonged, continuous NEVKP provides superior short-term outcomes following DCD kidney transplantation versus SCS or short additional NEVKP following SCS.

Abbreviations: AST, aspartate aminotransferase; BUN, blood urea nitrogen; DCD, donation after circulatory death; DGF, delayed graft function; ECD, extended criteria donor; EMS, exsanguinous metabolic support; EVNP, *ex vivo* normothermic perfusion; HMP, hypothermic machine perfusion; HPF, high-power fields; HTK, histidine–tryptophan–ketoglutarate solution; IM, intramuscular; IRI, ischemia–reperfusion injury; IRR, intrarenal resistance; LDH, lactate dehydrogenase; NEVKP, normothermic *ex vivo* kidney perfusion; NGAL, neutrophil gelatinase-associated lipocalin; PNF, primary nonfunction; pod, postoperative day; RPM, rounds per minute; SCS, static cold storage

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Introduction

Kidney transplantation represents the "gold standard" for the treatment of end-stage renal disease, providing superior outcomes in quality of life, and reduced morbidity and mortality when compared to dialysis (1). The worldwide severe organ shortage resulted in prolonged waiting times, which is associated with decreased long-term graft survival (2). To enlarge the donor pool, grafts of lower quality recovered from extended criteria donors (ECD) and donation after circulatory death (DCD) are increasingly used for transplantation (3). However, several studies demonstrate that transplantation of ECD and DCD kidney grafts can lead to increased rates of primary nonfunction (PNF), delayed graft function (DGF), and reduced long-term outcomes (4–6).

Poorer outcomes after ECD and DCD kidney transplantation are linked to their poor tolerance to cold ischemia (7–9). To address this, several approaches are being explored to reduce ischemia–reperfusion injury (IRI) in renal grafts, including changes in donor management (10), graft preconditioning (11), improvement of hypothermic preservation techniques (12–14), postconditioning, and regenerative techniques following transplantation (15).

Normothermic *ex vivo* organ perfusion provides oxygen and nutrition to maintain organ function during storage in contrast with cold storage, which suspends cell metabolism. It has been shown to improve posttransplant outcomes for heart (16), lung (17), and liver transplantation (18,19). In kidney transplantation, 1 additional hour of normothermic perfusion following cold storage reduced DGF of ECD human grafts significantly (20). However, no data are currently available as to whether better outcomes are achieved by a short period of normothermic preservation as compared with prolonged normothermic *ex vivo* kidney perfusion (NEVKP) with close to complete replacement of hypothermia.

Recently, we have shown the feasibility and safety of replacing hypothermic preservation in standard criteria donor grafts with 8 h of continuous NEVKP (21). Furthermore, we demonstrated its superiority over static cold storage (SCS) in DCD pig kidney transplantation (22). The aim of the present study was to investigate whether an additional, short period of normothermic kidney perfusion following SCS or prolonged, continuous NEVKP is superior in DCD kidney transplantation.

Methods

Study design

We compared prolonged, continuous pressure-controlled NEVKP versus various combinations of SCS and subsequent NEVKP. Following renal warm ischemia of 30 min, grafts were exposed to (A) 16 h of SCS, (B) 15 h of SCS + 1 h of NEVKP, (C) 8 h of SCS + 8 h of NEVKP, and (D) 16 h of NEVKP (Figure 1). After contralateral kidney resection, preserved grafts were autotransplanted and animals followed for 8 days. Markers of renal function and graft injury were assessed during preservation and follow-up. The Animal Care Committee of the Toronto General Research Institute, Ontario, Canada, approved the study.

Animals

Male Yorkshire pigs (30 kg) were utilized. Species-adapted housing with water and food *ad libitum* was provided. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care of Laboratory Animals" published by the National Institutes of Health.

Kidney retrieval

Porcine kidney retrieval was performed as previously described by our group (23). Briefly, ketamine (20 mg/kg; Bimeda-MTC Animal Health Inc., Cambridge, Canada), atropine (0.04 mg/kg; Rafter 8 Products, Calgary, Canada), and midazolam (0.3 mg/kg; Pharmaceutical Partners of Canada Inc., Richmond Hill, Canada) were applied intramuscularly to induce anesthesia. Following intubation, 2.0% isoflurane (Pharmaceutical Partners of Canada Inc.) was administered by inhalation. A permanent central venous catheter (9.5 French; Cook Medical Company, Bloomington, IN) was placed for administration of fluids, medication, and postsurgical blood collection. Following midline incision, right kidneys were dissected and renal artery and vein clamped with vascular clamps for 30 min to induce warm ischemia. After graft resection, artery (1.6 in, LivaNova PLC, London, UK), vein ($^1/_4 \times {}^1/_8$ in, LivaNova PLC), and ureter (feeding tube) were quickly cannulated and kidneys flushed with 4°C cold histidine–tryptophan–ketoglutarate (HTK) solution (300–500 mL) containing 10 000 IU/L heparin (Sandoz Canada Inc., Toronto, Canada) at a pressure of 100 cm H_2O. The abdominal wall was closed and the pigs recovered from surgery.

Static cold storage

Recovered grafts in group A, B, and C were submerged in HTK solution and statically stored on ice in a sterile organ bag (CardioMed Supplies Inc., Lindsay, Canada) for 16, 15, or 8 h until autotransplantation or NEVKP, respectively.

Normothermic ex vivo *kidney perfusion (NEVKP)*

NEVKP was performed as previously described by our group (24). An S3 heart–lung machine and neonatal cardiopulmonary bypass equipment

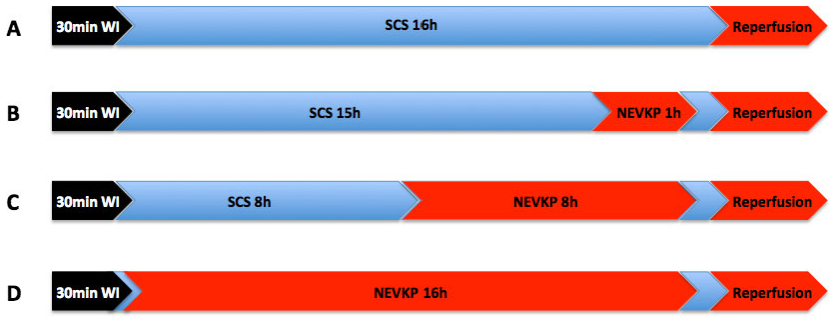

Figure 1: Study design. Animals were housed prior to planned procedures. Prior to recovery, renal grafts underwent 30 min of warm ischemia (WI). Following 4°C cold flush, renal grafts were preserved with (A) static cold storage (SCS) for 16 h, (B) SCS for 15 h + 1 h of normothermic *ex vivo* kidney perfusion (NEVKP), (C) SCS for 8 h + 8 h of NEVKP, or (D) for 16 h of NEVKP. Following NEVKP, grafts were flushed with 4°C cold preservation solution for autotransplantation and reperfused *in situ*.

