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OVERCOMING SEVERE RENAL ISCHEMIA: THE ROLE OF EX VIVO WARM PERFUSION

LAUREN BRASILE,[1,2] BART M. STUBENITSKY,[1,5] MAURITS H. BOOSTER,[1] SUSANNE LINDELL,[3] DORIAN ARANEDA,[4] CORINNE BUCK,[4] JOHN BRADFIELD,[4] CARL E. HAISCH,[4] AND GAUKE KOOTSTRA[1]

Department of Surgery, P Debyelaan 25, 6202 AZ Maastricht, The Netherlands; Breonics, Inc., Schenectady, New York 12305; Department of Surgery, University of Wisconsin, Madison, Wisconsin; and Departments of Surgery & Comparative Medicine, East Carolina University, Greenville, North Carolina 27834

Background. **The ability to effectively utilize kidneys damaged by severe (2 hr) warm ischemia (WI) could provide increased numbers of kidneys for transplantation. The present study was designed to examine the effect of restoring renal metabolism after severe WI insult during ex vivo warm perfusion using an acellular technology. After warm perfusion for 18 hr, kidneys were reimplanted and evaluated for graft function.**

Methods. **Using a canine autotransplant model, kidneys were exposed to 120 min of WI. They were then either reimplanted immediately, hypothermically machine perfused (4°C) for 18 hr with Belzer's solution, or transitioned to 18 hr of warm perfusion (32°C) with an acellular perfusate before implantation.**

Results. **Warm perfused kidneys with 120 min of WI provided life-sustaining function after transplantation, whereas the control kidneys immediately reimplanted or with hypothermic machine perfusion did not. The mean peak serum creatinine in the warm perfused kidneys was 3.7 mg/dl, with the mean peak occurring on day 2 and normalizing on day 9 posttransplant.**

Conclusions. **These results indicate that 18 hr of ex vivo warm perfusion of kidneys is feasible. Furthermore, recovery of renal function during warm perfusion is demonstrated, resulting in immediate function after transplantation. The use of ex vivo warm perfusion to recover function in severe ischemically damaged kidneys could provide the basis for increasing the number of transplantable kidneys.**

For the past decades the organ donor pool has consisted largely of the ICU-based, heart-beating, cadaver donor, which represents a limited pool of potential donors. Despite concerted efforts, the number of organs available for transplantation has remained stagnant over the past 10 years (*1*, *2*). Recent attempts to provide more kidneys have focused on using non-heart-beating (NHB) donors with limited warm ischemia (≤30 min). Unfortunately the posttransplant outcomes with these kidneys are characterized by a high incidence of both delayed graft function and primary nonfunction, thereby hampering effective use of the NHB donor kidney. The major obstacle preventing any substantial expansion of the donor pool is warm ischemia (WI) combined with cold preservation (*3*). Although it has been proposed that the kidney can tolerate as much as 2 hr of WI before the damage becomes irreversible, the added cold ischemia used to preserve the kidney during the period of tissue typing, matching, and transportation has made WI of greater than 30 min an insurmountable obstacle in transplantation today.

Although the WI insult is unavoidable in using kidneys from the NHB donor, an alternative to traditional hypothermic inhibition of metabolism may be feasible. Our hypothesis was that an acellular warm perfusion after WI could reestablish metabolism, restore vascular integrity, and re-institute cell volume regulation ex vivo sufficiently to render severely damaged kidneys capable of providing life-sustaining function when reimplanted.

To test this hypothesis, a canine autotransplant model consisting of an initial WI insult of 120 min was employed. Exsanguinous metabolic support (EMS) technology was used for ex vivo warm perfusion (32°C) for 18 hr.

MATERIALS AND METHODS

Animals and Surgical Protocol

The autotransplantation experiments were performed on foxhounds weighing 20–30 kg and approximately 2 years old. 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 were mobilized. If two renal arteries were present, the right kidney was used. Ten minutes before excision, 200 mg/kg of mannitol was administered intravenously. The excised kidney was exposed to 120 min of WI while remaining in the abdominal cavity (37°C). Kidneys were then divided into three groups:

Group 1 (n=2). Controls: 120 min WI followed by reimplantation;

Group 2 (n=2). Controls: 120 min WI followed by 18 hr hypothermic machine perfusion (MP) at 4°C and reimplantation;

Group 3 (n=5). Test: 120 min WI followed by 18 hr warm perfusion (32°C) and reimplantation.

The mean anastomosis time was 27 min and ranged from 20 to 30 min. Fifteen minutes before reperfusion, mannitol (200 mg/kg) and verapamil (0.1 mg/kg) were administered. Contralateral nephrectomy was performed before reperfusion of the preserved kidney. Group 1 kidneys were flushed of blood before reimplantation with the same solution that was used to pump the test kidneys.

