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NOS: The Underlying Mechanism Preserving Vascular Integrity and During *Ex Vivo* Warm Kidney Perfusion

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Research involving metabolically active and functioning organs, maintained *ex vivo* in culture-like conditions, could provide numerous opportunities for medical innovations and research. We report successful perfusion of isolated canine and human kidneys *ex vivo* at near physiologic temperature for 48 h. During the perfusions parameters of metabolism and function remained stable. Nitric oxide synthase (NOS) was identified as the underlying mechanism preserving vascular integrity. Most importantly, when the canine kidneys were reimplanted there was immediate normal renal function. This report highlights the potential significance of whole organ culture using a warm temperature *ex vivo* perfusion and discusses medical applications that could be developed.

Key words: Human kidneys, longterm warm perfusion, kidney transplantation, NOS, vascular integrity

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Introduction

Isolated organ perfusion

One of the first researchers to recognize the potential significance of being able to maintain intact organs was the Nobel Laureate, Alexis Carrel, at the turn of the 20th century. Using intermittent blood perfusion alternatively with saline, Carrel reported being able to maintain viability in organ blocks consisting of heart, lung, gut, pancreas and kidney (1). However, the organs within the block were never reimplanted to demonstrate subsequent life-sustaining function nor was there any histologic evidence or functional data to support viability.

Subsequent to Carrel many researchers have reported successful *ex vivo* warm perfusion for various periods using in most cases a blood perfusion, or less frequently cell-free perfusates (2–12). Successful *ex vivo* warm perfusion has been reported for various periods, usually a few hours in the case of kidneys and hearts and as much as 72 h for livers. A common feature in warm perfused kidneys has been a generalized loss of barrier functions of the vasculature leading to edema, which results in rising perfusion pressures and diminished vascular flow rates following several hours of *ex vivo* perfusion (13). However, none of these reports has demonstrated life-sustaining function following *ex vivo* warm perfusion of greater than 24 h by reimplanting the organ and demonstrating normal function (2–12). Currently, the only reliable means of maintaining organ viability once an organ is removed from the body is to suppress the metabolic rate via profound hypothermia (14–17).

New approach: exsanguineous metabolic support

Our hypothesis in developing organ culture based upon the acellular near-normothermic exsanguineous metabolic support (EMS) was that the retention of normal barrier functions of the blood vessel wall could be preserved and cellular metabolism could be adequately supported. If feasible, then it would be possible to maintain organ integrity and function during an *ex vivo* near-normothermic and acellular perfusion. Rather than employing a standard electrolyte solution supplemented with a few ingredients, as used in traditional isolated perfusion of kidneys, we instead utilized a cell culture perspective. This approach represented the coupling of organ perfusion technology with cell culture techniques to maintain an isolated whole organ. Nutrient delivery consisted of all the needed raw materials for synthesis, substrates for energy and ongoing metabolism and bulk inorganic ions for both catalytic and physiologic functions. Physiologic parameters such as temperature, pH, O_2-tension and perfusion pressures were kept within a narrow range. Therefore, the organ culture system is dynamic, and individual components are interactive in order to support adequate basal metabolism.

In previous EMS experiments we have reported successful maintenance of canine kidneys in a metabolically active condition at near physiologic temperature (32 °C). When these canine kidneys were reimplanted following 18 h of organ culture, they provided immediate normal renal function (18). The EMS organ culture has also previously been shown to support resuscitation of oxidative metabolism in kidneys damaged by 2 h of warm ischemia, and when transplanted provided for immediate life sustaining function (19). However, in order for this technology to become clinically relevant it is necessary that longer *ex vivo* periods be achieved.

We now report organ culture of human kidneys for up to 48 h at near-normothermic temperature with stable oxidative metabolism and perfusion characteristics. As the human kidneys could not be transplanted, viability following long-term *ex vivo* warm perfusion was evaluated in parallel canine transplant studies. The canine kidneys demonstrated stable rates of oxidative metabolism, perfusion characteristics and function comparable to the results observed with human kidneys. Normal renal function was observed when the 48-h warm perfused canine kidneys were reimplanted, demonstrating the preservation of renal viability during *ex vivo* warm perfusion. Furthermore, we present the initial evidence for the underlying mechanisms of vascular regulation during the period of organ culture.

