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Lowering Perfusate Temperature From 37°C to 32°C Diminishes Function in a Porcine Model of Ex Vivo Kidney Perfusion

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Background. Ex vivo perfusion (EVP) is a novel method of preservation. However, optimal perfusion conditions remain undetermined. Reducing the temperature of the perfusate to subnormothermia may be beneficial during EVP and improve early graft function. The aim of this study was to investigate whether subnormothermia would influence the conditioning effect of EVP when compared with normothermic perfusion, and standard cold static storage (CS). **Methods.** Porcine kidneys underwent static CS for 23 hours followed by 1 hour of EVP using leukocyte-depleted blood at a mean temperature of 32°C or 37°C. After this, kidneys were reperfused with whole autologous blood at 37°C for 3 hours to assess renal function and injury. These were compared with a control group that underwent 24 hours CS. **Results.** During EVP, kidneys perfused at 37°C had a higher level of renal blood flow and oxygen consumption compared with EVP at 32°C ($P = 0.001$, 0.002). During reperfusion, 32°C EVP kidneys had lower creatinine clearance and urine output than control ($P = 0.023$, 0.011) and a higher fractional excretion of sodium, serum potassium, and serum aspartate transaminase than 37°C EVP kidneys ($P = 0.01$, 0.023, 0.009). **Conclusions.** Tubular and renal functions were better preserved by a near-physiological temperature of 37°C during 1 hour of EVP, when compared to EVP at 32°C or cold storage.

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The principle of therapeutic hypothermia—organ protection through induction and maintenance of a subnormal core temperature—has seen increasing clinical application. Considerable evidence exists for a neuroprotective effect after traumatic brain injury, neonatal hypoxic encephalopathy and major cardiac ischemia or operations[1]; accordingly, protocols for out-of-hospital cardiac arrest, some cardiac surgery, and traumatic brain injury now advocate the use of therapeutic hypothermia ranging from 32°C to 35°C.[2-4]

It is thought that hypothermia exacts a cytoprotective effect predominantly against secondary injury, through numerous pathways including: decreased oxidative metabolism and associated oxidative damage, attenuated proinflammatory mediator release and potency, reduced vascular permeability, and improved cellular integrity.[1] Because many of these protective mechanisms are highly conserved, it has been hypothesized that hypothermia may similarly prevent or abate renal injury. There further have been historical reports of hypothermia conditioning against ischemia, and to a lesser extent reperfusion, in a murine model.[5]

Furthermore, a randomized dual-center study recently documented a significantly lower incidence of delayed graft function in recipients receiving kidneys from deceased donors that had been cooled to 34°C to 35°C before retrieval.[6]

Our group has developed an ex vivo perfusion (EVP) circuit designed to assess, condition, and treat marginal organs to improve graft outcomes. The kidney is placed on a modified pediatric heart-lung bypass circuit where it is perfused at normal core body temperature with an oxygenated, autologous blood-based solution. This normothermic EVP circuit has been used to condition and assess[2,7] marginal kidneys and has the potential to allow targeted drug delivery to the isolated organ.

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Our EVP system has previously used a temperature of 37°C to 38°C, defined here as near-normothermia, based on favorable outcomes as compared to cold storage (CS)[8]; a comparable system operating at 32°C, defined here as subnormothermia, has been reported. Brasile et al[9,10] have used this subnormothermic perfusion with an acellular, oxygen-carrying perfusate to report cytoprotective gene induction and reduced reperfusion injury in a canine model of donation after cardiac death (DCD) organ retrieval.

The aim of this study was to investigate whether subnormothermia would influence the conditioning effect of EVP, when compared to near-normothermia and standard static CS.

MATERIALS AND METHODS

This study used a porcine model of controlled DCD procurement kidneys that underwent a fixed length of warm ischemia (WI) and CS before undergoing either further CS, normothermic (EVP 37°C) or subnormothermic (EVP 32°C) perfusion. They then underwent a period of reperfusion on the isolated organ perfusion system to assess function and injury.

Female Landrace pigs weighing approximately 50 kg were terminated under Schedule 1 of the Home Office Scientific Act (1986) regulations. After exsanguination and collection of blood into a sterile receiver containing 25 000 units of heparin the kidneys were exposed to 10 minutes of warm ischemia before being flushed with 500 ml of Soltran (hyperosmolar citrate solution, Baxter, UK) at 4°C. Kidneys were then stored on ice for either 23 or 24 hours in further Soltran preservation solution.

