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ARTICLES

PRETRANSPLANTATION PROGNOSTIC TESTING ON DAMAGED KIDNEYS DURING EX VIVO WARM PERFUSION[1]

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Background. **Further expansion of the donor pool with ischemically damaged kidneys will be predicated on the ability to develop prognostic testing. Using a well-established canine autotransplantation injury model, we assessed whether actual restoration of renal metabolism by ex vivo warm perfusion could be used to predict the status of an organ before transplantation.**

Methods. **Kidneys were subjected to 30 min of warm ischemia followed by 24 hr of static storage in ViaSpan at 4°C. After warm ischemia and static storage the kidneys were transitioned to 3 hr of warm perfusion using Exsanguinous Metabolic Support technology. During this period, parameters indicative of renal metabolism and vascular function were used to predict outcomes prospectively. Parameters included measures of oxidative metabolism, perfusion characteristics, and vascular condition. A Viability Score (VS) was calculated as the sum of the three parameters mentioned above. Results were grouped by a VS>2 and a VS<2.**

Results. **A clear association between the severity and duration of graft dysfunction and the VS was observed. Organs with a VS>2 had a significantly milder period of acute tubular necrosis, with both a less severe rise in serum creatinine (mean of 4.4 vs. 11 mg/dl) and a shorter recovery period (mean of 8 vs. 18 days) than those with a VS<2.**

Conclusions. **Results indicate the possibility of utilizing warm perfusion to evaluate kidneys before transplantation. The VS developed demonstrated efficacy in classifying the severity of the acute tubular necrosis and the occurrence of primary nonfunction, offering a sensitive assay for prospective organ testing.**

The success in renal transplantation has resulted in an increased demand for transplantable organs. Procurement of kidneys from heartbeating or living-related donors has not kept pace with demand. Although a more liberal legislation together with a more positive attitude toward organ donation might increase the effectiveness of these conventional procurement programs, the number of suitable donor organs is unlikely to ever meet the demand. This growing shortage of transplantable organs has renewed the interest in kidneys obtained from non-heartbeating (NHB) donors, thereby offering an untapped source of renal grafts.

Unfortunately the extent of ischemic damage suffered, resulting in a higher incidence of delayed- and primary nonfunction (PNF) after transplantation, hampers effective use of these kidneys. It is known that, due to variation in the resistance to ischemia, the duration of the insult does not always correlate with graft function after transplantation. Successful expansion of the existing donor pool with NHB donors will, therefore, be dependent upon the development of prospective organ evaluation during preservation.

From the beginning of kidney transplantation, attempts have been made to prospectively determine organ viability. Viability is determined by the integrated metabolism of all cells in an organ (*1*). However, metabolism is severely inhibited during the hypothermia used in organ preservation (*2, 3*), making transplantation the only way to determine the status of a kidney (*4*).

It seems logical to consider that a measurement in an environment that mirrors physiological metabolism would provide the best means of distinguishing between viable and nonviable kidneys before reimplantation. The development of a warm perfusion technology could provide the opportunity for ex vivo restoration of metabolism, thereby creating a window for prognostic testing (*5*).

The aim of this study was to develop a sensitive Viability Score (VS) using and combining parameters measured during ex vivo warm perfusion, which could be used to predict the severity of acute tubular necrosis (ATN) within an experimental group subjected to the same ischemic injury.

MATERIALS AND METHODS

Animals and surgical protocol. The autotransplantation experiments were performed on mixed foxhounds weighing 35–40 kg and approximately 2 years old (n=10). The animals received standard kennel food, routine lighting cycle and room temperature, and 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

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were mobilized. If two renal arteries were present, the right kidney was used. Ten minutes before excision, 1000 U of heparin and 200 mg/kg of mannitol were administered intravenously. The excised kidney was exposed to 30 min of warm ischemia (WI) while remaining in the abdominal cavity (37°C). After the warm ischemic period the kidney was flushed and statically stored for 24 hr in ViaSpan (4°C). The kidneys were then transitioned to warm perfusion (32°C) with an acellular perfusate for 3 hr before reimplantation. Fifteen minutes before to reperfusion, mannitol (200 mg/kg) and verapamil (0.1 mg/kg) were administered. Contralateral nephrectomy was performed before reperfusion of the preserved kidney.