were used for *ex vivo* graft perfusion (LivaNova PLC). Data were recorded with the data management system (LivaNova PLC). During *ex vivo* perfusion, kidneys were positioned in a customized, heated, double-walled chamber to provide normothermia and sterility. Based on baseline measurements in 20 healthy pigs, the perfusate solution was composed to provide a physiologic environment in terms of acid–base and electrolyte homeostasis, hemoglobin concentration, osmolarity, and oncotic pressure, as previously described by our group (Table S1) (21). For blood collection, additional pigs served as non–blood group–matched donor animals. The recovered whole blood was repeatedly washed to obtain leukocyte-depleted, plasma-free erythrocytes, as previously described. Prior to perfusion, the circuit was primed with Ringer's lactate (200 mL), Steen solution^{TM} (150 mL) to provide physiological oncotic pressure, washed erythrocytes (125 mL) to provide physiologic hemoglobin values, double reverse osmosis water (27 mL) for optimal osmolarity, sodium bicarbonate (8.4%, 8 mL) to adjust pH and electrolyte levels, calcium gluconate (10%, 100 mg/mL, 1.8 mL) for optimal calcium concentration, and heparin (1000 IU) to prevent coagulation (Table S2) (21). After priming the circuit, perfusate samples were collected to perform blood gas analyses and assess markers of kidney function and injury (lactate, aspartate aminotransferase [AST], lactate dehydrogenase [LDH]) at baseline. Following renal graft recovery, flush with HTK (group D), and additional cold storage (groups B and C), kidneys were connected to the primed perfusion circuit, and perfused at 37°C with an initial arterial pressure of 75 mmHg. Following graft rewarming, pressures dropped and were kept stable at 65 mmHg with minimal need of rounds per minute (RPM) regulation. During perfusion, oxygen (2 L/min, 95% O_2, 5% CO_2), nutrition (amino acids and glucose [1 mL/h]), and insulin (5 IU/h), and verapamil (0.25 mg/h) for vasodilation were administered continuously. Urine production and evaporation were replaced by Ringer's lactate infusion to provide a stable perfusate composition with physiologic ranges for hemoglobin and electrolytes (Table S2). Hourly perfusate and urine samples were collected to assess trends of the abovementioned parameters. Samples were frozen down for further investigation at −80°C following centrifugation. At the end of NEVKP, grafts were flushed with 300–500 mL HTK and stored on ice in a sterile organ bag until transplantation.

Kidney transplantation

Recovered pigs were re-anesthetized using intravenous administration of propofol. Following intubation, anesthesia was continued with 1.5% isoflurane per inhalation and 15 mL/h propofol intravenously. Following repeat laparotomy, contralateral kidneys were resected, grafts removed from ice, and renal anastomoses (vein end-to-side to cava, artery end-to-side to aorta, ureter side-to-side) sewed. Perioperative procedures, drug administration, and follow-up of the pigs were conducted as previously described (23). After follow-up of 8 days, pigs were sacrificed under anesthesia.

Whole blood, serum, and urine measurements

Blood gas analyses (RAPIDPoint 500 Systems; Siemens AG, Berlin, Germany) were performed hourly during NEVKP and daily after kidney transplantation. Perfusate samples were analyzed for AST and LDH during NEVKP and serum samples for measurement of creatinine and blood urea nitrogen (BUN)/urea (Piccolo Xpress, Union City, Canada) for follow-up after transplantation. Serum samples were also frozen at −80°C until enzyme-linked immunoassay (ELISA) analysis. Neutrophil gelatinase-associated lipocalin (NGAL), a specific renal injury marker, was measured using a porcine (NGAL) ELISA kit (Bioporto, Hellerup, Denmark) at baseline, day 3, and day 5 after kidney transplantation. Twenty-four-hour urine collection was performed using a metabolic cage to investigate the creatinine clearance before transplantation (day 0), on postoperative day 3 (pod 3), and postoperative day 8 (pod 8). Further serum and urine analyses were performed in the Core Laboratory on the Abbott Architect Chemistry Analyzer using the manufacturer's reagents (Abbott Laboratories, Abbott Park, IL).

Histology

On pod 8, pigs were anesthetized and intubated. Following repeat laparotomy, wedge biopsies of the transplanted kidneys were taken and placed in 10% neutral buffered formalin and transferred to 70% alcohol after 36–48 h. Following paraffin-embedding, sectioning, and staining, 3-μm periodic acid–Schiff–stained sections were used to score tubular injury and interstitial inflammation on a scale of 0 to 3 (0 representing no changes, 1 mild, 2 moderate, and 3 severe changes) blinded to the experimental group as previously described (21). Tubular injury, including brush border loss, tubular dilatation, epithelial vacuolation, thinning and sloughing, and luminal debris, was scored in 30 high-power fields (HPF) and averaged to assess overall tubular injury. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining was performed according to standard protocol, and the numbers of apoptotic cells were counted in 50 HPF and averaged.

Statistical analysis

SPSS software version 23.0 (IBM, Armonk, NY) was used to perform statistical analysis. Fisher's exact test was used for calculation of differences in mortality (two-sided). The Shapiro–Wilk test was used to test variables for normal distribution. One-way analysis of variance was chosen to compare differences of parametric continuous variables. Post hoc tests were performed using Bonferroni correction. Kruskal–Wallis test was used for ordinal and nonparametric data between groups. To test for differences of normally distributed continuous parameters over time within the same group, a paired t-test was utilized. Significance was defined as $p < 0.05$.

Results

Animal demographics, times for preservation, and times for anastomoses were similar

Animal weights in group A (16 h SCS), group B (15 h SCS + 1 h NEVKP), group C (8 h SCS + 8 h NEVKP), and group D (NEVKP 16 h) were similar (A 30.7 ± 3.3 kg, B 29.7 ± 1.8 kg, C 32.6 ± 0.9 kg, and D 30.2 ± 2.4 kg, with $t(17) = 1.449$, and $p = 0.299$). There was no significant difference in the total preservation times between the groups with 968 ± 32 min in group A, 984 ± 21 min in group B, 979 ± 12 min in group C, and 979 ± 12 min in group D ($t(19) = 0.476$, $p = 0.703$). Total times for anastomoses were also similar with 26.8 ± 1.6 min in group A, 30.6 ± 15.7 min in group B, 33.8 ± 1.5 min in group C, and 25.4 ± 1.9 min in group D ($t(19) = 1.137$, $p = 0.364$). Group B had a higher standard deviation of 15.7 min because one animal required a redo of the venous anastomosis prior to reperfusion because of a tear. The graft was kept cold with ice during anastomosing and posttransplant results were in line with other animals from group B.