Materials and Methods

Exsanguineous metabolic support (EMS) technology (Breonics Inc., Schenectady, NY) was used for *ex vivo* warm perfusion (32 °C); a temperature range previously shown to adequately support oxidative metabolism and cellular reparative processes (18–24). Both human and canine kidneys were placed on a perfusion system including an oxygenator and a pulsatile pump, retrofitted with controllers to maintain PAO_2, $PACO_2$, pH and temperature. The renal artery was cannulated and perfusion was initiated with a pulsatile perfusion pressure of 50/30 mmHg. The volume of the circulating medium was 500 mL for the canine and 750 mL for the human kidneys. The EMS medium consisted of an enriched tissue culture-like solution (Table 1). The acellular EMS medium was supplemented with pyridoxylated bovine hemoglobin (6-g percentage) (Ezon Inc., Piscataway, NJ) to obtain a target PAO_2 of 200 mmHg to support continued oxidative metabolism in the range previously shown to correlate with adequate (nondamaging) oxygen delivery (20). In order to minimize the risk of microbial infection and to avoid changes in osmolarity, the urine produced during the period of warm perfusion was recirculated. The circulating medium was exchanged (10%) every 4 h. PAO_2 analysis of prerenal and postrenal samples was performed using an ABL5 blood gas analyzer (Radiometer Medical A/S, Copenhagen, Denmark). O_2-consumption (mL/min/g) was calculated using the following formula:

$$[(PAO_2\ \text{Art} \times \text{percentage saturation}) - (PAO_2\ \text{Ven} \times \text{percentage saturation}) \times \text{flow}]/\text{weight}$$

Biopsies were not taken before initiating perfusion because small specimens would not be representative of the whole kidney and large biopsies would alter the subsequent perfusion characteristics. Instead postperfusion biopsies were taken from the human kidneys for histologic evaluation.

Hypothermic perfusion and cold storage

Control kidneys in groups 3 and 4 were flushed and placed into a MOX-100 perfusion machine (Water's instruments, MN) with 500 mL of Belzer's Machine Perfusate used as a circulating solution. After the kidneys were connected to the perfusion system, perfusion was set to a systolic pressure of 50 mmHg. After 1 h of machine perfusion, if needed, the systolic pressure was readjusted to 50 mmHg. Flow and pressure were continuously monitored.

Control kidneys in groups 5 and 6 were flushed and submerged in cold ViaSpan™. The kidneys were then triple-bagged and packed in ice until reimplantation.

Human kidneys

Human kidneys were procured from heartbeating donors with negligible warm ischemic time for the purpose of transplantation, and were later determined to be nontransplantable by institutional criteria that included the presence of a tumor on the paired kidney, atherosclerotic plaque, age of the donor, and the period of cold ischemia. The kidneys were stored in ViaSpan at 4 °C (mean cold storage time of 38 h) before their release for research use. The kidneys were weighed and the renal artery of each kidney was cannulated. The kidneys were flushed with approximately 250 cc of organ culture medium warmed to 32 °C to remove the hypothermic solution from within the vasculature. During the organ culture at 32 °C, the kidneys were evaluated for oxidative metabolism, vascular dynamics and organ function. The organ parameters were tested hourly. Human kidneys were perfused for 12 h (n = 2), 24 h (n = 2) or 48 h (n = 2).

Animals and surgical protocol

The autotransplantation experiments were performed on foxhounds weighing 20–30 kg. The animals demonstrated normal renal function before 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. Kidneys were then divided into six groups:

