CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.1470065

Available online at: http://www.iajps.com *Research Article*

PROLONGED NORMOTHERMIC EX VIVO KIDNEY PERFUSIONSAFE PRESERVATION TECHNIQUE FOR KIDNEY TRANSPLANTATION

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Abstract:
Kidney transplantation is the treatment of choice for patients with end-stage renal disease as it results in lower morbidity and mortality rates when compared to dialysis [2-4]. However, the limited number of deceased donor grafts available represents a severe problem worldwide. Deceased standard criteria donors (SCD) represent the main source for kidney transplantation. The organ shortage has triggered interest in expanding the pool of available kidneys by using renal grafts from donors with higher age, hypertension, increased terminal serum creatinine levels of >1.5 mg/dL, or death from cerebrovascular accident (extended criteria donors; ECD) [75] to increase the number of available donors. Our study has several limitations. Our model did not include kidneys recovered in donation after brain death or kidneys with severe preservation injury. Thus, possible protection against deleterious effects of brain death or preservation injury was not investigated. In addition, in the absence of severe preservation injury, we did not assess mechanisms of graft injury such as cytokine release, infiltration of inflammatory cells, or ATP depletion. Furthermore, keeping the preservation time to less than 8 hours may be impossible in organ procurement regions where prolonged storage periods are sometimes required. To address the question of safety, we on purpose chose a short preservation time with minimal injury in the control group. This might explain that there was no significant difference in peak serum creatinine and BUN in between both groups. Future studies will focus on grafts with severe kidney injury, such as kidneys recovered after circulatory death.

Keywords: *Kidney transplantation, static cold storage, hypothermic machine perfusion.*

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Please cite this article in press Mahum Imran et al., ***Prolonged Normothermic Ex Vivo Kidney Perfusionsafe Preservation Technique for Kidney Transplantation****., Indo Am. J. P. Sci, 2018; 05(10).*

INTRODUCTION:

Current renal graft preservation techniques prior to transplantation include static cold storage (SCS) and hypothermic machine perfusion (HMP). The principle of hypothermic techniques is based on the reduction of the tissue's metabolic activity to reduce tissue injury. Unfortunately, ECD and DCD grafts tolerate cold anoxic storage only poorly and the detrimental impact of hypothermia has been demonstrated in several studies [96,15]. Even in SCD heart-beating donor (HBD) kidney transplantation DGF rates of up to 20-50% have been reported in particular after prolonged hypothermic preservation [29,30,19]. DGF in HBD kidney transplantation is known to result in decreased long-term outcomes and increased health care costs [17,18]. Solid evidence supports the concept that even modest lengthening of cold ischemia times may worsen outcome in kidney transplantation of HBD grafts [19]. In a recently published study, Debout et al. investigated the relationship between cold ischemia time and post transplant outcomes in 3839 adult recipients of a first heart-beating deceased donor kidney transplant between 2000 and 2011. The results demonstrated a significant increase in the risk of graft failure and mortality for each additional hour of cold ischemia [20].

Awareness of detrimental effects of hypothermia has resulted in the exploration of alternative storage techniques. A novel approach to solid organ preservation is normothermic *ex vivo* machine perfusion with avoidance of prolonged cold storage. Cypel and colleagues have demonstrated favorable outcomes in experimental models and clinical practice using normothermic *ex vivo* lung perfusion. Others noted similar benefits using subnormothermic *ex vivo* liver perfusion in experimental models [5,22]. In renal transplantation, Brasile, Stubenitsky, Kootstra et al. investigated acellular, low pulsatile pressure perfusion at subnormothermic temperatures of 32 °C demonstrating promising results in canine models in the early 2000s [14,15,17]. Recently, Hosgood and colleagues demonstrated improved outcomes for ECD kidney grafts by an additional application of one hour of normothermic *ex vivo* kidney perfusion prior to transplantation in clinical practice [15].

We report here a novel technique of continuous, pressure-controlled, erythrocyte-based normothermic *ex vivo* kidney perfusion (NEVKP). Our ultimate goal is to entirely replace cold storage with NEVKP as the preservation method. The aim of our study was to determine feasibility and safety of replacing cold storage with normothermic *ex vivo* kidney perfusion in a cattle model of SCD HBD kidney transplantation.

MATERIALS AND METHODS:

Study Design

Heart-beating donor kidney retrieval was performed and the grafts were either stored for 8 h in cold histidine-tryptophan-ketoglutarate (HTK) solution, or preserved using 8 h of NEVKP (n=5 in each group). Then, kidney autotransplantation was performed in both groups with 10 d of follow-up. Perfusion characteristics, graft injury, and graft function after transplantation were determined.