Grafts demonstrated physiologic perfusion characteristics during NEVKP

As previously described, NEVKP was initiated at an arterial pressure of 75 mmHg and a venous pressure between 0 and 3 mmHg was secured. Following graft

rewarming, the pressure dropped to a physiological value of 65 mmHg. In case the pressure dropped further, the RPM of the centrifugal pump were adjusted to keep the pressure at 65 mmHg (Figure 2A). The pressure in the renal vein was maintained at 0–3 mmHg by height regulation of the venous reservoir in relation to the renal vein of the graft. The lower the inflow is positioned in relation to the renal vein, the more negative the pressure becomes (24). Following graft rewarming, flow rates reached physiologic values within 1 h after initiation of NEVKP. Flow rates at final hour of perfusion reached 142 ± 25 mL/min (group B), 198 ± 23 mL/min (group C), and 215 ± 30 mL/min (group D) (Figure 2B). Intrarenal resistance (IRR) decreased in all groups after graft rewarming and reached physiologic values within 1 h. Decrease between initial IRR and final IRR values were significant in all groups (group B 1.09 ± 0.37 vs. 0.47 ± 0.08 mmHg/mL/min with $p = 0.013$; group C 0.92 ± 0.21 vs. 0.33 ± 0.04 with $p = 0.002$; group D 1.27 ± 0.38 vs. 0.30 ± 0.04 with $p = 0.012$) (Figure 2C). Cumulative urine production reached 3 ± 1 mL in group B, 29 ± 25 mL in group C, and 161 ± 103 mL in group D.

NEVKP was associated with maintenance of biochemical parameters in the perfusate

The renal grafts viability and metabolic activity were assessed by hourly blood gas analyses. Acid–base homeostasis was maintained without administration of bicarbonate after initiation of NEVKP. The pH, hydrogen carbonate, base excess, and electrolytes were stable and maintained in physiologic range in all groups (data not shown).

NEVKP preserved grafts demonstrated low injury markers and high lactate clearance

Injury markers, such as AST and LDH, were assessed hourly during NEVKP. AST increased from baseline until end of perfusion in group B ($AST_{baseline}$ 2 ± 1 U/L vs. $AST_{last\ hour}$ 24 ± 7 U/L, $t(3) = -6.262$ with $p = 0.008$), group C ($AST_{baseline}$ 2 ± 1 U/L vs. $AST_{last\ hour}$ 16 ± 2 U/L, $t(4) = -13.370$ with $p < 0.001$), and group D ($AST_{baseline}$ 1 ± 0.5 U/L vs. $AST_{last\ hour}$ 37 ± 28 U/L, $t(4) = -2.893$ with $p = 0.044$) (Table 1). Similarly, LDH increased from baseline until end of perfusion in group B ($LDH_{baseline}$ 2.3 ± 2.9 U/L vs. $LDH_{last\ hour}$ 40.8 ± 10.3 U/L, $t(3) = -5.911$ with $p = 0.01$), group C ($LDH_{baseline}$ 17.2 ± 8.7 U/L vs. $LDH_{last\ hour}$ 37.2 ± 20.9 U/L, $t(4) = -1.836$ with $p = 0.140$), and group D ($LDH_{baseline}$ 3.0 ± 4.7 U/L vs. $LDH_{last\ hour}$ 39.3 ± 14.9 U/L, $t(3) = -3.908$ with $p = 0.03$) (Table 1). Although AST and LDH increased in all groups, overall these injury markers remained low during NEVKP in all groups.

Lactate significantly decreased from baseline until the end of perfusion in the prolonged perfusion groups C ($lactate_{baseline}$ 10.14 ± 0.76 mmol/L vs. $lactate_{last\ hour}$ 1.62 ± 0.79 mmol/L, $t(4) = 13.05$ with $p < 0.001$) and D ($lactate_{baseline}$ 9.89 ± 0.38 mmol/L vs. $lactate_{last\ hour}$ 1.47 ± 0.70 mmol/L, $t(2) = 24.676$ with $p = 0.002$) only. Lactate did not decrease significantly in group B using only 1 additional hour of NEVKP following SCS ($lactate_{baseline}$ 9.48 ± 0.45 mmol/L vs. $lactate_{last\ hour}$ 8.36 ± 2.40 mmol/L, $t(3) = 0.796$ with $p = 0.484$) (Table 1).

NEVKP resulted in significantly improved kidney function and reduced injury

Grafts preserved with NEVKP alone (group D) demonstrated significantly lower serum creatinine values following heterotopic renal autotransplantation when compared to SCS only (group A) and compared to different combinations of SCS and NEVKP (groups B and C). Overall, differences were significant at hour 10 ($p = 0.003$), day 1 ($p < 0.001$), day 2 ($p < 0.001$), day 3 ($p < 0.001$), and day 4 ($p = 0.002$) after transplantation. For group D, post hoc Bonferroni testing demonstrated significantly lower values at hour 10 versus groups A and C. On pod1, pod2, and pod3 group D had significantly lower creatinine values versus all other groups, and on pod4 versus group A (Figure 3A). For BUN, overall differences were significant on pod 2, 3, 4, and 5. Post hoc Bonferroni testing demonstrated significantly lower values for group D versus all other groups on pod2, and versus groups A and B on pod3 and 4 (data not shown).

Peak serum creatinine levels were highly significantly different between the groups with $F(3, 19) = 17.403$ and $p < 0.001$. Post hoc Bonferroni testing demonstrated significant differences between group D versus all other groups (Figure 3A). Peak BUN was also significantly lower between the groups with $F(3, 19) = 7.352$ and

Figure 2: (A) Renal artery pressure during normothermic *ex vivo* kidney perfusion. Values are presented as mean ± standard deviation (SD) in mmHg. Dashed line and gray area represent mean systemic blood pressure and SD measured invasively *in situ* in 30 anesthetized pigs by placing a catheter into the carotid artery. (B) Renal artery flow during normothermic *ex vivo* kidney perfusion. Values are presented as means ± SD in mL/min. Dashed line and gray area represent mean flow rate with SD measured *in situ* in 30 anesthetized pigs; upper and lower lines represent maximal and minimal renal artery flow rates in these pigs. The measurements were performed in control pigs following laparotomy and minimal dissection of the right renal artery with a flow probe. (C) Intrarenal resistance during normothermic *ex vivo* kidney perfusion. Values are presented as mean ± SD in mmHg/mL/min. Dashed line and gray area represent mean IRR with SD based on measurements performed *in situ* in 30 anesthetized pigs. The IRR decreased significantly in all groups from baseline to last hour of perfusion ($p < 0.05$, respectively). IRR, ischemia–reperfusion injury; NEVKP, normothermic *ex vivo* kidney perfusion; SCS, static cold storage.