1) Group 1 (n = 6): 24-h organ culture (32 °C) and reimplantation

2) Group 2 (n = 3): 48-h organ culture (32 °C) and reimplantation

3) Group 3 (n = 3): 24-h hypothermic perfusion (4 °C) and reimplantation

4) Group 4 (n = 3): 48-h hypothermic perfusion (4 °C) and reimplantation

5) Group 5 (n = 3): 24-h cold storage (4 °C) and reimplantation

6) Group 6 (n = 3): 48-h cold storage (4 °C) and reimplantation

The mean anastomosis time was 28 min and ranged from 22 to 32 min. Contralateral nephrectomy was performed before reperfusion of the preserved kidney. The contralateral kidneys were used in the nitric oxide synthase (NOS) blocking studies. NOS was not inhibited (control; n = 3), inhibited by L-NAME (100 μM; n = 3); inhibited by iNOS (inducible NOS) specific with dexamethasone (15 μM; n = 3), or inhibited by iNOS specific L-NIL (100 μM; n = 3). Values are expressed as the mean with standard deviation for each experimental group. The concentration of nitrate in the EMS medium was determined by analysis on a Dionex DX-500 ion chromatograph using an AS-15 column and KOH eluant that varied in concentration by a programmed gradient. All samples were analyzed in duplicate sets diluted by a factor of 11. The concentrations of nitrate in the various EMS media specimens were within the standard calibration range.

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). Serum creatinine was considered normal with values below 2.0 mg/dL.

Results

EMS organ culture of human kidneys

Human kidneys were placed in organ culture at 32 °C and resulting metabolism and function were evaluated (Figure 1). Evaluation of the kidneys at 1, 4, 8, 16, 24, 36

Table 1: Composition of basal exsanguineous metabolic support 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_2O	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 Monoolate	0.04 g/L
Glycine	0.10 g/L	Pyridoxal HCl	0.00005 g/L
L-Histidine HCl H_2O	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 $2H_2O$	0.265 g/L
DL-Theonine	0.12 g/L	Ferric Nitrate $9H_2O$	0.00144 g/L
DL-Tryptophan	0.04 g/L	Magnesium Sulfate (anhydrous)	1.2 g/L
L-Tyrosine 2Na	0.11532 g/L	Potasium 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		

and 48 h demonstrated that human kidneys could be maintained stably *ex vivo* in the acellular environment of organ culture. During the culture period, O_2-consumption remained stable. Similarly, perfusion pressures and vascular flow rates remained stable throughout the experiment. Urine flow was in all cases reestablished during organ culture, and in every human kidney the ending diuresis increased over the initial rate. The histologic findings on the human kidneys post-EMS perfusion were similar with no significant abnormalities including no significant hypercellularity, necrosis, karyorrhexis or crescent formation. There was also no evidence of destructive hyalinosis or vasculitis in the renal vessels. These results demonstrate the feasibility of maintaining isolated human kidneys in organ culture for 48 h.

Transplantation following EMS organ culture

To demonstrate the results obtained with the culture of human kidneys represented preservation of renal cellular and functional integrity, a canine kidney autotransplant model was used (Figure 2). Group 1 and 2 kidneys were placed in organ culture for 24 or 48 h at 32 °C. All kidneys reperfused well upon reimplantation, with restored urine flow within minutes of reperfusion. All dogs survived with

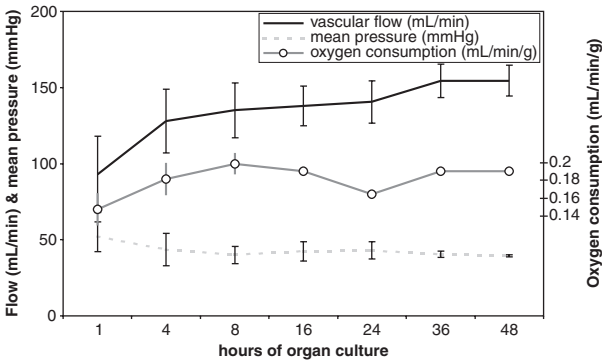

Figure 1: Perfusion characteristic of the human kidneys (n = 6) following upto 48 h of whole organ culture.

normal serum chemistries, normal urine and histologically normal kidneys. The dogs transplanted with Group 3 and 4 kidneys that were hypothermically perfused (4 °C) with Belzer's machine perfusate and Group 5 and 6 that were cold stored in ViaSpan also survived with normal serum chemistries. However, in these control dogs the peak serum creatinine values were higher than in the warm perfused test kidneys ($p < 0.5$). These results demonstrate the feasibility of maintaining intact isolated organs in a metabolically active state in the cell culture-like organ system.