Normothermic *Ex Vivo* Kidney Perfusion

A neonatal cardiopulmonary bypass circuit was used for the *ex vivo* kidney perfusion at 37°C. The customized perfusion circuit consisted of a venous reservoir and an oxygenator (D100 neonatal venous reservoir and oxygenator; arterial filter (D130 neonatal arterial filter; Sorin Group Inc.), P.h.i.s.i.o coated PVC tubing (Sorin Group Inc.), and a customized double-walled organ chamber. The centrifugal pump of an S3 heart-lung- machine (Sorin Group Inc.) was used to perfuse the oxygenated perfusion solution through the circuit and the kidney graft. Perfusion circuit parameters such as temperature, arterial and venous pressure, and arterial flow were recorded continuously with the Data Management System (Sorin Group Inc.). Bubble and reservoir level sensors were used to prevent air embolism (**Figure 1**).

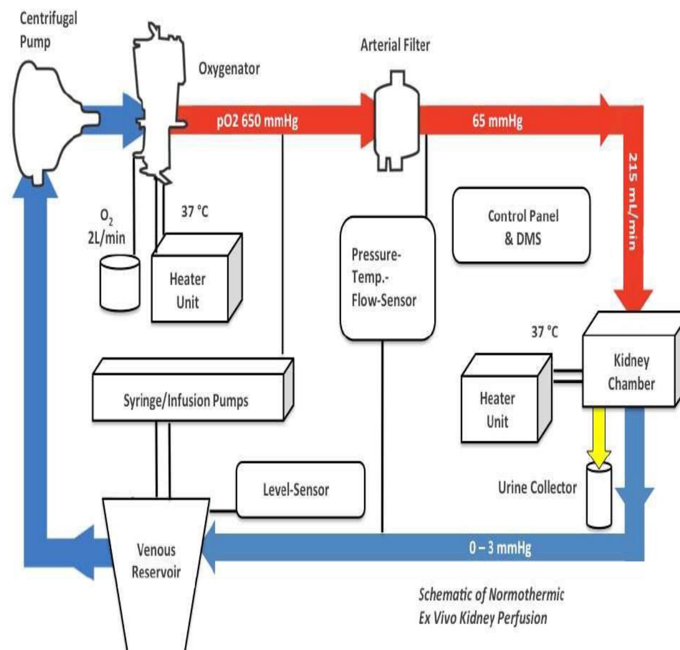

Figure 1: Schematic of the NEVKP Circuit. The circuit consists of neonatal cardiopulmonary bypass technology. The perfusion solution is collected in the venous reservoir. A centrifugal pump propels the solution into the oxygenator, where it is enriched with oxygen and warmed to 37°C. After passing the arterial filter, the perfusate is driven with a pressure of 65 mmHg through the renal artery into the graft located in the customized double-walled kidney chamber. The venous outflow (0 – 3 mmHg) leads the perfusate back into the venous reservoir. Syringe and infusion pumps secure the supply with additional compounds. The urine is collected throughout the perfusion. Control panel and Data Management System (DMS) indicate and record perfusion parameters continuously.

Prior to graft retrieval, whole blood was collected from a separate donor animal and passed through a leukocyte filter. Following centrifugation, the isolated erythrocytes were washed in sterile saline solution to avoid contamination with leukocytes or plasma. Heart-lung machine and perfusion circuit were prepared and primed with a physiologic perfusion solution just prior to initiation of the retrieval procedure. The composition of the perfusion solution is displayed in **Table 5.1**. It consisted of leukocyte-depleted washed erythrocytes (125 mL), mixed with STEEN

Composition of the perfusate solution

Ingredient	Amount / Rate
Stock solution	
Ringer's lactate	200 mL
STEEN solution™	150 mL
Erythrocytes (leukocyte-depleted)	125 mL Double reverse osmosis (DRO) filtered
water	27 mL Sodium bicarbonate 8 mL
Calcium gluconate	1.8 mL
Heparin	1000 IU
Continuous administration	
Ringer's lactate	Replacement of produced urine and evaporation
Amino acids and glucose (intravenous)	Target glucose concentration: 5 – 15 mmol/L
Insulin (intravenous)	5 IE/h
Verapamil (intraarterial)	0.25 mg/h

Table 5.1: Ingredients in perfusate solution and amount or rate administered.

pH	7.46 ± 0.06	7.37 ± 0.02
pCO_2	43.5 ± 7.3 mmHg	36.1 ± 5.5 mmHg
pO_2	47.5 ± 7.3 mmHg	633 ± 21 mmHg
HCO_3^-	30.3 ± 2.4 mmol/L	20.2 ± 2.8 mmol/L
Hb	104 ± 10 g/L	105 ± 14 g/L
O_2 Sat	-	99.9 %
Na^+	137 ± 3.9 mmol/L	142 ± 0.8 mmol/L
K^+	3.9 ± 0.5 mmol/L	3.5 ± 0.1 mmol/L
Ca^{2+}	1.25 ± 0.10 mmol/L	1.36 ± 0.15 mmol/L

Table 5.2: Blood gas analysis, osmolarity, and oncotic pressure measured at baseline in Yorkshire cattle and at the start of normothermic *ex vivo* kidney perfusion.