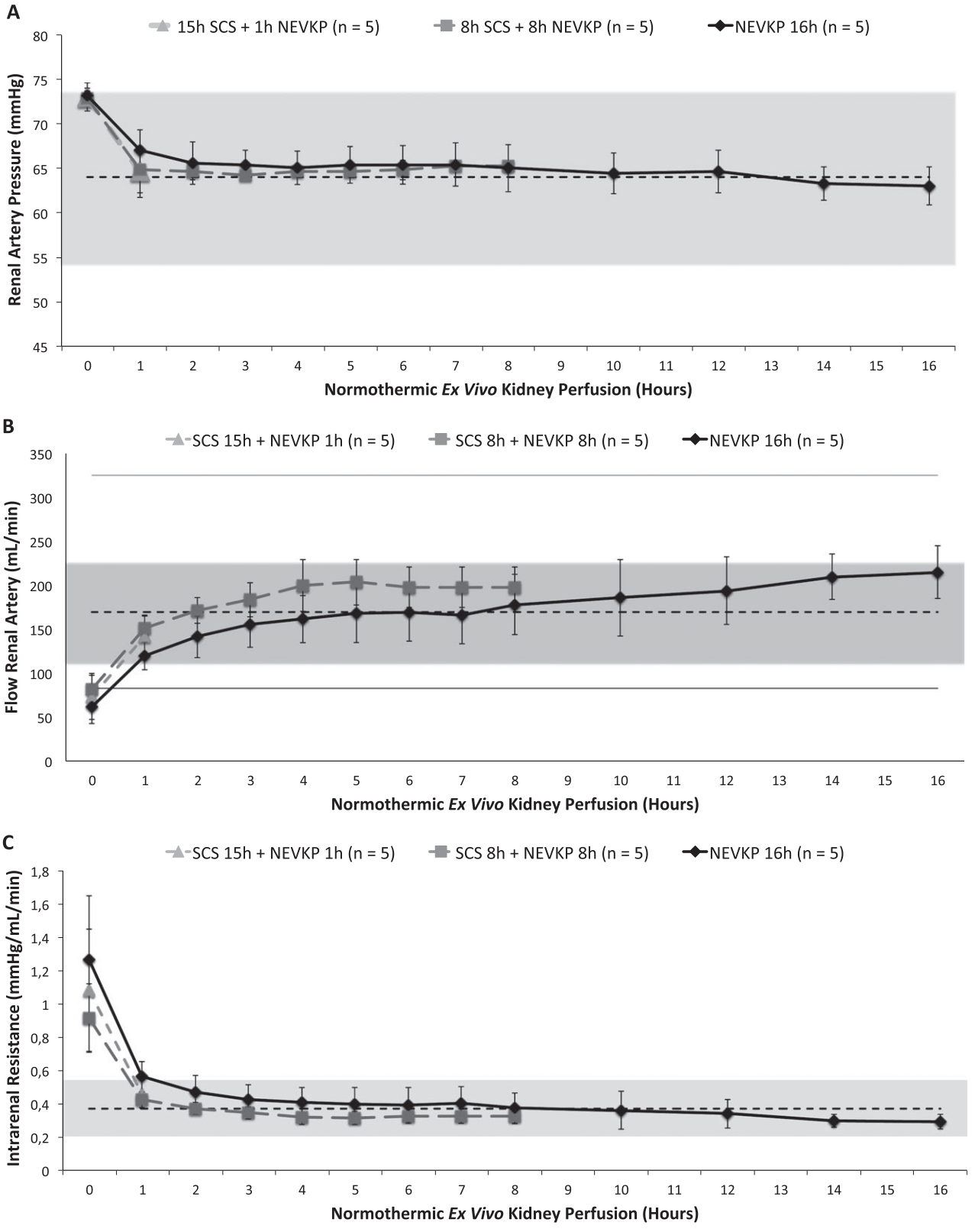
A
15h SCS + 1h NEVKP (n = 5)
8h SCS + 8h NEVKP (n = 5)
NEVKP 16h (n = 5)
Renal Artery Pressure (mmHg)
80
75
70
65
60
55
50
45
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Normothermic *Ex Vivo* Kidney Perfusion (Hours)
B
SCS 15h + NEVKP 1h (n = 5)
SCS 8h + NEVKP 8h (n = 5)
NEVKP 16h (n = 5)
Flow Renal Artery (mL/min)
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Normothermic *Ex Vivo* Kidney Perfusion (Hours)
C
SCS 15h + NEVKP 1h (n = 5)
SCS 8h + NEVKP 8h (n = 5)
NEVKP 16h (n = 5)
Intrarenal Resistance (mmHg/mL/min)
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Normothermic *Ex Vivo* Kidney Perfusion (Hours)

Table 1: Injury markers aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) as well as lactate clearance were tested hourly during perfusion

Marker	Group	Baseline of NEVKP	Last hour of NEVKP	p-value
AST (U/L)	A	–	–	–
	B	2 ± 1	24 ± 7	0.008
	C	2 ± 1	16 ± 2	<0.001
	D	1 ± 0.5	37 ± 28	0.044
LDH (U/L)	A	–	–	–
	B	2.3 ± 2.9	40.8 ± 10.3	0.01
	C	17.2 ± 8.7	37.2 ± 20.9	0.14
	D	3.0 ± 4.7	39.3 ± 14.9	0.03
Lactate (mmol/L)	A	–	–	–
	B	9.48 ± 0.45	8.36 ± 2.40	0.484
	C	10.14 ± 0.76	1.62 ± 0.79	<0.001
	D	9.89 ± 0.38	1.47 ± 0.70	0.002

Although AST and LDH levels were significantly higher at end of perfusion (hour 1 for group B, hour 8 for group C, hour 16 for group D) versus baseline (NEVKP after priming without connection of graft to circuit), these injury markers remained low. Lactate decreased significantly until the end of perfusion in groups C and D. NEVKP, normothermic *ex vivo* kidney perfusion.

$p = 0.003$. Post hoc Bonferroni testing demonstrated significantly lower values for group D versus groups A and B (data not shown).

Serum NGAL, one of the earliest and most robustly induced proteins that is recently becoming more often used to detect acute renal injury (25), demonstrated significant differences between all groups at day 5 after transplant ($p < 0.001$). Post hoc Bonferroni testing resulted in significantly lower values for group D (1103 ± 485 ng/mL) versus B (7437 ± 1941 ng/mL); differences between group D versus group A (3679 ± 1945 ng/mL), and group D versus group C (3142 ± 1856 ng/mL) were not significant. Interestingly, NGAL was significantly lower in all groups versus group B on day 3 after transplant (Figure 4).

Twenty-four-hour creatinine clearance was highly significant between groups on pod3 after transplant with $p < 0.001$. Post hoc Bonferroni testing demonstrated significantly higher creatinine clearance in group D versus all other groups (Figure 3B).

Renal tissue biopsy samples were compared among the four groups on day 8 following renal transplantation. Results for tubular injury and interstitial inflammation were lowest for prolonged 16-h NEVKP (group D) without reaching significance. TUNEL staining demonstrated significant differences between all groups, with lowest numbers of apoptotic cells for group D ($p = 0.008$) (Table 2; Figure 5).

To address the question of whether the exclusion of SCS or a prolonged period of NEVKP itself contributed to the beneficial effects seen in group D, posttransplant serum creatinine values were also compared to a group in which grafts were perfused for 8 h after 30 min of warm ischemia followed by autotransplantation. The results demonstrated a trend towards lower postoperative serum creatinine values after 16 h compared to 8 h of NEVKP (Figure 6). Thus, beneficial effects of prolonged NEVKP not only seem to result from exclusion of SCS but possibly also from repair mechanisms provided by prolonged NEVKP itself.