Kidneys were randomly assigned into 1 of 3 groups (n = 6 per group), control; 24 hours of static CS, normothermic; 23 hours of CS followed by 1 hour EVP at 37.0°C, or subnormothermic temperature; 23 hours of CS followed by 1 hour EVP at 32.0°C.

Ex Vivo Perfusion

The system was designed using pediatric cardiopulmonary bypass technology (Bioconsole 550, Medtronic, Watford, UK) as previously described.[11] The circuit is primed with a perfusate solution followed by autologous whole blood which was depleted of leukocytes using a white cell filter (LeukoGuard RS; Pall Medical, Portsmouth, UK) to give a total circulating volume of approximately 1 L. The perfusate was oxygenated with 95% O_2/5% CO_2 at 0.2 L/min. The renal artery, vein and ureter were cannulated with soft silastic catheters (Pennine, UK) and kidneys were flushed with 100 ml of Ringer lactate solution at 4°C immediately before perfusion to remove the high-potassium preservation solution. Kidneys were placed in the kidney chamber and perfused at a mean arterial pressure (MAP) of 75 mm Hg for 1 hour at a normothermic or subnormothermic temperature. Supplements, detailed in Table 1, were infused into the venous reservoir and arterial arm of the circuit to provide a protective environment.

Ex Vivo Reperfusion Model

The isolated organ perfusion system was also used to reperfuse the kidneys in both groups after the preservation period to assess renal function and injury. After 1 hour of EVP the kidneys were removed from the system and flushed with 100 ml of Ringer lactate solution at 4°C to remove the blood and cool the kidney.

The reperfusion model differs from the normothermic EVP in that whole autologous blood is used (containing all blood components) and with certain protective ingredients omitted (Table 1). The system has no ability to metabolically produce creatinine, therefore 1000 μmol creatinine (Sigma-Aldrich, Steinheim, Germany) was added to the circuit so that decrements in serum creatinine and creatinine clearance could be measured as markers of renal function. Kidneys were placed on the circuit and reperfused at a near-to-normal[12] MAP of 85 mm Hg for a period of 3 hours at 38°C. Renal blood flow (RBF) and MAP were recorded continuously and intrarenal resistance (IRR) calculated (MAP/RBF). Urine output was also measured during reperfusion and hourly serum and urine samples collected. Creatinine clearance ($[creatinine_{urine}] \times Vol_{urine}/[creatinine_{plasma}]$) and fractional excretion of sodium $[(Na_{urine} \times Vol_{urine})/(\text{glomerular filtration rate} \times Na_{plasma}) \times 100)]$ were calculated.

Blood gas analysis (Blood Gas Analyser; Rapidlab 248, Bayer Corp, East Walpole, MA) was used to record arterial and venous partial pressures of oxygen (P_aO_2 and P_vO_2 respectively) carbon dioxide (P_aCO_2) and acid-base homeostasis. Oxygen consumption [(P_aO_2 – venous P_vO_2) × flow rate/weight] was also calculated.

Cortical wedge biopsies, taken at 3 hours before the end of the reperfusion, were fixed in 10% formalin before sectioning and staining with hematoxylin and eosin (H&E). A blinded observer (TDA) then scored 10 fields per sample under light microscopy at 40× magnification for evidence of tubular damage, according to criteria outlined in Table 2.

TABLE 1.

Infusions and supplements added to the perfusion circuit during EVP and reperfusion

Infusion solution	Additives	Duration	Infusion rate
1000 mL Ringer lactate	0.116 g Creatinine (Sigma-Aldrich, Steinheim, Germany)	EVP and reperfusion	Priming solution
(Baxter Healthcare Ltd, UK)	5 g Mannitol (Sigma-Aldrich, Steinheim, Germany)		Further Ringer lactate titrated to rate of urine production to maintain circulating volume
	12 mL Sodium Bicarbonate 8.4%		
	Dexamethasone 3.3 mg/1 mL	EVP Only	
500 mL Synthamin 10%	100 IU Insulin	EVP and Reperfusion	20 mL/h
Amino Acid Infusion (Baxter Healthcare Ltd, UK)	15 mL 8.4% Sodium Bicarbonate		
1000 mL glucose 5%		EVP and Reperfusion	5 mL/h
100 mL 0.9% sodium chloride	Epoprostenol 10 μg	EVP Only	5 mL/h

TABLE 2.