Cl^- Glucose	101 ± 1.9 mmol/L	108 ± 2 mmol/L
Lactate	4.7 ± 2.5 mmol/L	4 ± 0.4 mmol/L
	0.94 ± 0.19 mmol/L	10.38 ± 0.76 mmol/L
Osmolarity	282 ± 2 mosmol/L (n = 6)	286 ± 4 mosmol/L (n = 5)
Oncotic pressure	14 ± 0.8 mmHg (n = 6)	11 ± 0.9 mmHg (n = 5)

In the NEVKP group, kidney grafts were retrieved and flushed with 300-500 mL Ringer's lactate solution. Meanwhile, arterial (1.6", Sorin Group Inc.) and venous cannulas (1/4" x 1/8", Sorin Group Inc.) were fixed with 2-0 silk ties (Covidien, Mississauga). Immediately after the flush, grafts were connected to the perfusion circuit for preservation under normothermic conditions. Perfusion was started with a mean arterial pressure of 70 mmHg; following graft adaption to the system, a physiologic arterial pressure of 65 mmHg was targeted (**Figure 2**).

After the first hour of perfusion, the pressure was stable without the need to adapt the speed of the centrifugal pump. The urine was continuously replaced with Ringer's lactate (**Table 5.1**). Blood gas parameters, concentration of lactate, and potential cell injury markers aspartate aminotransferase (AST) [207] and lactate dehydrogenase (LDH) [208] were measured in the perfusate hourly
Perfusate samples were collected hourly and frozen at -80°C after centrifugation for further assessment. Following NEVKP, the grafts were flushed with 4°C cold histidine-tryptophan- ketoglutarate (HTK; Metapharm Inc., Bratford) and kept cold for the duration of the vascular anastomosis. A detailed visualized description of the NEVKP technique has recently been published by our group .

Static Cold Storage
In the control group, kidney grafts were retrieved and flushed with 300-500 mL of 4 °C cold HTK solution with a pressure of 100 cmH2O. In a sterile organ bag (CardioMed Supplies Inc., Lindsa), grafts were submerged in preservation solution and placed on ice until autotransplantation.

Kidney Retrieval and Transplantation
A porcine model of heterotopic renal autotransplantation was chosen to further investigate the technique of normothermic *ex vivo* kidney perfusion. Anesthesia was administered as an intramuscular injection of ketamine. For administration of fluids and medication, a permanent venous catheter (9.5 French; Cook Medical Company, Bloomington, US) was placed into the right internal jugular vein using the Seldinger technique. After a midline incision, dissection of the right kidney and its adherent structures was performed, the renal artery and vein were clamped, and the graft was resected. Immediately, the renal artery was cannulated with a 1.6" cannula (Sorin Group Inc., Italy) and kidneys were either flushed with Ringer's lactate and placed on pump (NEVKP; study group), or flushed with 4 °C cold HTK solution and placed on ice (SCS; control group) for preservation, respectively. Following abdominal closure, the cattle recovered from surgery. After graft preservation time (8h), cattle were re-anaesthetized with propofol was administered every 8 hours following surgical procedure for at least 2 postoperative days. The decision to administer further

analgesia was based on clinical behavior of the cattle but was rarely necessary. Throughout retrieval and transplant procedure and recovery in the evening, animals received in total 3 L of intravenous fluids. During the initial postoperative course, about 200 mL of intravenous fluids were given in the morning and the evening when sampling the animals until full recovery. Animals were also permitted to drink ad libitum. A visualized description of the heterotopic renal autotransplantation technique has recently been published by our group (Kaths et al., The Journal of Visualized Experiments, in press).

Whole Blood, Serum, and Urine Measurements

Perfusate and whole blood were sampled for blood gas analysis (RAPIDPoint 500 Systems, Siemens AG, Berlin, Germany) hourly during NEVKP, or at baseline and each morning during follow up of the transplanted cattle, respectively. Serum samples were collected for analysis of AST and LDH (Vitros DT60 II, Johnson & Johnson, Markham) for baseline and each morning during follow up. 24h urine collection was performed using a metabolic cage to investigate the creatinine clearance before transplantation (day 0), and on postoperative day 10. Further serum and urine analyses were performed in the core lab using Abbott Architect Chemistry Analyzer using the manufacturer's reagents (Abbott Laboratories, Abbott Park, IL, USA).

Histology

Ten days after transplantation, the abdomen was opened under anesthesia, a wedge of renal tissue retrieved and placed into 10% neutral buffered formalin for histology and immunohistochemistry analyses. Fixed kidney tissue was paraffin-embedded, sectioned and stained. 3-µm periodic acid-Schiff (PAS) stained sections were used to score tubular injury, edema, fibrosis, and interstitial inflammation on a scale of 0 to 3 as previously described by us and others blinded to the experimental group. Histopathologic changes representing tubular injury including brush border loss, tubular dilatation, epithelial vacuolation, thinning and sloughing, and luminal debris were scored in 10 high power fields (HPF) and averaged to assess overall tubular injury [206 210 211]. Interstitial inflammation was scored in 10 low power fields and averaged. TUNEL staining was performed according to standard protocol. Because of the low rate of TUNEL-positive cells, the total number of positive cells were manually counted in 25 HPF and averaged.

