

Hypothermia – a Limiting Factor in Using Warm Ischemically Damaged Kidneys

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A study was performed to determine the limiting factors to expanding the donor pool with warm ischemically (WI) damaged kidneys. Canine kidneys were damaged by 30 min of WI, and then either cold stored (CS) in ViaSpan (4°C) for 18 h, or warm perfused with exsanguineous metabolic support (EMS) technology (32°C) for 18 h, or subjected to combinations of both techniques. The kidneys were autotransplanted with contralateral nephrectomy. In kidneys with WI and CS alone, the mean peak serum creatinine value was 6.3 mg/dL and took 14 days to normalize. In contrast, kidneys where renal metabolism was resuscitated *ex vivo* during 18 h of warm perfusion demonstrated mild elevations in the serum chemistries (2.6 mg/dL). The damage in kidneys CS for 18 h was ameliorated with 3 h of subsequent warm perfusion and eliminated by 18 h of warm perfusion. In contrast, reversing the order with CS following WI and 18 h of warm perfusion resulted in a time-dependent increase in damage. These results identify hypothermia as a major limiting factor to expanding indications for kidney donation. While hypothermia represents the foundation of preservation in the heart-beating donor, its use in WI damaged organs appears to represent a limiting factor.

Key words: Cold storage, exsanguineous metabolic support, renal ischemia

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Introduction

Society may soon be faced with the public healthcare issue of sustaining the costs associated with transplantation when relatively few patients will ever have the opportunity to be transplanted. The persistent organ shortage remains one of the major obstacles facing the field of clinical transplantation

today. The end-stage renal disease patient population being maintained with dialysis continues to grow, with the numbers of newly diagnosed patients each year exceeding healthcare group projections and doubling every decade (1–3). In contrast, there has been no comparable increase in the number of available allografts.

The disparity between the availability of kidney allografts and the number of patients in need of renal transplantation has led several groups to readdress the use of kidneys from non-heart-beating (NHB) donors. These efforts have largely focused on procurement in an intensive care unit (ICU) setting from patients who cannot be resuscitated, and has led to an increase in donors in the centers where this approach is practiced (4,5). The result has been the procurement of kidneys from NHB donors with relatively limited warm ischemic exposure, usually no more than 30 min prior to any form of intervention. The major barrier to more widespread use of these NHB donor kidneys is the high rates of delayed graft function and primary nonfunction.

In sharp contrast to this small ICU patient population, the largest number of potential NHB donors expire at the site of injury or in the ambulance and are almost never considered as organ donors. The warm ischemic insult encountered in this population has represented an insurmountable barrier to organ procurement. Despite the technical hurdles that must be addressed in using kidneys from NHB donors, the effective utilization of organs from NHB donors represents the best option for a near-term solution to the organ shortage.

A study was undertaken with the intention of defining the factors affecting the ability to recover function in kidneys with warm ischemic damage. Using a warm perfusion technology, that can reestablish cellular metabolism *ex vivo*, in varying combinations with traditional hypothermic preservation, we studied the impact of subsequent preservation on warm ischemically damaged kidneys.

Materials and Methods

Animals and surgical protocol

Autotransplantation experiments were performed on 2-year-old foxhounds weighing 20–30 kg. The animals received standard kennel food, routine lighting cycle and room temperature, and demonstrated normal renal function prior to the start of the study. All experiments were performed following the principles of laboratory animal care according to the NIH standards. Kidneys were exposed through a midline incision, and the left renal artery,

vein, and ureter were mobilized. If two renal arteries were present, the right kidney was used. Ten minutes prior to excision, 200mg/kg of mannitol was administered intravenously. The excised kidneys were exposed to 30min of warm ischemia (WI) while remaining in the abdominal cavity (37°C). Subsequent to the warm ischemic insult, the kidneys were divided into six groups, each with n = 4:

Group 1: 30min WI followed by 18h cold storage (CS) in Viaspan™ (4°C);

Group 2: 30min WI followed by 18h warm perfusion (32°C);

Group 3: 30min WI followed by 18h CS and then 3h warm perfusion;

Group 4: 30min WI followed by 18h CS and then 18h warm perfusion;

Group 5: 30min WI followed by 18h warm perfusion and then 12h CS;

Group 6: 30min WI followed by 18h warm perfusion and then 24h CS.