Posttransplantation graft function. Blood samples for blood urea nitrogen and serum creatinine levels were taken each morning and analyzed using an ACE analyzer (Schiapparelli Biosystems, Inc., Fairfield, NJ). Animals deemed to be in poor condition, with unlikely chance of recovery from the ATN, were classified as PNF. Canines with PNF were euthanized. For the purpose of grading the severity of the ATN, the area under the curve (AUC) of the posttransplantation serum creatinine level was determined (*6*). AUC was calculated as the area under the curve above a normal value of 2.0 mg/dl. In addition to the AUC, the peak serum creatinine level was measured and the day the serum creatinine normalized was determined. Serum creatinine level was considered normal once the curve fell below 2.0 mg/dl.

Ex vivo warm perfusion. Exsanguinous Metabolic Support (EMS) technology (Breonics, Inc., Schenectady, NY) was used for ex vivo warm perfusion. After the period of cold ischemia, the kidneys were temperature transitioned to 32°C by flushing and then placed on a pressure-controlled 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 connected to the perfusion circuit and perfusion was begun. The mean arterial pressure was kept at approximately 35, aiming for a pulsatile perfusion pressure of 50/30 mmHg. The volume of the circulating perfusate was 0.5 L. Both flush solution and perfusate consisted of a highly enriched tissue culture-like solution that contained amino acids, lipids, carbohydrates, metabolites, inorganic ions, serum proteins, lipids, hormones, nitrogen bases, vitamins, reducing agents, a buffering system, trophic factors, vasodilators, radical scavengers, and adenine compound substrates adjusted to a pH of 7.4. The warm flush/perfusion solution is a proprietary technology available through Breonics, Inc. (*7*).

The acellular perfusate was supplemented with pyridoxylated bovine hemoglobin (6 g %) (Ezon, Inc., Piscataway, NJ) to provide adequate oxygen (PaO_2 of 200 mmHg) to support ongoing renal metabolism (*5*).

Testing during EMS perfusion. Oxidative index: The initial oxygen consumption during actual EMS perfusion was determined after 15 min of perfusion. Within 60 min of starting EMS perfusion, the oxygen consumption of all kidneys stabilized and remained constant during the perfusion period. This stabilized oxygen consumption was calculated as the average oxygen consumption during subsequent hours of EMS perfusion. The blood gas analyzer used was an ABL5 (Radiometer Medical A/S, Copenhagen, Denmark). Both initial and average oxygen consumption (ml/min/100 g) were calculated using the following formula:

$$\{[(PaO_2art - PaO_2ven) \times flow]/weight\} \times 100$$

The initial oxygen consumption was then divided by the average oxygen consumption to yield an index indicative of the initial impairment in the ability to restore oxidative metabolism after injury. The range of the index was between 0 and 1, because initial O_2 consumption could at best be as good as the average O_2 consumption and at worst be zero. Threshold value for the average O_2 consumption for this calculation was 10 ml/min/100 g; if the O_2 consumption of a kidney was not capable of reaching 10 ml/min/100 g, a score of zero was given.

Perfusion index: The ability to regain normal perfusion pressures of approximately 50/30 mmHg and flow rates of 80–120 ml/min (1–2 ml/min/g), as determined in previous experiments (*8, 9*), was assigned with a score of either 1 or 0, with 1 if the perfusion characteristics normalized or 0 if the perfusion characteristics remained out of the normal range.

Vascular index: Although many parameters could be used to assess vascular function, for the purpose of this study, we selected a well-standardized measurement for evaluation of vascular integrity. By determining the ratio of the platelet concentration in the perfusate after 15 min of EMS perfusion in comparison to the platelet concentration after 3 hours of perfusion, an overall assessment of the vascular condition was approximated. The difference in platelet count was believed to be indicative of the status of the vasculature, in that normal, nondamaged kidneys maintain stable platelet counts during the course of EMS perfusion. Increased platelet count was thought to be indicative of inadequate vascular flushing due to constricted or clogged microvessels and was assigned a decreasing value as the platelet count incrementally increased (Table 1). Platelet counts were performed using an ACT Diff analyzer (Beckman Corp., Miami, FL) Coulter counter.

The viability score. A VS was calculated as the sum of the three parameters listed above, resulting in the formula:

The oxidative index, perfusion index, and the vascular index all had a score ranging from 0 of 1. Therefore the relative weight of each parameter within the VS was 33.3%.

Histologic evaluation. At autopsy the implanted kidneys were bisected for gross macroscopic examination. Wedge-shaped biopsy specimens were taken and fixed in 4% neutral-buffered formalin, dehydrated, and paraffin embedded. Four-micron sections from each kidney were made and stained using hematoxylin and eosin for light macroscopic evaluation. A minimum of five sections per kidney were studied. The morphological characteristics of the kidneys were determined by blinded histologic evaluation.