Statistical Analysis

Statistical analysis was performed using SPSS software version 23.0 (IBM, Armonk, NY, USA). Differences in mortality were calculated with Fisher's exact test (2-sided). Variables were tested for normal distribution using Kolmogorov-Smirnov Test / Shapiro-Wilk Test. Students t-test was used to compare differences of continuous values between the two groups. A paired t-test was used to calculate differences between paired continuous values. To compare ordinal, non- parametric data the Mann Whitney U test was used. Significance was defined as $p < 0.05$.

Results

Demographics

The weights of the cattle were not different between both groups (NEVKP 30.4 ± 1.9 kg vs. SCS 32.7 ± 1.9 kg, p = 0.092). Preservation and anastomoses times in NEVKP groups vs. SCS group were similar with 452.4 ± 22.7 vs. 461.0 ± 20.7 minutes (p = 0.549) and 33.4 ± 3.8 vs. 36.2 ± 8.8 minutes (p = 0.533), respectively.

Perfusion Characteristics during NEVKP

Normothermic *ex vivo* kidney perfusion was initiated with an arterial pressure set to 70 mmHg. After rewarming of the graft, the pressure was adjusted to 65 mmHg throughout the whole perfusion; this is identical to the mean systemic pressure measured invasively in anesthetized healthy cattle (**Figure 2A**). Venous pressure was maintained at around 2 mmHg by height regulation of the venous reservoir (**Figure 2B**). An initial renal artery blood flow rate of 114 ± 18 mL/min was measured. After rewarming of the renal graft flow rates of around 200 mL/min were attained. The achieved flow rates were above the mean values of anesthetized healthy cattle, which were measured in situ using flow probes (**Figure 2C**) as described previously by our group. Intrarenal resistance (IRR) was calculated by dividing the arterial pressure by the arterial flow. Baseline IRR at the start of the NEVKP was 0.63 ± 0.1 (mmHg/mL/min) which decreased during NEVKP below the mean values that were measured in situ in 30 cattle.

Comparison between baseline IRR and IRR at last hour of perfusion demonstrated a significant decrease (p = 0.003) (**Figure 2D**). The urine output during NEVKP is displayed in **Figure 3**.