The kidneys requiring cold storage according to the experimental protocol, were flushed with 120mL of Viaspan™ (4°C) at 50mmHg. Reimplantation time varied between 20 and 30min, with a mean reanastomosis time of 27min. Fifteen minutes before reperfusion, mannitol (200mg/kg) and verapamil (0.1mg/kg) were administered. Contralateral nephrectomy was performed prior to reperfusion of the preserved kidney.

Post-transplant graft function

Blood samples for BUN and serum creatinine were taken each morning and analyzed using an ACE analyzer (Schiapparelli Biosystems, Inc., Fairfield, NJ, USA). For the purpose of grading the severity of the renal impairment the area under the curve (AUC) was calculated for the post-transplant serum creatinine curves above the normal range (<2.0mg/dL) (6). In addition to the AUC, the peak serum creatinine (Peak-Scr) and the day serum creatinine normalized (D-norm) were determined. Serum creatinine was considered normal once the curve fell below 2.0mg/dL. All values are reported as the mean along with the calculated standard error. For statistical analysis of differences in obtained data between the experimental groups the Student t-test was used.

Ex vivo warm perfusion

Exsanguineous metabolic support (EMS) technology (Breonics, Inc., Schenectady, NY, USA), was used for *ex vivo* warm perfusion (32°C). Following treatment as listed above for each group, the kidneys requiring warm perfusion according to the experimental protocol, were flushed (120mL) at 50mmHg and then placed on a pressure-controlled perfusion system. The perfusion system included an oxygenator and a pulsatile pump, retrofitted with controllers to maintain PaO₂, PaCO₂, pH and temperature. The renal artery was cannulated and connected to the perfusion circuit. The pulsatile perfusion pressure was targeted to 50/30mmHg and the circulating perfusate volume was 500mL. Both flush solution and perfusate consisted of a highly enriched tissue culture-like medium that con-

Table 1: Composition of basal EMS medium

DL-Alanine	0.12 g/L	Menadione (Na Bisulfate)	0.00003 g/L
L-Arginine HCl	0.14 g/L	Myo-Inositol	0.0001 g/L
DL-Aspartic Acid	0.12 g/L	Niacinamide	0.00005 g/L
L-Cysteine HCl H ₂ O	0.00022 g/L	Nicotinic Acid	0.00005 g/L
L-Cystine 2HCl	0.52 g/L	Para-Aminobenzoic Acid	0.0001 g/L
DL-Glutamic Acid	0.2672 g/L	D-Pantothenic Acid Ca	0.00002 g/L
L-Glutamine	0.20 g/L	Polyoxyethylenesorbitan Monooleate	0.04 g/L
Glycine	0.10 g/L	Pyridoxal HCl	0.00005 g/L
L-Histidine HCl H ₂ O	0.04376 g/L	Pyridoxine HCl	0.00005 g/L
L-Hydroxyproline	0.02 g/L	Retinol Acetate	0.00028 g/L
DL-Isoleucine	0.08 g/L	Riboflavin	0.00002 g/L
DL-Leucine	0.24 g/L	Ribose	0.001 g/L
L-Lysine HCl	0.14 g/L	Thiamine HCL	0.00002 g/L
DL-Methionine	0.06 g/L	Thymine	0.0006 g/L
DL-Phenylalanine	0.10 g/L	Uracil	0.0006 g/L
L-Proline	0.08 g/L	Xanthine HCl	0.00069 g/L
DL-Serine	0.10 g/L	Calcium Chloride 2H ₂ O	0.265 g/L
DL-Theonine	0.12 g/L	Ferric Nitrate 9H ₂ O	0.00144 g/L
DL-Tryptophan	0.04 g/L	Magnesium Sulfate (anhydrous)	1.2 g/L
L-Tyrosine 2Na	0.11532 g/L	Potassium Chloride	0.40 g/L
DL-Valine	0.10 g/L	Sodium Acetate (anhydrous)	0.10 g/L
Adenine Hemisulfate	0.02 g/L	Sodium Chloride	6.8 g/L
Adenosine Triphosphate 2Na 2Na	0.002 g/L	Sodium Phosphate Monobasic (anh)	0.224 g/L
Adenylic Acid	0.0004 g/L	D-Glucose	2.0 g/L
Alpha Tocopherol Phosphate 2Na	0.00002 g/L	Insulin	0.01 g/L
Ascorbic Acid	0.001 g/L	Bovine Serum Albumin	30 g/L
D-Biotin	0.00002 g/L	Sodium Bicarbonate	4.4 g/L
Calciferol	0.0002 g/L	Pyruvate	0.22 g/L
Cholesterol	0.0024 g/L	Transferin	0.10 g/L
Choline Chloride	0.001 g/L	Serum	10 mL
Deoxyribose	0.001 g/L	B-cyclodextrin	0.50 g/L
Folic Acid	0.00002 g/L	Chondroitin sulfate B	0.004 g/L
Glutathione (reduced)	0.0001 g/L	Fibroblast growth factor	0.02 g/L
Guanine HCL	0.0006 g/L	Heparin	0.18 g/L
Hypoxanthine	0.0006 g/L		