Underlying mechanism preserving vascular integrity

Nitric oxide (NO) plays a fundamental role in hemodynamics. During organ culture, retention of the normal barrier functions of the blood vessel wall has been previously shown to represent the essential component that prevents deterioration in the vascular dynamics (19) (Figure 3). During the culture of kidneys, the perfusion pressures and vascular flow rates remain stable and the organs do not become edematous (<15% net weight gain). We evaluated if NOS played a regulatory role during organ culture.

One of the stable by-products of NOS isoforms, NO_3^-, was found to accumulate in the medium over the course of the culture period, suggesting the continued flux of NO during the organ culture. Blocking the induction of NOS by adding L-NAME (N^6–nitro-L-arginine methyl ester; 100 μM) at the start of the organ culture led to the development of interstitial edema with a mean net weight gain of 350% and significant increases in perfusion pressures. In contrast to the control kidneys where NO_3^- was found to accumulate in the culture medium, the addition of L-NAME significantly inhibited NO_3^- accumulation.

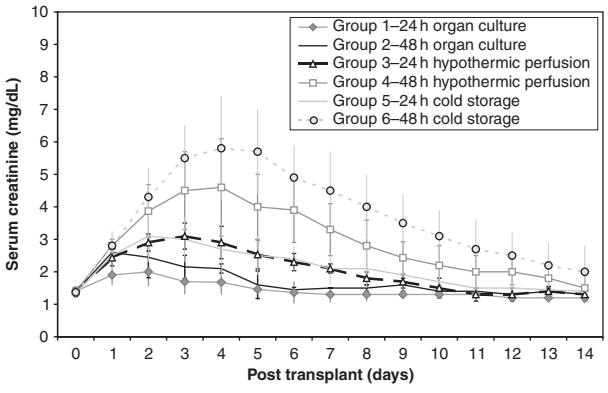

Figure 2: Serum creatinine concentration following renal transplantation of kidneys subjected to 24 h (group 1) and 48 h (group 2) of whole organ culture, and control kidneys that were hypothermically perfused for 24 h (group 3) and 48 h (group 4) and simply cold stored for 24 h (group 5) and 48 h (group 6). Values are expressed as the mean with standard deviation for each experimental group.

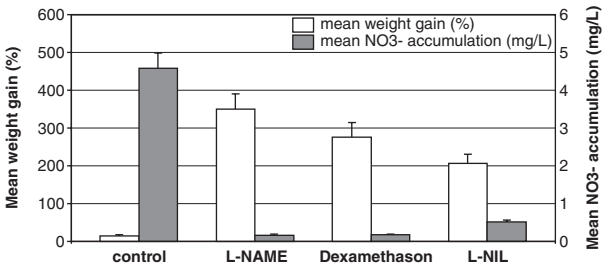

Figure 3: Mean net weight gain of kidneys and mean NO3- accumulation in the perfusate seen following 24 h of whole organ culture. Nitric oxide synthase was not inhibited (control; n = 3), inhibited by L-NAME (100 μM; n = 3); inhibited by iNOS-specific dexamethason (15 μM; n = 3), or inhibited by iNOS specific L-NIL (100 μM; n = 3).

Additional studies using the iNOS specific inhibitors L-NIL [L-N – (1-iminoethyl lysine hydrochloride; 100 μM)] and dexamethasone, which has been shown to suppress iNOS gene expression by interring with the binding of the essential transcription factors, such as nuclear factor, NF-?B (25), confirmed a central role for NOS during the culture of intact organs. Similar to the results using L-NAME, dexamethasone and L-NIL blockade of iNOS also resulted in marked edema (276% and 206%, respectively). In contrast to L-NAME, introduction at the start of the culture period did not affect perfusion pressures or vascular flow rates.

The absence of edema along with the mean NO_3^- accumulation of 4.6 ± 0.4 mg/L in the control kidneys following 24 h of organ culture was in contrast to the test kidneys where the L-NAME, dexamethasone and L-NIL groups were edematous and NO_3^- accumulation was lower (0.16 ± 0.1 mg/L; 0.18 ± 0.1 mg/L; and 0.51 ± 0.2 mg/L, respectively, $p < 0.05$). These results suggest a central role for NOS in the preservation of vascular integrity during organ culture.