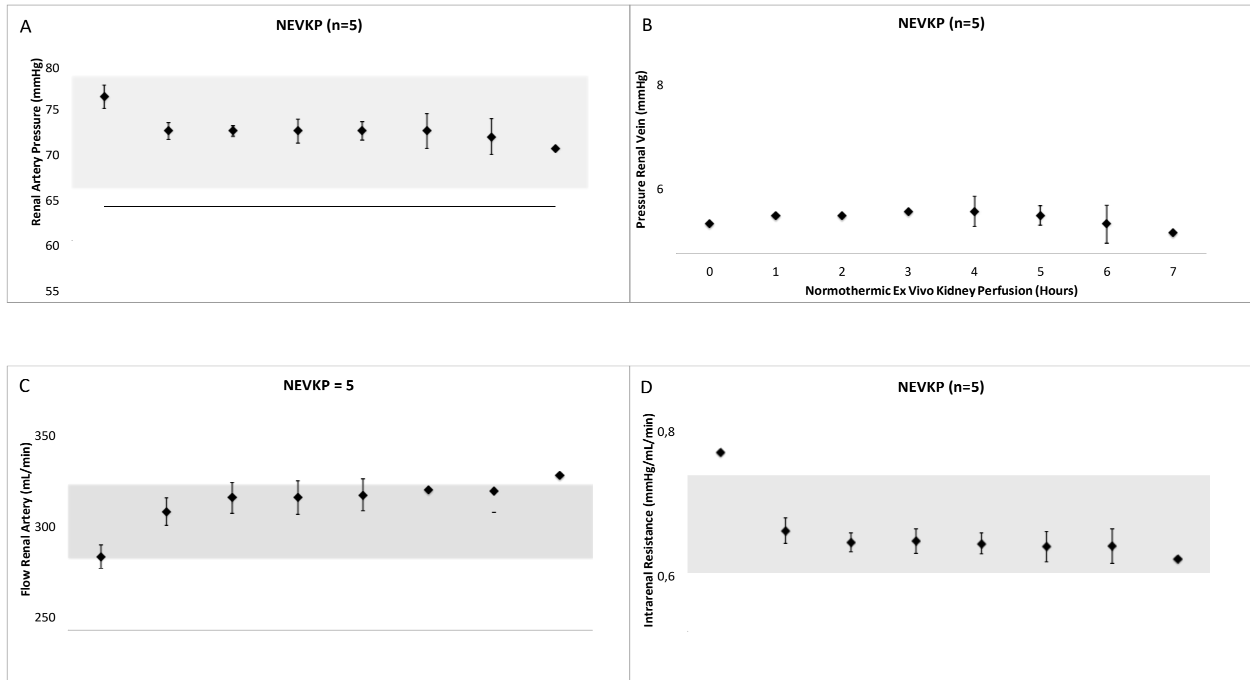

Figure 2A: Renal artery blood pressure during Normothermic *Ex Vivo* Kidney Perfusion. Values presented as mean ± SD in mmHg. Dashed line and grey area represent mean systemic blood pressure and SD measured invasively in situ in 30 anesthetized cattle by placing a catheter into the carotid artery. **Figure 5.2B: Pressure in the renal vein during Normothermic *Ex Vivo* Kidney Perfusion.** Values presented as mean ± SD in mmHg. **Figure 2C: Renal artery flow during Normothermic *Ex Vivo* Kidney Perfusion.** Values presented as mean ± SD in mL/min. Dashed line and grey area represent mean flow rate with SD measured in situ in 30 anesthetized cattle; upper and lower lines represent maximal and minimal renal artery flow rates in these cattle. The measurements were performed in control cattles following laparotomy and minimal dissection of the right renal artery with a flow probe. **Figure 2D: Intrarenal resistance during Normothermic *Ex Vivo* Kidney Perfusion.** Values presented as mean ± SD in mmHg/mL/min. Dashed line and grey area represent mean IRR with SD based on measurements performed in situ in 30 anesthetized cattles.

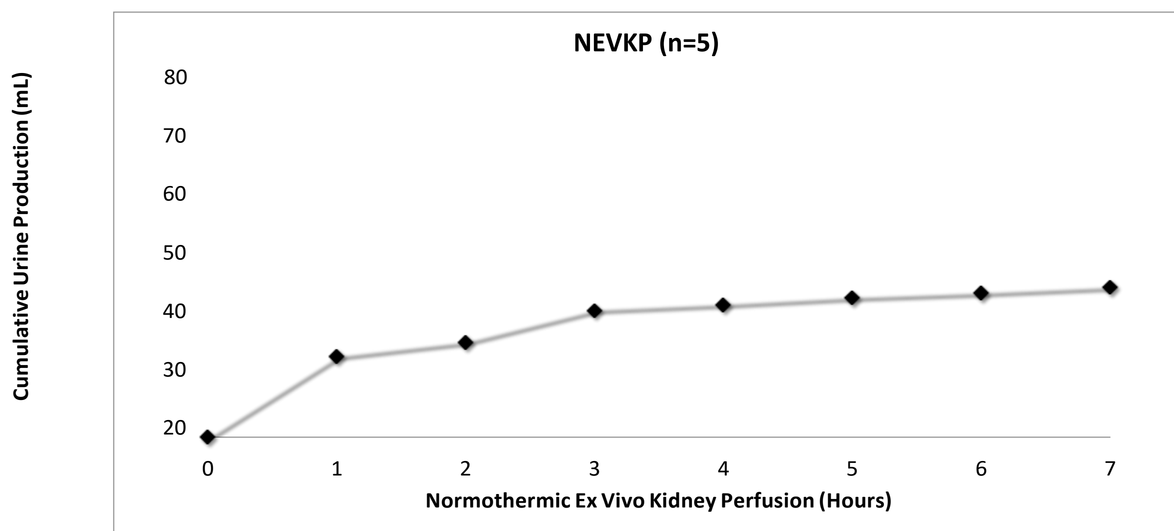

Figure 3: Cumulative urine output during Normothermic *Ex Vivo* Kidney Perfusion.
Values presented as mean ± SD in mL.

NEVKP was associated with maintenance of physiologic biochemical parameters in the perfusate
Blood gas analyses were performed at baseline (**Table 1**) and then hourly during normothermic *ex vivo* perfusion. Measures of acid-base homeostasis, including pH and bicarbonate concentration, remained stable during preservation time and were physiologic when compared to basal values observed in 20 healthy control cattle (**Figure 4A and 4B**).

Tissue injury during NEVKP was assessed by hourly measurement of AST and LDH levels in the perfusate. AST and LDH were below the detectable analyzer measurement limits of 4 U/L and 100 U/L, respectively. Lactate levels were measured in the perfusate as an indicator of graft injury during *ex vivo* perfusion. Perfusate lactate levels decreased from baseline until the last hour of NEVKP (10.38 ± 0.76 vs. 1.22 ± 0.26 mmol/L, p = 0.0001) (**Figure 5**).