Histological scoring system for assessing tubular injury in cortical wedge biopsies taken after 3 hours reperfusion

	Percentage of field affected			
Category	**0-24%**	**25-49%**	**50-74%**	**≥75%**
Epithelial flattening	0	1	2	3
Tubular dilatation				
Vacuolation				
Tubular debris				
Interstitial edema				
Cumulative score		0 - 15		

Statistical Analysis

Normally distributed data is presented as mean ± SD and nonparametric data presented as median (range). Levels of continuous variables such as RBF were plotted against time and the area under the curves (AUC) for individual perfusion experiments were calculated using Excel software (Microsoft, Reading, UK) and GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). Mean, AUC and raw values were compared using an unpaired *t* test with Welch correction (GraphPad Prism).

RESULTS

Ex Vivo Perfusion

The temperatures achieved were 37.1 ± 0.4°C in the normothermic group and 31.0 ± 0.6°C in the subnormothermic group.

The RBF was significantly higher and IRR lower during EVP 37°C compared with EVP 32°C (P <0.0001; Table 3, Figure 1). Oxygen consumption was also significantly higher in the 37°C group compared with the 32°C group. The amount of urine produced was numerically higher in the EVP 37°C kidneys (P = 0.074; Table 3).

Reperfusion

The AUC RBF was significantly higher and IRR significantly lower in the EVP 32°C kidneys compared with the control (P = 0.005, 0.002; Figures 1A and B, respectively). The level of oxygen consumption was also significantly higher at 3 hours in the EVP 32°C kidneys compared with the control (Control 20.8 ± 5.2, EVP 32°C 46.1 ± 15.8, EVP 37°C 42.6 ± 19.5 mL/min per gram; P = 0.029).

Renal function was significantly improved in the control and the EVP 37°C groups compared with the EVP 32°C. The control group produced significantly more urine and had a higher level of creatinine clearance compared to the EVP 32°C (P = 0.011; Figure 1D, Table 4), (total urine output; control 467 ± 223, EVP 32°C 168 ± 155 vs EVP 37°C 317 ± 104 mL; P = 0.023). The levels of serum creatinine were significantly lower in the EVP 37°C kidneys (P = 0.026; Figure 1C). The EVP 32°C kidneys had a lower level of tubular function with a significantly higher AUC fractional excretion of sodium compared to the EVP 37°C (P = 0.001; Figure 1E).

The EVP 37°C kidneys had a significantly lower level of potassium compared the control (P = 0.023; Figure 1F) and lower level of aspartate transaminase (AST) compared with the EVP 32°C (P = 0.009; Figure 1G). Serum levels of lactate dehydrogenase (LDH) were significantly higher in the control kidneys compared with the EVP 37°C (P = 0.001; Figure 1H).

Histology from 3-hour reperfusion biopsies demonstrated all groups sustained tubular damage, without significant different between cumulative scores in the control (mean, 7.36; SD, 2.17), EVP 32°C (mean, 7.71; SD, 1.98), and EVP 37°C (mean, 8.34; SD, 2.36) groups. However, there was significantly greater epithelial flattening in the EVP 32°C group versus Control (mean difference, –0.42; P = 0.0075; 1-way analysis of variance with Holm-Sidak post hoc test) and interstitial edema in the EVP 37°C group compared with EVP 32°C (mean difference, 0.75; P = 0.0002 as above).

DISCUSSION

In our model of uncontrolled DCD kidney reperfusion, results indicate that subnormothermia does not improve the conditioning effect of EVP upon kidneys subjected to 10 minutes warm and 23 hours cold ischemia, and indeed may be inferior to CS.