NEVKP did not compromise animal survival

All animals survived until day 8 after transplant. Animal survival was therefore similar in all groups.

Discussion

Normothermic *ex vivo* kidney perfusion represents a novel preservation technique for renal grafts. Recently, we have demonstrated the safety and feasibility of continuous NEVKP for 8 h in heart-beating donor pig kidney transplantation (21) and its superiority over SCS in DCD pig kidney transplantation (22). In the present study, we show that the outcome of porcine DCD kidney transplantation is improved with decreasing times of SCS and increasing duration of NEVKP. Short (1 h) NEVKP following prolonged SCS (group B) had minor beneficial effects on outcome, while prolonged (16 h) NEVKP without SCS (group D) significantly improved the graft function after transplantation. Renal grafts were safely perfused at normothermic temperatures for up to 16 h, demonstrating low values of intrarenal resistance, low injury markers, and high clearance of lactate. Following renal autotransplantation, serum creatinine and BUN values were significantly lower and creatinine clearance significantly higher in the prolonged NEVKP group when compared to various combinations of SCS and NEVKP. Histological assessment demonstrated numerically less tubular injury and significantly lower rates of apoptotic cells after 16 h of NEVKP (group D). Furthermore, 16 h NEVKP versus 8 h NEVKP demonstrated a trend towards lower serum creatinine following transplantation, suggesting that prolonged NEVKP might provide potential benefits itself, not only by exclusion of SCS.

In a porcine model of kidney transplantation, Hosgood et al applied 2 h of additional *ex vivo* normothermic kidney perfusion (EVNP) using a plasma free red-cell-based solution after hypothermic machine perfusion (HMP). The results indicated the feasibility of 2 h of EVNP following HMP, but did not show significantly improved functional outcomes posttransplant (26). In another set of experiments, porcine kidneys were warm perfused for a maximum of 6 h to compare different arterial perfusion pressures (27), and whole blood versus leukocyte-depleted perfusate compositions (28). However, grafts were only assessed during preservation as no transplants

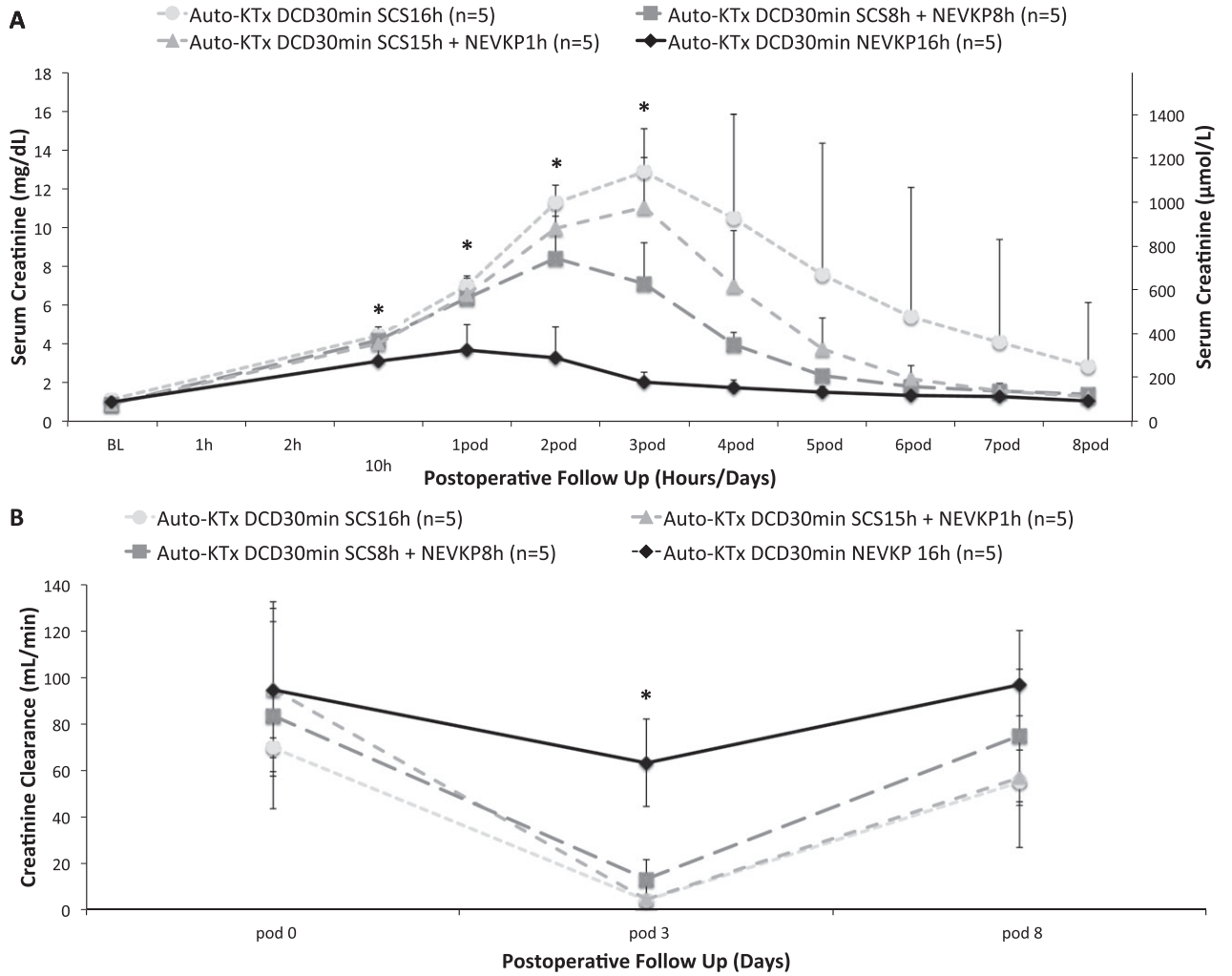

Figure 3: (A) Serum creatinine of transplanted animals during 8 days follow-up. Values presented as mean ± standard deviation (SD) in mg/dL and µmol/L. Overall, significant differences were observed at hour 10 posttransplant and days 1–4 between groups. Peak serum creatinine was significantly lower in the NEVKP 16-h group when compared to all other groups ($p < 0.001$). (B) Creatinine clearance of transplanted animals during 8 days follow-up. Values presented as mean ± SD in mL/min. The creatinine clearance between groups was significantly different on postoperative day 3 ($p < 0.001$) and significantly higher in the NEVKP 16-h group versus all other groups. NEVKP, normothermic *ex vivo* kidney perfusion; KTx, kidney transplant; DCD, donation after circulatory death; SCS, static cold storage; pod, postoperative day.

were performed, and outcomes were not compared to cold-preserved grafts. None of the studies compared short, additional versus prolonged, continuous perfusion periods with near to complete exclusion of hypothermia.