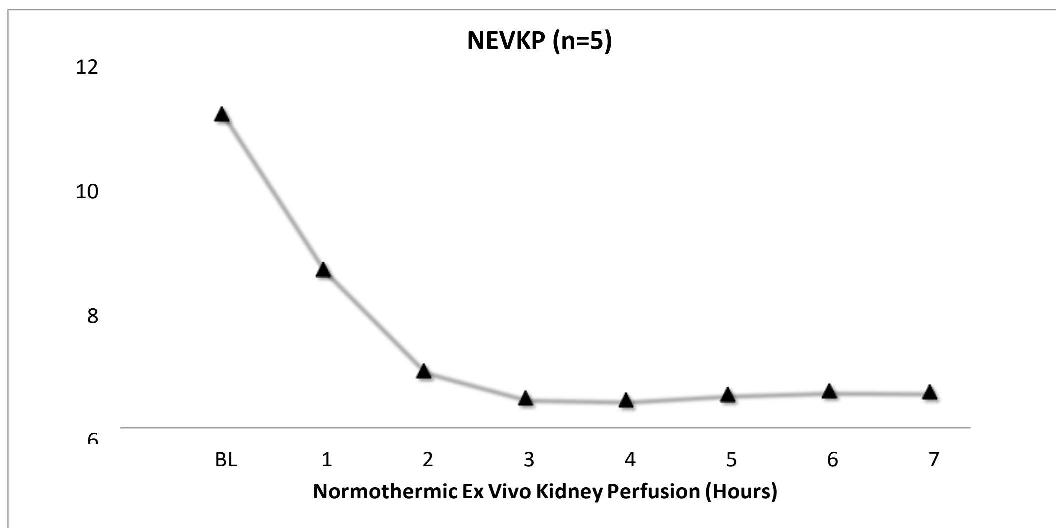

Figure 5: Lactate levels in renal perfusate during Normothermic *Ex Vivo* Kidney Perfusion. Values presented as mean ± SD in mmol/L.

NEVKP vs. SCS results in comparable graft function and injury after kidney transplantation
During the 10-day postoperative follow-up, daily comparison of serum creatinine and BUN demonstrated a trend to lower values in NEVKP perfused grafts compared to cold storage kidneys (**Figure 6A and 6B**). Peak creatinine and BUN levels in the NEVKP vs. SCS group after transplantation were 2.0 ± 0.5 mg/dL vs. 2.7 ± 0.7 mg/dL (p = 0.114) and 19 ± 3.5 mg/dL vs. 24 ± 6.7 mg/dL (p = 0.159), respectively. Interestingly, 10 days after transplantation, creatinine (1.1 ± 0.2 at day 10 vs. 1.0 ± 0.2 mg/dL at baseline; p = 0.49) and BUN (8 ± 1 at day 10 vs. 8 ± 3 mg/dL at baseline; p = 0.59) levels in the NEVKP group were comparable to their baseline values. In contrast, SCS preserved kidneys had significantly higher creatinine (1.5 ± 0.4 vs. 0.9 ± 0.1 mg/dL; p = 0.01) and BUN (10 ± 3 vs. 6 ± 1 mg/dL; p = 0.03) values on day 10 after transplant when compared to baseline. Serum potassium values during follow up and estimation of 24-hour creatinine clearance on postoperative day 10 were similar to baseline values (**Figure 6C and 6D**). No hematuria was observed.