During subnormothermic EVP, kidneys were less well perfused with significantly higher vascular resistance and correspondingly lower RBF when compared to kidneys undergoing normothermic EVP. This may reflect the modulatory influence of temperature on vascular smooth muscle contractility.[13] The significant reduction in oxygen consumption seen in the subnormothermic group may represent a reduction in temperature-sensitive adenoside triphosphate–dependent ion transporter function, and therefore oxidative metabolism, a mechanism that leads to a well-documented reversible tubular dysfunction.[14-16] The globally high rates of urine output seen during EVP may represent a low oncotic pressure that results from dilution of the red-cell base with Ringer lactate.

During the reperfusion phase, kidneys from the subnormothermic group produced a smaller quantity of more dilute, natriuretic urine than kidneys from the normothermic group. These findings, coupled with higher indices of renal damage (AST and LDH), suggest that upon reperfusion, subnormothermic kidneys have a higher initial burden of

TABLE 3.

Perfusion parameters during EVP. RBF, IRR, oxygen consumption, and total urine output

Value	Subnormothermic	Normothermic	*P*
RBF, mL/min per 100 g	90 ± 23	246 ± 61	<0.0001
IRR, mm Hg/mL per min per 100 g	5.12 ± 0.47	2.31 ± 0.54	<0.0001
Oxygen consumption, mL/min per g	27.5 ± 3.9 53.7 ± 19.0	0.007	
Urine output (ml)	151 ± 57	245 ± 76 0.074	