RESULTS

Posttransplantation graft function. The posttransplantation serum creatinine concentrations of all kidneys are illustrated in Figure 1. One dog was symptomatic of uremia and was euthanized on day 7 after transplantation with a serum creatinine of 12.5 mg/dl. It was classified as a PNF. In one case a double renal artery was missed leading to a prolonged period of ATN. Although the dog survived and the kidney recovered normal renal function on day 18 after transplantation, the viability measurements were excluded from the study. The AUC, the marker for the severity of ATN, together with the peak serum creatinine level and the day serum creatinine normalized for each dog are listed in Table 2.

Testing during EMS perfusion. Values from each kidney comprised of the oxidative, the perfusion, and the vascular index, were combined to yield the VS and are listed in Table 3.

$$VS = \text{oxidative index} + \text{perfusion index} + \text{vascular index}$$

TABLE 1. Vascular index

Platelet increase (K/μL) during EMS perfusion	Vascular index scoring
0–<2000	1.0
2000–<3000	0.75
3000–<4000	0.50
4000–<5000	0.25
>5000	0.0

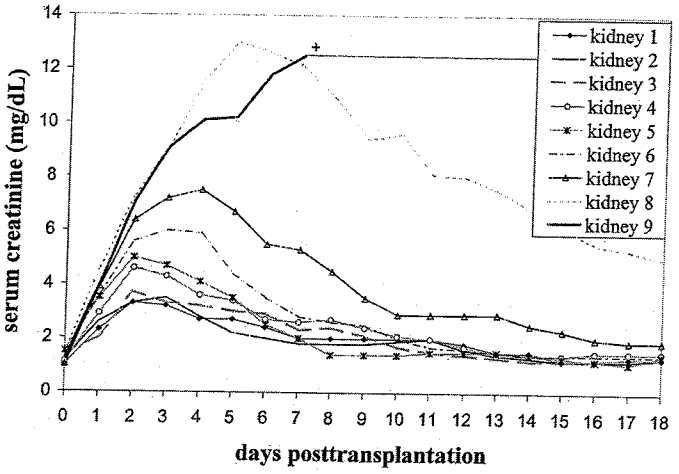

FIGURE 1. Posttransplantation survival. +, death.

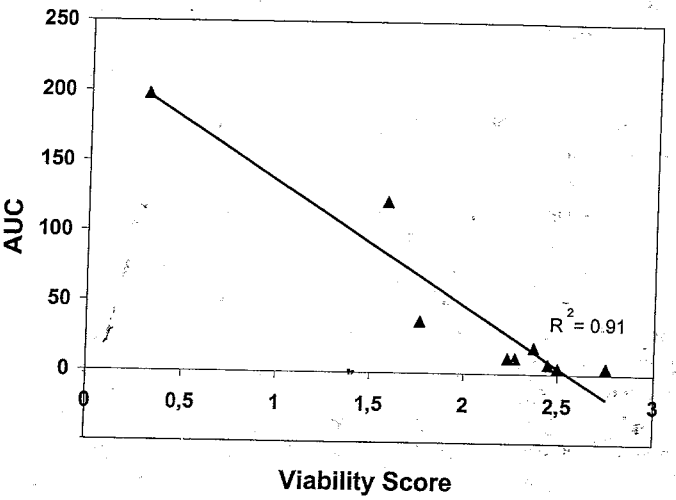

FIGURE 2. Correlation between VS and AUC.

TABLE 2. Posttransplantation graft function

Kidney number	AUC	Peak-Scr (mg/dl)	D-norm (days)
1	4.6	3.3	7
2	4.4	3.5	6
3	6.9	3.7	9
4	11.3	4.6	11
5	11.3	5	7
6	18.9	6	10
7	36.9	7.5	16
8	121.8	13	18[a]
9	197.8	12.5	18[a]

Abbreviations: Peak-Scr, peak serum creatinine; D-norm, day serum creatinine normalized (<2.0 mg/dl).

[a] Surpassed study period of 18 days.