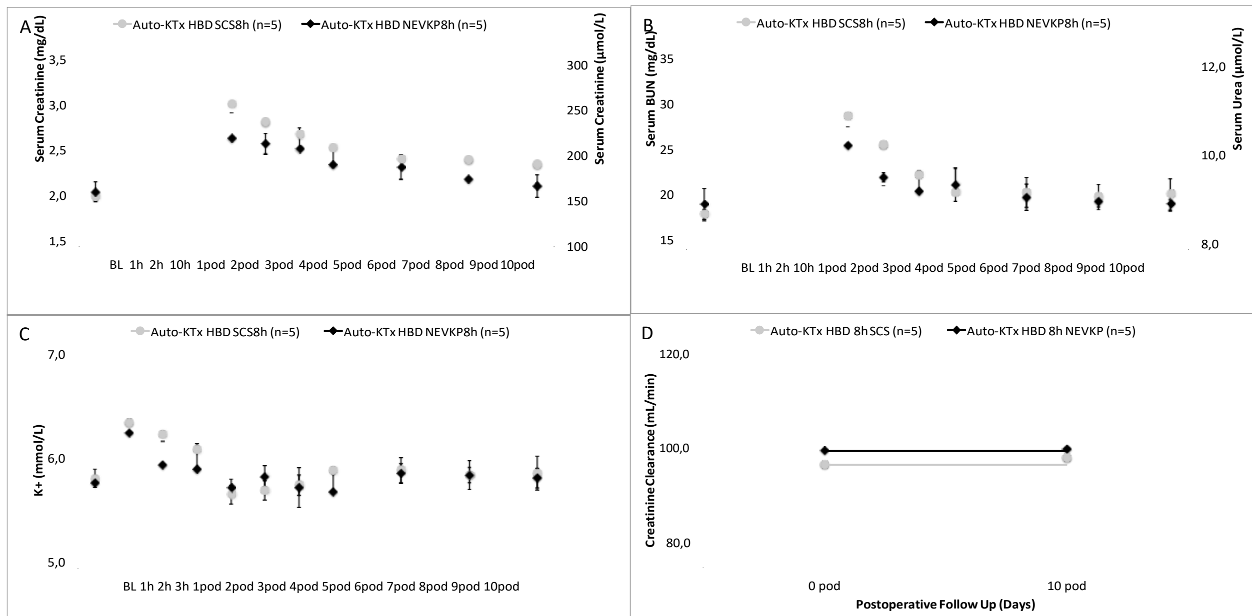

Figure 6A: Serum creatinine of the transplanted animals during 10 day postoperative follow-up for autologous kidney transplantation following SCS and NEVKP. Values presented as mean ± SD in mg/dL and µmol/L. **Figure 6B: Serum BUN/urea during 10 day postoperative follow-up for autologous kidney transplantation following SCS and NEVKP.** Values presented as mean ± SD in mg/dL and µmol/L. **Figure 6C: Serum potassium during 10 day postoperative follow-up for autologous kidney transplantation following SCS and NEVKP.** Values presented as mean ± SD in mmol/L. **Figure 6D: 24-hour creatinine clearance during 10 day postoperative follow-up.** Values presented as mean ± SD in mL/min.

Kidney histology was assessed 10 days after transplantation. Between NEVKP vs. SCS preserved kidneys, there were no significant differences for tubular injury (0.5 (0.5 – 1.5) vs. 0.5 (0 – 1.5), p = 0.690), interstitial inflammation (1.0 (0.5 – 2.0) vs. 1.0 (0.5 – 2.0), p = 0.841),
edema (0 (0 – 0) vs. 0 (0 – 1), p = 0.690), or fibrosis (0 (0 – 0) vs. 0 (0 – 1), p = 0.310) (**Table 5.3**). In all cases, the majority of glomeruli were mildly shrunken. TUNEL staining showed extremely low levels of apoptotic cells with no differences between NEVKP and SCS preserved kidneys (data not shown).

Table 5.3: Histological findings ten days after transplantation assessed by H&E / PAS staining. Biopsies were scored on a scale of 0-3 for tubular injury, inflammation, edema, and fibrosis. Data represent median (range) of 30 fields.

Histological Findings

	NEVKP	**SCS**	**p-value**
Tubular injury	0.5 (0.5 – 1.5)	0.5 (0 - 1.5)	0.690
Inflammation	1.0 (0.5 – 2.0)	1.0 (0.5 – 2.0)	0.841
Edema	0 (0 – 0)	0 (0 – 1.0)	0.690
Fibrosis	0 (0 – 0)	0 (0 – 1.0)	0.310

DISCUSSION:

This is the first demonstration that NEVKP can be performed for at least 8 hours in HBD grafts under physiologic conditions, resulting in excellent early outcomes after kidney transplantation. During NEVKP physiologic perfusion parameters were maintained, high *ex vivo* metabolic activity, low intrarenal resistance, and no markers of tissue injury were seen. Further assessment after renal graft autotransplantation demonstrated comparable kidney function for NEVKP when compared to cold storage control during 10 days follow up.

Our findings are in keeping with those of Brasile, Stubenitsky, and Kootstra who investigated *ex vivo* kidney perfusion using a canine model of subnormothermic (32°C), acellular, low pulsatile pressure (MAP 35 mmHg) perfusion in the early 2000s. Following application of 30 minutes of renal warm ischemia, grafts were stored statically on ice (4 °C), perfused at subnormothermic temperatures (32 °C), or subjected to a combination of both preservation techniques. Following renal autotransplantation, grafts preserved with subnormothermic perfusion demonstrated lower serum creatinine values, while grafts stored statically on ice had a significantly reduced renal function. Only prolonged rather than short subnormothermic perfusion following SCS was capable to fully recover renal function [14]. These findings demonstrated (1) the detrimental effects of cold ischemic preservation, (2) the option of replacing SCS continuously with perfusion at subnormothermic temperatures, and (3) the potential of prolonged subnormothermic perfusion to repair grafts following SCS. Some key differences exist between our experimental set-up and that of Brasile et al. We utilized healthy porcine HBD grafts in our study whereas Brasile used canine DCD kidneys. Furthermore, Brasile et al. used a different perfusion set up with an acellular, low pulsatile pressure perfusion at a subnormothermic temperature of 32 °C [14]. Despite these promising results, this system has never been reported to have been successfully translated to clinical trials for the preservation of human kidney grafts and subsequent transplantation.