Discussion

These results demonstrate that it is feasible to maintain intact human kidneys during an acellular perfusion with a cell culture-like medium administered via the vascular bed for 48 h. While the kidneys were maintained *ex vivo* at 32 °C, stable metabolism, vascular perfusion and organ function were observed. Moreover, when canine kidneys that were similarly warm perfused for 48 h with comparable metabolic rates, perfusion characteristics and function were reimplanted, the kidneys provided immediate life-sustaining function.

The canine kidney model represents a long-established methodology for developing and testing organ preservation technologies. The canine kidney was effectively utilized for

the development of all the perfusate solutions used clinically, including ViaSpan™, Belzer Machine Perfusate, Collins, Eurocollins, HSA-based, cryoprecipitated plasma, and Sacks solutions (26–36). Likewise, the canine kidney model has also been instrumental in the development of supporting preservation technologies such as perfusion preservation, static storage and in establishing their relative efficacy (37–39). The canine kidney model has also been employed to assess immunologic complications and injury following preservation, potential pharmacologic interventions and substrate requirements (40–48).

Most importantly, the various organ preservation technologies developed using the canine kidney model were, in every case, successfully transferred to clinical use without the necessity of reformulation or substantial optimization.

Although these human kidneys could not be transplanted, the results of the canine kidney transplant studies demonstrated the viability and functional status of kidneys following 48 h of near-normothermic perfusion. To our knowledge this is the first report of long-term *ex vivo* perfusion of kidneys at near normothermic temperature following reimplantation in which normal life-sustaining function is demonstrated.

Protective mechanisms involved in the organ culture appear to include the continued flux of NO by constitutive NOS, as well as activation of the inducible isoform, iNOS. Blockade of NOS by L-NAME, dexamethasone or L-NIL resulted in profound edema after several hours of organ culture. The literature is replete with reports of the versatility of this critical signaling messenger with demonstrations of both protective and adverse effects. It appears likely that NO generated by both the constitutive isoform found in endothelial cells and inducible isoform of NOS provide important signaling pathways during organ culture. The continued function of the constitutive NOS isoform during organ culture is suggested by the increased dysfunction observed with L-NAME blockade that targets multiple NOS isoforms in comparison with specific blockade of iNOS. L-NIL is approximately 28-fold more selective for iNOS in comparison with the constitutive endothelial NOS isoform (49). Similarly, dexamethasone inhibits the induction of iNOS without affecting the constitutive isoforms (50,51). While the edema was more severe in the kidneys perfused with the addition of L-NAME, blockade of iNOS also leads to profound edema supporting the function of iNOS during organ culture.

The induction of iNOS may be attributable a generalized modulatory mechanism when organs are removed from their physiologic setting and adapt to an altered state of equilibrium (52). However, the NO produced during the period of organ culture was not cytotoxic, as the canine kidneys following *ex vivo* organ culture supported normal serum chemistries with immediate resumption of urine flow upon reimplantation.

The ability to study the heterogeneous cell populations that constitute complex tissues with preservation of normal cell-cell and cell-extracellular matrice integrity holds the potential to better understand cellular interactions. Among the potential medical applications that may be developed, one of the most intriguing would be the possibility of repairing damaged organs. The application of cell-culture principles to an isolated organ in combination with adequate growth factor signaling could provide the basis for cellular repair mechanisms; as an optimized cell culture system can support a population doubling in $<$24h. The repair during *ex vivo* perfusion would be distinct from the physiologic processes involved in wound repair, because wound repair consists of the primary steps of coagulation and inflammation, along with a migratory/adhesion phase. As organ culture is acellular and *ex vivo*, any repair represents the cellular recovery that occurs with restoration of metabolism during cell culture.

Other potential medical applications would include using *ex vivo* metabolism to modulate allograft immunogenecity preventing allosensitization by rendering grafts nonimmunogenic. In the near term, the ability to perform prognostic testing during organ culture may well form the basis for expanding the organ donor pool with organs from marginal and nonheartbeating donors.

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