TABLE 3. Measurements during EMS perfusion

Kidney number	Oxidative (I/A)[a] and index		Perfusion index	Vascular index	VS
1	(16/16)	1.00	1	0.75	2.75
2	(16/16)	1.00	1	0.50	2.50
3	(14/14)	1.00	1	0.50	2.50
4	(12/16)	0.75	1	0.50	2.25
5	(13/17)	0.76	1	0.50	2.26
6	(13/15)	0.87	1	0.50	2.37
7	(11/14)	0.76	1	0.00	1.76
8	(5/15)	0.33	1	0.25	1.58
9	(5/17)	0.30	0	0.00	0.30

[a] I, initial O_2 consumption (ml/min/100 g); A, average O_2 consumption (ml/min/100 g); O_2 consumption calculated by {[(PaO_2art − PaO_2ven) × flow]/weight} × 100.

Viability score. The VS was used as prognostic index to predict of the severity of the ATN and was expressed as the AUC. There seemed to be a continuous relationship between the VS and the outcome measure AUC, in that the dogs with the least severe ATN were found to have the highest VS. Similarly, the kidneys with the lowest VS had poorer outcomes. The one dog with PNF was found to have the lowest VS. Regression analysis demonstrated a high level of significance between the VS and the AUC as shown in Figure 2.

For the purpose of determining statistical significance of the VS in predicting graft outcomes, results were grouped by score, where VS>2 represented mild ATN and VS<2 severe ATN. Only one case of PNF occurred, and the result of using a VS demarcation of 2.0 grouped this case with the cases of severe ATN. Table 4 gives the statistical comparison between the two groups. Because the standard deviations increased with the mean concentration, the data was transformed by a logarithmic transformation (*10*). The difference is expressed as the ratio of the geometric means. A clear association between the severity of graft dysfunction and the VS was observed in that a greater peak value and a longer period needed for recovery was seen in organs with a VS<2 than in those with a VS>2 ($P<0.05$).

TABLE 4. Correlation between the VS and posttransplantation outcomes

	AUC	Peak-Scr (mg/dl)	D-norm (days)
Mild ATN			
Arithmetic mean (SD)	9.6 (5)	4.4 (1)	8.3 (2)
Geometric mean	8.1 (1.7)	4.2 (1)	8.0 (1.3)
Severe ATN			
Arithmetic mean (SD)	118.8 (80)	11 (3)	17.3 (1)
Geometric mean	92.8 (2.3)	10.5 (1.3)	17.6 (1)
Ratio of geometric mean	11.5	2.5	2.2
95% confidence interval	2–60	1.3–4.5	1.7–2.7
P value	<0.05	<0.05	<0.05

Peak-Scr, peak serum creatinine; D-norm, day serum creatinine normalized (<2.0 mg/dl); SD, standard deviation.

Histology. In the eight EMS perfused kidneys exhibiting a reversible ATN, the histologic evaluation of the kidney sections provided evidence of widespread, intermittent cystic dilation of tubules with flattened or regenerating tubular epithelium. There was mild, multifocal mineralization of the cortical tubules. Mild inflammatory infiltrates were found to be associated with areas of regeneration, repair, and mineralization. The blood vessels appeared to be normal.

In the dog with PNF, the kidney sections revealed a markedly different histologic evaluation. There was moderate, segmental, acute glomerulonephritis with glomerular thrombosis, neutrophil infiltrates, and occasional mesangial proliferation. There was also marked interstitial hemorrhage and tubular necrosis. Several wedge-shaped hemorrhagic infarcts

extending from the medulla were observed together with focal arteriolar thrombosis.

DISCUSSION

Viability testing in clinical transplantation has gained renewed interest with the resurgence of the NHB donor. Historically, evaluation of organ viability has focused on the determination of the energy charge, various enzymes and electrolyte releases, perfusion characteristics, and the functional and morphologic status of the kidney. Despite these efforts there is currently no objective method or assay to evaluate the potential function of a kidney (*4*). An impediment to developing viability testing is the inhibition of metabolism that occurs during hypothermia. Hypothermia merely slows the ongoing ischemic damage leading to cell death (*11*) making evaluation of the integrated metabolic capacity, needed for actual determination of the viability, virtually impossible (*1*).

With the development of a more physiologic preservation technology that supports adequate metabolism at a warm temperature, it seems feasible to develop viability testing that could distinguish between viable and nonviable organs (*5*, *12–14*).

Two important issues play a major role in the viability of an organ. First, the ability to support and maintain an adequate and sufficient metabolic state. Second, the maintenance of the integrity and normal barrier function of the vasculature. Ischemia impairs both the cellular metabolic capacity and the vascular functions within a kidney. In view of this, three parameters for the prognostic testing of a kidney during ex vivo warm perfusion were developed:

- initial and overall oxygen consumption
- ability to normalize the perfusion pressures and flow rate
- estimation of the vascular condition as determined by the release of platelets during warm perfusion.