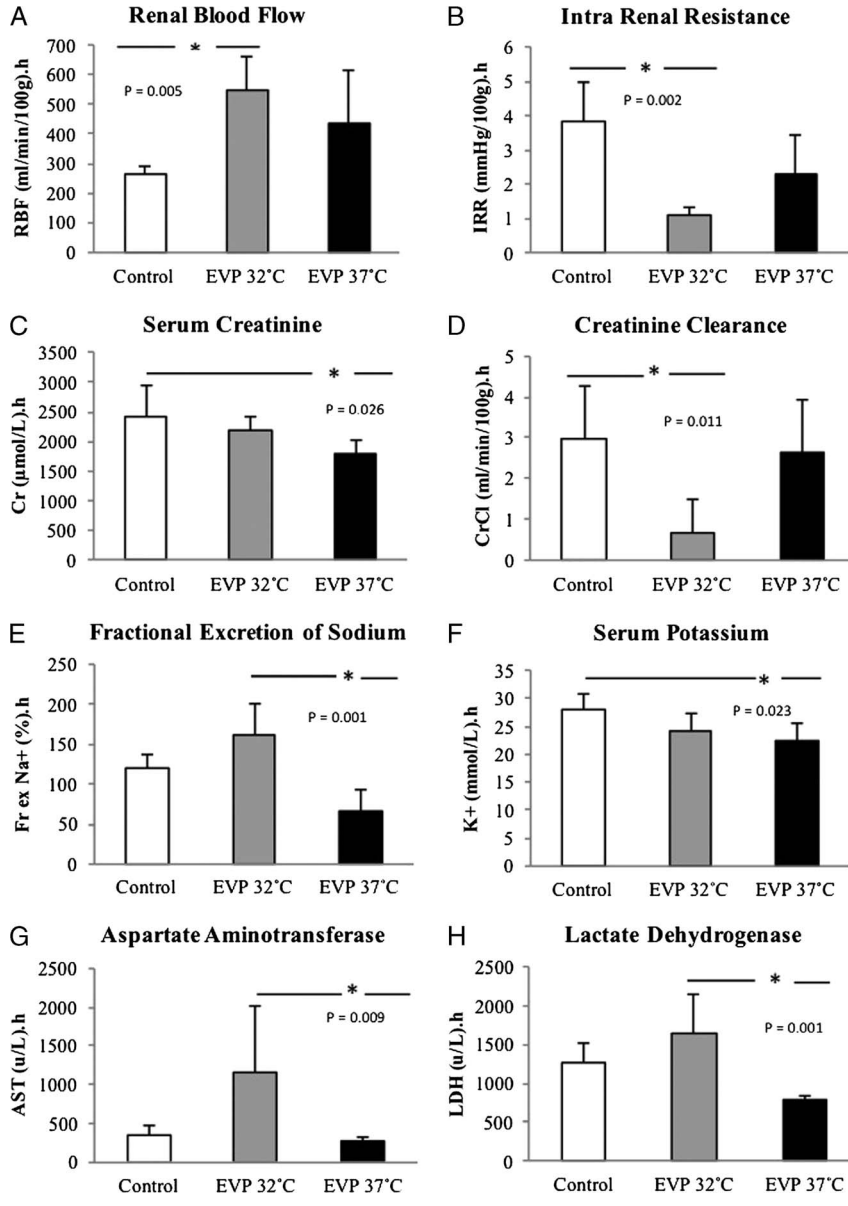

FIGURE 1. A-H, The AUC (A) RBF, (B) IRR, (C) serum creatinine, (D) creatinine clearance, (E) fractional excretion of sodium, (F) serum potassium, (G) aspartate aminotransferase and (H) LDH, during 3 hours of reperfusion in the Control, EVP 32°C and EVP 38°C groups.

tubular injury. After 3 hours of reperfusion, kidneys from all groups demonstrated histological evidence of tubular injury. Higher values for certain indices of injury in subnormothermia versus control appear to support the poorer creatinine clearance during reperfusion.

Our results would appear to reflect the heterogeneous clinical literature on whether transplant kidney injury can be ameliorated by hypothermia. Although Niemann et al[6] documented a significantly lower incidence of delayed graft function in recipients receiving kidneys from deceased donors that had been cooled to 34°C to 35°C before retrieval, a correlation between cooling and improvement in donor renal function was not conclusively shown.

Differences between our subnormothermic perfusion outcomes and those performed by Brasile et al[9,10] may represent methodological distinctions: whereas we have perfused porcine kidneys with an autologous red-cell-based perfusate, Brasile et al perfuse canine kidneys with an acellular fluid; the longer warm ischemic time favored by Brasile et al, is more analogous to an uncontrolled, rather than controlled, DCD retrieval. Despite differences, our studies merit comparison due to the paucity of data available on this subject.

Two further potential issues related solely to EVP are that if renal function and proinflammatory mediators are reduced during hypothermia, this may reduce the capacity of the system to deliver targeted drug therapies, and may theoretically allow propagation of infective organisms present in the donor kidney.

The advantage of this study is that our model allows us to interrogate single aspects of physiology in a controlled environment that has direct and current clinical application. A longer reperfusion phase may reveal molecular markers of kidney injury that would provide a mechanistic foundation for our findings. A limitation of our study is the absence of a group of kidneys undergoing hypothermic machine perfusion; without this we cannot definitively characterize the

TABLE 4.

Parameters during ex vivo reperfusion at 1 and 3 hours

	Control		Subnormothermic		Normothermic	
	1 h	3 h	1 h	3 h	1 h	3 h
CrCL, mL/min per 100 g	2.6 ± 1.3	0.8 ± 0.5	0.5 ± 0.8[a]	0.2 ± 0.2[b]	1.9 ± 1.3	1.4 ± 0.6
Fr Ex Na+, %	74 ± 15	55 ± 8	108 ± 19[b]	69 ± 20[b]	49 ± 40	31 ± 19
Oxygen Con, mL/min per g	21 ± 6	21 ± 5[a]	38 ± 14	46 ± 16	36 ± 13	43 ± 20
K+, mmol/L	9.9 ± 1.5[c]	10.4 ± 1.0	8.1 ± 0.8	8.2 ± 3.6	7.6 ± 1.5	7.9 ± 1.9
AST (mmol/L)	104 ± 17	191 ± 97	223 ± 258	823 ± 412[a]	90 ± 51	156 ± 50[b]
LDH, mmol/L	437 ± 83[c]	487 ± 101	442 ± 125[b]	833 ± 266[b]	276 ± 45	320 ± 20

Levels of creatinine clearance (CrCl), fractional excretion of sodium (Fr Ex Na+), oxygen consumption, potassium (K+), AST, LDH.

[a] Subnormothermic vs control (P <0.05).

[b] Subnormothermic versus normothermic (P <0.05).

[c] control versus normothermic (P < 0.05).

influence of temperature over perfusion itself. It would further be desirable to undertake autotransplant experiments to confirm our ex vivo measurements with those posttransplant.

In conclusion, this study provides evidence that subnormothermic EVP of DCD kidneys is inferior to a near-physiological normothermic perfusion, which better preserved tubular and renal function.

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