In 2013, Nicholson and Hosgood published the first clinical trial investigating EVNP. Eighteen ECD kidney grafts were perfused at 34.6°C for 1 h following SCS directly prior to transplantation. The outcome following transplantation was compared to a control group of 47 ECD kidneys that were preserved statically on ice. Delayed graft function occurred in 1/18 patients (5.6%) whose grafts underwent a brief period of EVNP, and in 17/47 patients (36.2%) whose grafts were statically cold stored only ($p = 0.014$). Graft and patient survival at 12 months were not significantly different (20). These data demonstrate that adding short EVNP following SCS can improve renal graft function following kidney transplantation. Nicholson's findings support the results of our present study that also demonstrated improved renal function for short NEVKP following SCS. However, our data indicate that prolonged perfusion with avoidance of cold storage is more protective than short periods of NEVKP following SCS. In Nicholson's study it remains unclear whether the duration of perfusion impacts on graft function and injury.

Our findings are supported by studies from Brasile and Stubenitsky. In the late 1990s and early 2000s, this

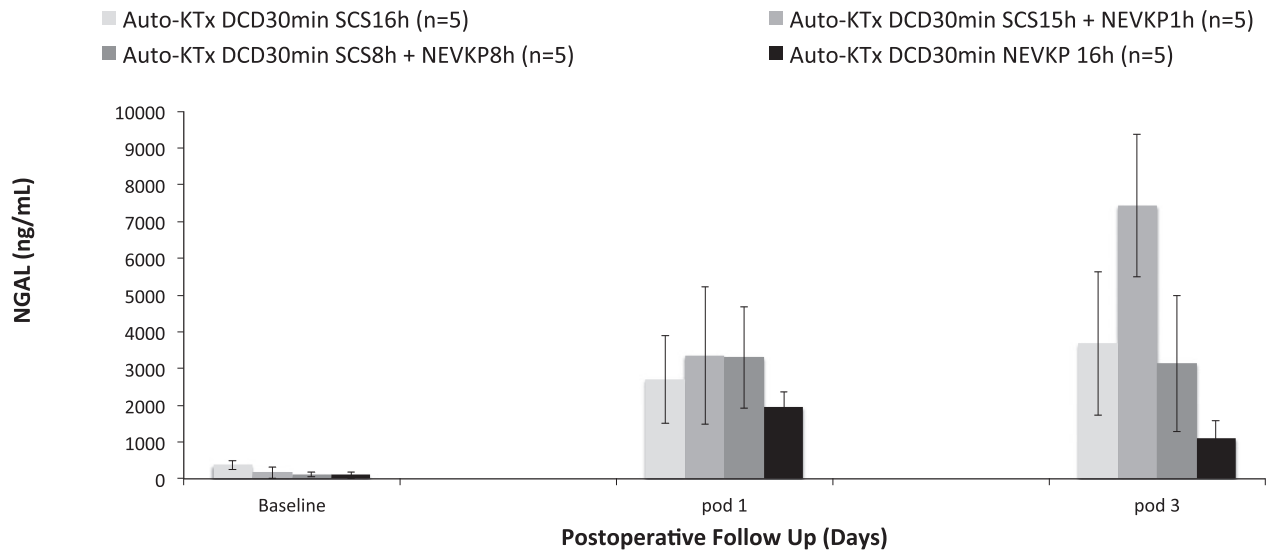

Figure 4: Serum NGAL of transplanted animals during 3 days follow-up. Values presented as mean ± standard deviation in ng/mL. The NGAL between groups was significantly different on postoperative day 3 ($p < 0.001$) and significantly higher in group B versus all other groups. NGAL, neutrophil gelatinase-associated lipocalin; KTx, kidney transplant; DCD, donation after circulatory death; SCS, static cold storage; pod, postoperative day.

Table 2: Histopathologic injury scores assessed on periodic acid–Schiff–stained slides in the four experimental groups 8 days after transplantation

	Histological findings				
	SCS 16 h (n = 5)	SCS 15 h + NEVKP 1 h (n = 5)	SCS 8 h + NEVKP 8 h (n = 5)	NEVKP 16 h (n = 5)	p-value
Tubular injury	1.5 (1–3)	1 (1–2)	1 (1–1.5)	1 (1–1)	0.079
Inflammation	1 (0.5–1.5)	0.5 (0.5–1.5)	1 (0.5–1.5)	0.5 (0.5–2)	0.965
TUNEL	16 (11–38)	4 (0–18)	5 (1–30)	0 (0–4)	0.008

Thirty high-power fields were scored on a scale of 0–3 for tubular injury and inflammation (0 representing no changes, 1 mild, 2 moderate, and 3 severe changes). Additionally, apoptotic cells were counted on TUNEL-stained slides in 50 high-power fields. Data are shown as median (range). SCS, static cold storage; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling; NEVKP, normothermic *ex vivo* kidney perfusion.

group investigated the potential to improve ischemically injured renal grafts using acellular exsanguinous metabolic support (EMS) at subnormothermic temperatures of 32°C in a canine autotransplantation model. Kidneys were exposed to 30 min of warm ischemia, preserved statically on ice for 24 h, and then either transplanted (control group) or primarily perfused for 3 h before reimplantation. Following transplantation, EMS-preserved grafts had lower 24-h posttransplant serum creatinine, lower peak serum creatinine, and improved survival rates (29). In a follow-up study, this group demonstrated the superiority of prolonged subnormothermic perfusion times. Following 30 min of warm ischemia, kidneys were exposed to various combinations of SCS and warm perfusion. Complete exclusion of SCS using EMS for 18 h demonstrated the best posttransplant renal function, followed by prolonged EMS perfusion after SCS (SCS 18 h + EMS 18 h). The lowest renal function was observed in the groups exposed to only SCS (18 h) or SCS followed by short times of EMS (SCS 18 h + EMS 3 h) (30).

SCS, HMP, and combinations of both are the clinical standards for renal graft preservation. Nonoxygenated HMP is performed at pulsatile, low perfusion pressures of 20–30 mmHg and has been shown to reduce the overall rate of DGF in randomized controlled trials when compared to SCS, especially in ECD grafts (31,32). In DCD kidney transplantation, two recently published studies, however, demonstrated controversial outcomes for HMP versus SCS. One trial, investigating the outcome of DCD III (controlled donation after circulatory death, Maastricht category III) kidneys preserved with HMP versus SCS demonstrated reduced rates of DGF (33); a parallel conducted trial did not show superior outcomes for HMP and was stopped prior to completion (15). Interestingly,