tained amino acids, carbohydrates, metabolites, inorganic ions, serum proteins, lipids, hormones, vitamins, reducing agents, a buffering system, trophic factors, radical scavengers, and adenine compound substrates adjusted to a pH of 7.4 (Table 1). The warm flush/perfusion solution is a proprietary technology of Breonics, Inc. The acellular perfusate was supplemented with pyridoxylated bovine hemoglobin (6g percentage) (Ezon, Inc., Piscataway, NJ, USA) to provide adequate oxygen (PaO₂ of 200mmHg) to support ongoing renal metabolism.

Histology

Biopsies of Group 1 and 2 kidneys were taken at 1 h post-reperfusion using a 15-gauge biopsy punch. The biopsies were fixed in 4% neutral-buffered formalin for histologic evaluation. Sections were made from each biopsy and stained with hematoxylin and eosin for light macroscopic evaluation. The morphologic characteristics of each kidney were determined by blinded histological evaluation.

Results

The AUC, a marker for the severity of the renal impairment; together with the Peak-Scr and the D-norm for each group are listed in Table 2.

Cold storage vs. warm perfusion

Figure 1: When kidneys were subjected to 30 min of WI followed by CS (Group 1) the mean peak serum creatinine

Table 2: Post-transplant graft function

	AUC	Peak- Scr (mg/dL)	D-norm (days)
Group 1	35	6.3	> 14
Group 2	2*	2.6*	5*
Group 3	18	4.9	13
Group 4	2*	2.8*	6*
Group 5	16	5	11
Group 6	43°	7.5°	> 14°

All values expressed as means; AUC = area under the curve; Peak-Scr = peak serum creatinine; D-norm = day serum creatinine normalized (<2.0 mg/dL); * = p < 0.05 compared to Group 1; ° = p < 0.05 as compared to Group 2.

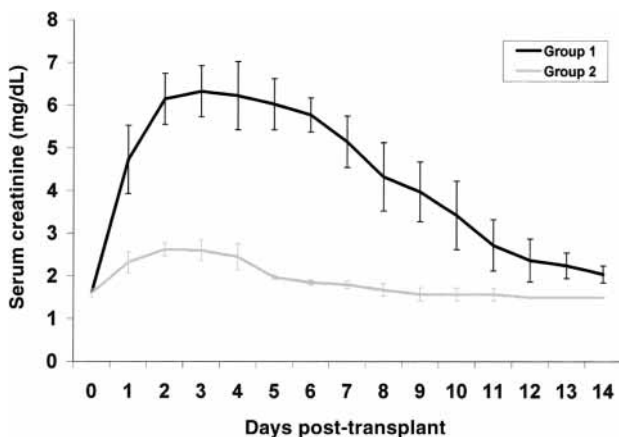


Figure 1: Mean serum creatinine concentration following renal transplantation of kidneys subjected to 30 min of warm ischemia followed by either 18 h of cold storage (Group 1) or 18 h of warm perfusion (Group 2).

was higher and the time to normalization of the serum chemistries longer than in kidneys that were instead warm perfused for 18h (Group 2). Group 1 dogs, demonstrated a mean peak serum creatinine value of 6.3mg/dL and took on average 14 days to normalize the serum chemistries. In contrast, Group 2 dogs demonstrated a mean peak serum creatinine value of 2.6mg/dL and the mean time to normalization of the serum chemistries was 5 days.