The three viability parameters were measured during 3 hr of warm perfusion following a known critical autotransplantation model (*15*). The injury model consisted of an initial insult of 30 min of WI followed by 24 hr of cold storage in Viaspan, after which the kidney was transitioned to warm perfusion using EMS technology (Breonics, Inc.). Addition of the 3 hr of warm perfusion to the WI and cold storage before transplantation did not adversely affect posttransplantation outcomes. In fact EMS technology proved to be beneficial in reducing the injury seen after WI and cold storage (*16*).

Oxidative index. The oxygen consumption has been described as a marker for renal damage (*17*, *18*). In a previous study we investigated the relative role of cold preservation on initial renal metabolism by varying the cold ischemia times (*8*). A cold ischemia-associated lag-phase in the restoration of metabolism was observed, where the longer cold preserved kidneys exhibit a lower initial rate of oxygen consumption. However, after 3 hr of EMS perfusion, oxygen consumption was found to be equal in kidneys cold stored for up to 24 hr. In the present experiments both the initial oxygen consumption and the average oxygen consumption were incorporated into the oxidative index. A lower initial oxygen consumption, indicative of more extensive damage, resulted in a lower oxidative index. Those kidneys found to have substantially reduced rates of oxygen consumption at the start of warm perfusion experienced longer periods of ATN. In two kidneys the initial oxygen consumption was severely impaired. One kidney represented the upper limit of ATN in this model with a peak serum creatinine level of 13.0 mg/dl on day 5 (kidney number 8). The other kidney with severely impaired initial oxygen consumption proved to be PNF (kidney number 9).

Perfusion index. Deterioration in hemodynamic characteristics have frequently been observed to occur during hypothermic perfusion and have been correlated with poor function after transplantation (*13*, *14*, *19–21*). Initial vasoconstriction or immediate dilation, when placed on EMS perfusion, were not found to correlate with outcomes. Rather, the ability to regain normal perfusion pressures of approximately 50/30 mmHg and flow rates of 80–120 cc/min was indicative of a reversible ATN. The only kidney that did not regain normalized perfusion during the period of EMS perfusion was the kidney with PNF.

Vascular index. During the WI period, depletion of energy-rich phosphates and inhibition of active transmembrane ion transport systems, leads to swelling of especially the endothelial cells (*22*, *23*) and rigidity of blood cells, resulting in obstruction in the capillaries (*24–26*).

During the course of EMS perfusion, blood cells trapped in the capillaries are flushed out and circulate in the acellular EMS perfusate. Since the amount of blood cells released is small in regard to the volume of the circulating perfusate, the only measurable and indicative blood cells were the platelets. The platelet release test used in this study, although representing a routine laboratory test, provided an effective means to assess one aspect of the vascular integrity. An increased platelet count during perfusion seemed to reflect areas of the kidney where damage resulting in edema and constriction prevented adequate flushing and preservation of the vasculature.

Viability score. Since numerous types of metabolically active cells govern organ viability, organ assessment during warm perfusion most likely would require multiple assays forming a score that could predict posttransplantation outcome better than one analysis alone (*1*, *5*). The aim of this study was to develop a sensitive VS, composed of the three parameters, that could be used to predict the variability in the innate resistance to ischemia between kidneys subjected to the same ischemic injury.

The three, previously established, parameters measured during the EMS perfusion were all given an equal share in the VS: 33.3%.

The time points used for the viability testing were carefully chosen. In previous attempts to evaluate organs during hypothermic preservation there still was a subsequent injury at reperfusion, confounding any possible correlation between the testing and the posttransplantation outcomes. Ex vivo restoration of sufficient metabolism seems to be a key factor in reducing the injury seen upon reperfusion (*16*). By measuring viability during warm perfusion, just before reimplantation, the injury normally seen at reperfusion is reduced substantially, making the viability testing more reliable.

Based on these results, we conclude that prognostic information collected before transplantation can effectively be linked to transplant outcomes. There seemed to be a direct correlation between a decreasing VS and an increase in the severity of the ATN, both in terms of the peak serum creatinine level and the time necessary for repair.

The results of this study indicate that it is possible to utilize the ongoing metabolism during the warm temperature EMS perfusion to assess organs before they are transplanted.

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