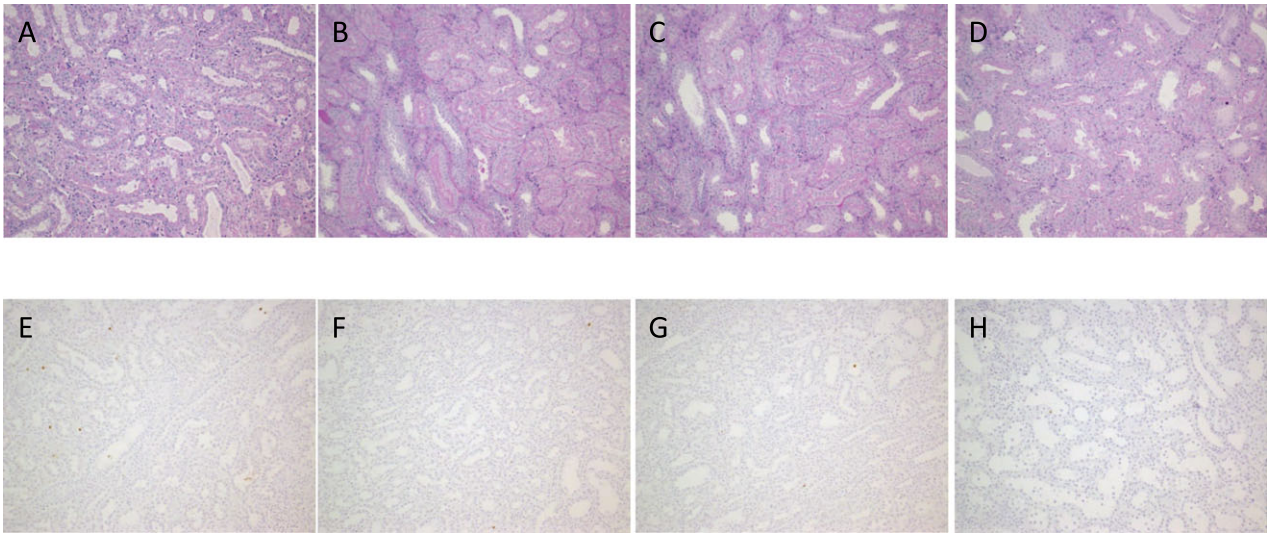

Figure 5: Tubular injury as identified by brush border loss, luminal ectasia, and sloughing is slightly worse in 16-h SCS pigs (A) compared to the other groups (B, C, and D) but not significantly different between the groups at 8 days after transplantation (periodic acid–Schiff, 100×); TUNEL staining shows many more apoptotic cells in 16-h SCS pigs (E) compared to the NEVKP groups (F, G, and H), with the fewest numbers in the 16-h NEVKP group (H) at the same time point (TUNEL stain, 100×). SCS, static cold storage; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling; NEVKP, normothermic *ex vivo* kidney perfusion.

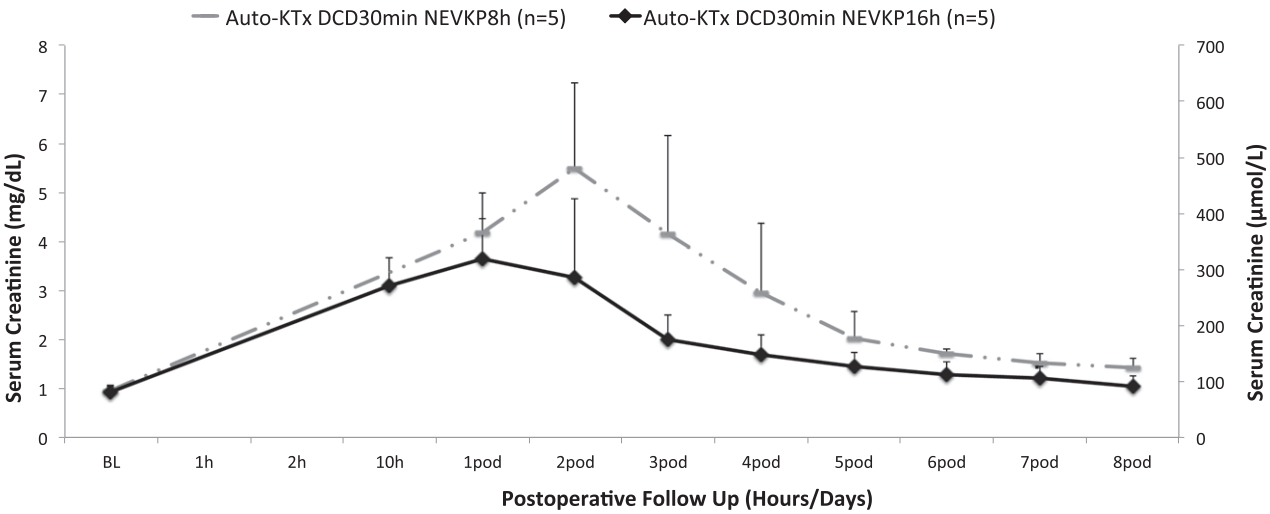

Figure 6: Serum creatinine of transplanted animals during 8 days follow-up. Values presented as mean ± standard deviation in mg/dL and µmol/L. KTx, kidney transplant; DCD, donation after circulatory death; NEVKP, normothermic *ex vivo* kidney perfusion; pod, postoperative day.

the first study used continuous HMP, while the second study combined initial SCS for transportation with brief HMP prior to transplantation. Experimental data from porcine models have demonstrated that short periods of HMP following long SCS times might not be capable of improving graft function following transplantation (12). Currently, no randomized clinical trials are registered that are comparing long, continuous versus short, additional HMP following SCS (3).

To date, prolonged, continuous perfusion at normothermic temperatures has not been investigated. The current study demonstrates that stepwise reduction of SCS using NEVKP leads to superior graft function and reduced renal injury in a porcine kidney transplant model; superior results are demonstrated for close to complete exclusion of hypothermia using NEVKP. These findings are in line with studies published for subnormothermic (34) and hypothermic perfusion (35–38). Pressure-controlled

perfusion leads to physiologic flow values and low IRR. Low IRR has been shown to be an independent risk factor for DGF in HMP (39,40). Acid–base homeostasis and electrolytes are maintained at physiologic levels during NEVKP. Markers of cell injury, such as AST and LDH, slightly increased from baseline until the end of the normothermic perfusion period, but remained low in all groups. Mild increased values were expected, as renal grafts underwent 30 min of warm ischemia. Furthermore, utilization of centrifugal pumps and cardiopulmonary bypass technology in combination with red blood cells are known to cause hemolysis, especially when long perfusions are performed (41). This may have caused an additional slight increase in AST and LDH levels in our perfusion system, especially in prolonged perfusion groups. Lactate clearance demonstrated a significant decrease between baseline and last hour of perfusion only in prolonged NEVKP (group C and D). In a systematic review, increased values of AST, LDH, and lactate, and changes in pH, all measured in hypothermic machine perfusate, were significantly associated in some studies with increased rates of DGF, PNF (for LDH), and reduced graft function (for LDH) (42). However, the significance of these markers measured during NEVKP needs further investigation. The fact that estimation of perfusate characteristics and biomarkers in our control group (A) is not feasible due to lack of perfusion impedes the comparison with SCS preservation. On a separate note, urine samples obtained during NEVKP were not further analyzed for additional injury marker as our NEVKP setup leads to rather low and inconsistent volumes of urine.