Amelioration of damage with warm perfusion

Figure 2: The damage observed following 30 min of WI with 18h of subsequent CS was ameliorated with 3h of subsequent warm perfusion (Group 3) and eliminated by 18h of warm perfusion (Group 4). The time-dependent benefit of EMS following combined warm and cold ischemia, led to a reduced mean peak serum creatinine and reduced the time to normalization of the serum chemistries by more than 50% (Table 2).

Detrimental effect of hypothermia

Figure 3: In contrast to the results above, CS following WI and 18h of warm perfusion resulted in a time-dependent increase in damage (Groups 5 and 6). The time-dependent damage that was associated with cold storage was demonstrated by both an increased mean peak serum creatinine and an increase in the number of days needed to normalize the serum chemistries (Table 2).

Histology

Biopsies taken at 1 h post-reperfusion from Group 1 and 2 kidneys highlighted a marked difference between kidneys reperfused from the cold and those reperfused following warm perfusion. Reperfusion directly from 18h of cold storage resulted in more tubular degeneration, with acute tubular epithelial damage observed in approximately 70% of the renal

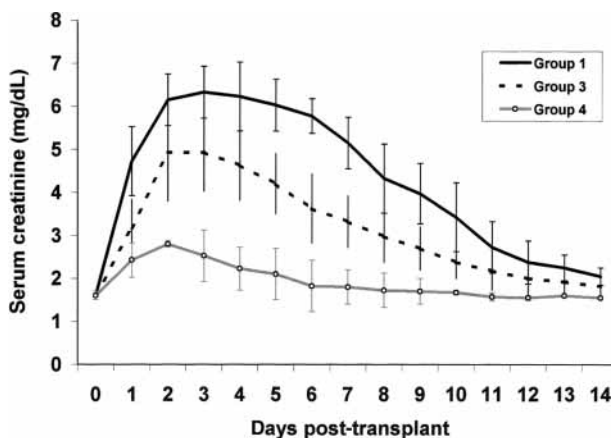


Figure 2: Mean serum creatinine concentration following renal transplantation of kidneys subjected to 30 min of warm ischemia followed by either 18h of cold storage (Group 1); 18h of cold storage and subsequent 3h of warm perfusion (Group 3); or 18h of cold storage and subsequent 18h of warm perfusion (Group 4).

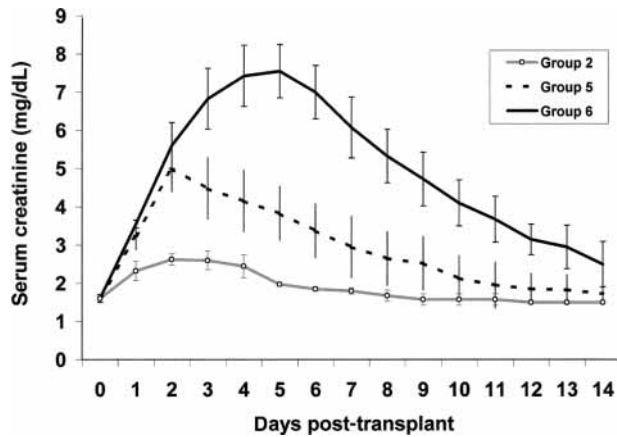


Figure 3: Mean serum creatinine concentration following renal transplantation of kidneys subjected to 30min of warm ischemia followed by either 18h of warm perfusion (Group 2); 18h of warm perfusion and subsequent 12h of cold storage (Group 5); or 18h of warm perfusion and subsequent 24h of cold storage (Group 6).

tubules. Renal tubules contained numerous intraepithelial neutrophils. Interstitial edema was pronounced with moderate numbers of neutrophil infiltrates. Likewise, neutrophil aggregates were evident within the glomerular tufts. Reperfusion directly from 18h of warm perfusion resulted in approximately 50% of the renal tubules having swollen epithelial cells. A small number of neutrophils were observed in the interstitium but were absent in the glomerular tufts. The results of the blinded histologic evaluations indicated that reperfusion of warm perfused kidneys resulted in less inflammatory cell components and the absence of neutrophil aggregates in the glomerular tufts.