Hosgood et al. previously investigated shorter periods of normothermic *ex vivo* kidney perfusion in a porcine model of renal autotransplantation. Kidneys were subjected to 30 minutes of warm ischemia and preserved with either 22 hours of hypothermic machine perfusion (HMP) or 20 hours of HMP followed by 2 hours of normothermic preservation (NP) using autologous blood. There was no significant difference in graft survival or kidney function, but lower levels of lipid peroxidation were measured in the NP group 60 min after transplantation. In a first clinical trial in 2013, *ex vivo* normothermic perfusion (EVNP) was investigated in ECD kidney grafts. Eighteen kidneys from extended criteria donors underwent one hour of EVNP immediately prior to transplantation. Comparing the outcome of these kidneys to 47 ECD kidneys that underwent SCS demonstrated a significant reduction in DGF (5.6% vs. 36.2%, respectively). Although Hosgood and Nicholson demonstrated benefits of short term *ex vivo* perfusion in marginal grafts, the effects of continuous perfusion and avoidance of cold storage were not assessed. In addition, possible negative effects of normothermic *ex vivo* perfusion on good quality HBD grafts were not investigated.

For renal grafts recovered from ECD or DCD the susceptibility for additional injury caused by hypothermia has been well described [93,96]. In addition, prolonged cold ischemia is also known to have detrimental effects on healthy HBD grafts. Delpech et al. demonstrated the detrimental impact of 24 hours cold storage in kidneys retrieved in a porcine heart-beating model. Three days, 7 days, and 3 month after autotransplantation, the creatinine clearance was still reduced and significantly lower when compared to baseline. Even kidneys in which the renal hilum was clamped in situ for 60 min without undergoing transplantation afterwards demonstrated better function and nearly returned to baseline after 3 month. As expected, the combination of 30 minutes of warm ischemia, 24 hours of cold storage, and autotransplantation demonstrated the lowest values of creatinine clearance.

In our study we demonstrated that NEVKP can replace hypothermic storage techniques and therefore offers the potential to avoid harmful effects of cold storage. We aimed to provide the most physiologic conditions for the renal graft during the *ex vivo* perfusion period. Thus, the arterial and venous pressures, the chosen temperature, and the composition of the perfusion solution were based on physiologic values obtained in healthy cattle (**Table 1 and 2, Figures 2**) [12,23] Leukocyte-depleted washed erythrocytes were used to provide an immunologically protected environment. No bicarbonate had to be given throughout the perfusion as the kidneys demonstrated physiologic function in keeping the acid-base hemostasis stable.

Verapamil was chosen for vasodilation as it allowed stable perfusions of 10 hours and more in our

recently conducted study [206]. Furthermore, the perioperative application of verapamil demonstrated improved renal graft function after transplantation [216] and reduced the incidence of acute tubular necrosis in a Cochrane Database systematic review [217]. Hourly, potential markers of renal graft injury (AST and LDH) were assessed [144 207,209] and demonstrated to be below analyzer range. The initial high values of lactate can be explained with the high content of Ringer's lactate in the perfusate solution. Biopsies were only taken at the end of the study period so as to avoid bleeding and to not compromise the animal outcome. In a former set of experiments, biopsies were taken immediately following 10 hours of NEVKP of good quality HBD grafts. These biopsy specimens demonstrated minimal changes and no necrosis [20].

Normothermic *ex vivo* kidney perfusion can offer several advantages in comparison to static cold storage or hypothermic machine perfusion. First, as described above, negative effects of cold storage can be avoided. Second, during the normothermic *ex vivo* perfusion period grafts are metabolically active and the function of the graft can be assessed. Third, graft repair strategies could be applied during the period of normothermic perfused preservation.

We chose a cattle model of heart-beating donation with a short preservation time to investigate the safety of continuous NEVKP. By keeping the storage time short and avoiding the deleterious effects of brain death we created a control group that would be expected to have excellent graft function, thus maximizing the probability that any potential deleterious effect of normothermic perfusion would be detected. The use of an autotransplantation model excluded rejection as a confounding factor and allowed us to evaluate exclusively the effects of storage and reperfusion. Clinical trials in the future will likely include acceptable kidney grafts for transplantation and demonstrating that the new technology is not detrimental to good quality grafts provides reassurance about the safety of this technique for future clinical trials. This information will also facilitate clinical trials of new therapeutic strategies such as stem cell therapy, gene transfer, or microRNA administration to modify grafts of which some are more likely to target standard criteria donor rather than ECD or DCD grafts.

Our study has several limitations. Our model did not include kidneys recovered in donation after brain death or kidneys with severe preservation injury. Thus, possible protection against deleterious effects of brain death or preservation injury was not investigated. In addition, in the absence of severe preservation injury, we did not assess mechanisms of graft injury such as cytokine release, infiltration of inflammatory cells, or ATP depletion. Furthermore, keeping the preservation time to less than 8 hours may be impossible in organ procurement regions where prolonged storage periods are sometimes required. To address the question of safety, we on purpose chose a short preservation time with minimal injury in the control group. This might explain that there was no significant difference in peak serum creatinine and BUN in between both groups. Future studies will focus on grafts with severe kidney injury, such as kidneys recovered after circulatory death.

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