Posttransplant, peak serum creatinine and peak serum BUN values were lower, with decreasing SCS and increasing NEVKP preservation time. NGAL is a protein that is released early in acute kidney injury (AKI). It can be investigated in serum and urine and represents a promising early biomarker to detect renal injury in AKI and kidney transplantation (43,44). Our results demonstrate the lowest values for NGAL for group D on days 1 and 3 after transplantation with a significant difference when compared to group B. Interestingly, NGAL in group B was significantly higher than in all other groups. Although promising, NGAL results need to be interpreted carefully. In specifically selected patients, such as pediatric patients undergoing cardiopulmonary bypass surgery, NGAL was rated a helpful marker of AKI (25). However, several studies including reviews indicate that using a novel biomarker remains cumbersome, especially in heterogeneous patient populations (45). Creatinine clearance on pod3 was significantly higher when kidneys were stored with prolonged NEVKP only (group D); in fact, creatinine clearance remained elevated and was similar to baseline. Core biopsies taken on day 8 following transplantation demonstrated lower tubular injury and significantly lower numbers of apoptotic cells in group D.

Besides the superior outcome provided by prolonged NEVKP over short additional normothermic perfusion presented in this study, prolonged NEVKP bears further benefits. Potential advantages of prolonged perfusion include improved assessment options. Only prolonged perfusion times will suffice to investigate potential functional and biological markers, such as IRR (14), AST (46), LDH (47), or more specific renal injury markers such as NGAL (46), kidney injury molecule-1, and IL-18 (29,48). However, additional studies will need to explore the potential of assessing renal function and injury during normothermic perfusion to allow prediction of whether or not a kidney is suitable for transplantation. Protein expression profiling might facilitate prediction of graft performance, as demonstrated in clinical *ex vivo* lung perfusion recently (49). Furthermore, repair strategies such as gene transfer, administration of micro RNAs, anti-inflammatory and immunomodulating drugs, or stem cell therapies will most likely need prolonged normothermic preservation periods for successful action. In lung transplantation, successful treatment of infected donor lungs (50) and functional repair strategies (51,52) applied during *ex vivo* lung perfusion have been demonstrated.

Thirty minutes of warm ischemia were applied in this study, as this most closely reflects the clinical scenario of DCD category III and IV kidney transplantation. In this model, blood flow in the renal artery and vein were completely blocked as opposed to clinical DCD, in which periods of reduced, low, and no flow alternate. Although provoking severe injury, renal grafts of these young pigs without any comorbidity all completely recovered. Despite full graft recovery, kidneys in control and brief NEVKP groups demonstrated severe injury with high elevation of serum creatinine. In comparable clinical settings, the patients experience DGF—which is the primary outcome measurement in most clinical studies investigating alternative preservation techniques—or even complete renal failure. However, future studies should specifically address more severely injured kidneys with longer warm ischemia times to investigate the full potential of NEVKP to recover renal grafts.

This study design postulates the availability of a portable normothermic kidney perfusion device that facilitates placing the graft on normothermic preservation right after retrieval. Currently, devices are commercially available for normothermic liver, lung, and heart, but not yet for renal graft preservation. Until further reliable data indicate the need for portable NEVKP systems, an initial phase of cold storage will be applied for renal graft transportation prior to NEVKP and transplantation. Therefore, future studies need to evaluate how long NEVKP periods should be performed following initial cold storage to obtain best results and facilitate graft assessment and repair.

This study has several limitations. Mechanisms of early reperfusion injury after SCS and NEVKP were not

investigated, as early tissue biopsies during NEVKP or at early time points after transplantation were not taken to prevent bleeding and to not compromise animal survival. It is likely that the avoidance of detrimental effects caused by cold ischemia, such as ATP depletion and cell swelling, is an important part of the beneficial effects of NEVKP. Our findings that the outcome after transplantation was better with reduction of SCS time indicates that minimizing cold ischemia is of great importance. However, prolonged NEVKP by itself demonstrated improved renal graft function following transplantation. Stable perfusion in a controlled, nonimmunogenic environment (leukocyte depleted, plasma free perfusate solution) might be key but needs further investigation. Follow-up studies will need to explore mechanistic pathways to clarify underlying differences between SCS, short NEVKP, and prolonged NEVKP. Of note, future strategies could include adding anti-inflammatory substrates to the perfusion solution to improve the repair or recovery potential of shorter NEVKP periods.

In addition, SCS was applied in the control group instead of HMP. Current data about the differential effects of HMP and SCS are ambiguous and further studies need to investigate whether HMP provides substantial benefits over SCS. Most centers in North America and Europe still utilize SCS rather than HMP for renal graft preservation (United States: SCS 55.1% vs. HMP 44.5% vs. unknown preservation technique 0.4%, United Network for Organ Sharing/Organ Procurement and Transplantation Network data from 2015). Furthermore, current animal studies investigate alternative HMP preservation techniques using active oxygenation or slow rewarming, for instance. Future trials will need to compare NEVKP versus pulsatile/nonpulsatile, versus oxygenated/nonoxygenated, versus prolonged hypothermic/slow graft rewarming setups, and so on to distinguish which factors are of importance.

Another limitation of our study involves the use of renal autotransplantation rather than allotransplantation. As immunosuppression is challenging in porcine models, autotransplantation was chosen to prevent graft rejection (53). Clinical signs of graft rejection such as increase in serum creatinine and histopathological hanges are similar to those seen in ischemia–reperfusion injury and might have confounded the results of our study. Although heterotopic renal autotransplantation models are widely used to investigate IRI and alternative preservation techniques (13,26,30,44), future studies exploring NEVKP in renal allotransplantation models should be conducted. Those would be more similar to clinical practice and incorporate the complex alloimmune responses seen in transplant-related ischemia–reperfusion injury.

In conclusion, this study demonstrates that continuous NEVKP provides superior outcome in DCD kidney transplantation when compared to SCS only, or when compared to combined SCS and NEVKP. Future clinical trials should aim to replace SCS with NEVKP and minimize cold storage periods. Short periods of NEVKP following SCS appear to have limited benefit in a model of porcine kidney transplantation.

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Author Contributions

Johann Moritz Kaths, Darius Bagli, Istvan Mucsi, Anand Ghanekar, David Grant, Lisa Robinson, and Markus Selzner participated in research design; Johann Moritz Kaths, Lisa Robinson, and Markus Selzner participated in writing the paper; Johann Moritz Kaths, Jun Yu Cen, Yi Min Chun, Juan Echeverri, Ivan Linares, and Sujani Ganesh participated in performing the experiments; Johann Moritz Kaths, Paul Yip, and Rohan John participated in data analysis.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1: Blood gas analysis (BGA), osmolarity, and oncotic pressure measured at baseline in serum of Yorkshire pigs and at the start of normothermic *ex vivo* kidney perfusion (NEVKP) in the perfusate solution. *Osmolarity and oncotic pressure were measured in five former NEVKP setups, using the same perfusate solution composition as in this study.

Table S2: Ingredients in perfusate solution and amount or rate administered.