Conclusion

The results of this study identify cold ischemia as the major obstacle to expanding indications for organ donation with warm ischemically damaged kidneys. Although only static storage with Viaspan was used for this study, previous work using this animal model has demonstrated the lack of significant differences in outcome when hypothermic machine perfusion with Belzer solution was used instead of static storage in Viaspan (7,8). While hypothermic technique represents the foundation of organ preservation in the heart-beating donor, its use in warm ischemically damaged organs appears to represent a major limiting factor.

One obvious explanation for this startling observation is that the relative degree of damage between warm and cold ischemia is in fact additive. In the early days of transplantation, using kidneys procured from NHB donors there was substantial warm ischemic insult during the period from the moment of death until the organs could be retrieved. The hypothermic preservation times were quite limited and there was a sense

of urgency to reimplant the kidneys in order to limit the preservation times, since preservation technology was in its infancy (9). Today in using kidneys from heart-beating donors we have the inverse situation. Warm ischemic times are now minimal and there is a prolonged hypothermic preservation time averaging 24h while organs are shipped to remote centers due to the organ shortage. The recent experience with the reintroduction of the NHB donor has necessitated combining the prolonging aspects from each era, substantial warm ischemia with prolonged hypothermic preservation. The effect of combining these two injuries is a much higher rate of delayed function, reported to range from 68 to 80%; a rate much higher than those reported for either the early years of transplantation using NHB donors or currently with heart-beating donors (10–14). As the period of cold ischemia becomes prolonged, there is a correlation with increased severity of the reperfusion injury, and at some point the hypothermic inhibition of metabolism becomes irreversible. Prolonged cold ischemia has been shown to lead to a lag-phase in the restoration of renal metabolism, with low initial rates of oxygen consumption (15).

A benefit of warm perfusion is that it ameliorates the reperfusion injury known to occur when a warm ischemically damaged and hypothermically stored organ is reperfused with blood (16). The acellular nature of EMS warm perfusion presents the opportunity to restore cellular integrity *ex vivo*, prior to contact with inflammatory components. It would likewise be expected that microvessel circulation would be superior, with less red cell sludging in warm kidneys than in kidneys undergoing the requisite lipid phase-change that occurs in cell membranes when a hypothermically stored kidney is reperfused (17). The results of 1-h biopsies support the amelioration of reperfusion injury in the EMS-perfused kidneys.

An additional benefit of warm perfusion, beyond simply ameliorating reperfusion injury, is the potential to substantially recover metabolism and function during *ex vivo* perfusion at temperatures approaching normothermia (32°C). It is logical to expect that a kidney with continued metabolic function, retention of normal barrier functions and restoration of cell-volume regulatory functions, maintained at near-normothermic temperature, would sustain less damage. The results of this study support this interpretation since longer periods of warm perfusion (3h vs. 18h of EMS perfusion) provided for enhanced recovery of renal function from both the warm and cold ischemic insults. Therefore, warm perfusion holds the potential to ameliorate reperfusion injury and recover function *ex vivo*, both functions being necessary to allow for effective prospective evaluation of damaged kidneys (18,19). These combined functions could support a more effective utilization of kidneys from NHB donors, and could have a positive effect on cost-containment issues by helping to reduce the period of delayed graft function.

The goal of this study was to address the obstacles to recovering renal function in warm ischemically damaged kidneys. Following warm ischemic insult, further inhibition of cel-

lular metabolism mediated by severe hypothermia appears to have a time-dependent, detrimental effect on subsequent graft function. In conclusion, the NHB donors represents the only near-term solution for making substantially increased numbers of kidney allografts available for transplantation. The results of this study suggest that hypothermia may be the major limiting factor to the effective utilization of NHB donor kidneys.

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