[1] UM Faculty of Medicine and azM Department of Surgery.
[2] Breonics, Inc.
[3] Department of Surgery, University of Wisconsin.
[4] Department of Surgery, East Carolina University.
[5] Address correspondence to: B.M. Stubenitsky, M.D., Ph.D., c/o G. Kootstra, M.D., Ph.D., UM Faculty of Medicine, Dr Tanslaan 10, 6229 ET Maastricht, The Netherlands.
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Hypothermic Machine Perfusion

After the period of WI, the kidneys in group 2 were flushed and placed into a MOX-100 perfusion machine (Waters instruments, MN) with 500 ml of Belzer's perfusate used as a circulating solution (*4*). After the kidney was connected to the perfusion system, perfusion was set to a systolic pressure of 50 mmHg. After 1 hr of MP, if needed, the systolic pressure was readjusted to 50 mmHg. Flow and pressure were continuously monitored.

Warm Perfusion

EMS technology (Breonics, Inc., Schenectady, NY) was used for ex vivo warm perfusion (32°C). After the period of WI, the test kidneys in group 3 were flushed and then placed on a pressure controlled perfusion system, including a siliconized membrane 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 aiming for a pulsatile perfusion pressure of 50/30 mmHg. The volume of the circulating perfusate was 500 ml. Both flush solution and perfusate consisted of a highly enriched tissue culture-like solution that contained amino acids, carbohydrates, metabolites, inorganic ions, serum proteins, lipids, hormones, vitamins, reducing agents, and a bicarbonate buffering system (Table 1). The warm flush/perfusion solution is a proprietary technology of Breonics, Inc. 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. Metabolism was assessed by measuring the renal oxygen consumption. PaO_2 analysis of prerenal and postrenal samples were performed using an ABL5 blood gas analyzer (Radiometer Medical A/S, Copenhagen, Denmark). Oxygen consumption (ml/min/g) was calculated using the following formula: [(PaO_2 art−PaO_2ven) × flow]/weight.

Posttransplant Graft Function

Blood samples for BUN and serum creatinine were taken each morning and analyzed using an ACE analyzer (Schiapparelli Biosystems, Inc., Fairfield, NJ). Animals deemed to be in poor condition with an unlikely chance of recovery from the delayed graft function were classified as primary nonfunction (PNF). Canines with PNF were euthanized. Serum creatinine was considered normal once the curve fell below 2.0 mg/dl.

Histologic Evaluation

The study protocol called for euthanizing surviving canines after the second posttransplant week if the serum creatinine values normalized to <2.0 mg/dl. Irreversibly damaged kidneys necessitated euthanizing the canines with clinical symptoms of renal failure and a serum creatinine >8.0 mg/dl according to our standard institutional animal use protocol. At autopsy the implanted kidneys were bisected for gross macroscopic examination. Wedge-shaped biopsies 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.

To address the extent of WI damage and the effect of 18 hr of subsequent warm or cold perfusion ex vivo, contralateral kidneys, subjected to the same protocol, were sectioned and fixed in 4% neutral-buffered formalin, dehydrated, and paraffin-embedded.

RESULTS

Testing During Perfusion

Group 2. In the kidneys that were hypothermically MP for 18 hr, the initial perfusion pressure that was set at 50 mmHg resulted in a mean flow rate of 12 ml/min. After 60 min of

TABLE 1. Composition of basal EMS medium

DL-Alanine	0.12 g/L
L-Arginine HCI	0.14 g/L
DL-Aspartic Acid	0.12 g/L
L-Cysteine HCI H_2O	0.00022 g/L
L-Cystine 2HCI	0.52 g/L
DL-Glutamic Acid	0.2672 g/L
L-Glutamine	0.20 g/L
Glycine	0.10 g/L
L-Histidine HCI H_2O	0.04376 g/L
L-Hydroxyproline	0.02 g/L
DL-Isoleucine	0.08 g/L
DL-Leucine	0.24 g/L
L-Lysine HCI	0.14 g/L
DL-Methionine	0.06 g/L
DL-Phenylalanine	0.10 g/L
L-Proline	0.08 g/L
DL-Serine	0.10 g/L
DL-Theonine	0.12 g/L
DL-Tryptophan	0.04 g/L
L-Tyrosine 2Na	0.11532 g/L
DL-Valine	0.10 g/L
Adenine Hemisulfate	0.02 g/L
Adenosine Triphosphate 2Na 2Na	0.002 g/L
Adenylic Acid	0.0004 g/L
Alpha Tocopherol Phosphate 2Na	0.00002 g/L
Ascorbic Acid	0.001 g/L
D-Biotin	0.00002 g/L
Calciferol	0.0002 g/L
Cholesterol	0.0024 g/L
Choline Chloride	0.001 g/L
Deoxyribose	0.001 g/L
Folic Acid	0.00002 g/L
Glutathione (reduced)	0.0001 g/L
Guanine HCL	0.0006 g/L
Hypoxanthine	0.0006 g/L
Menadione (Na Bisulfate)	0.00003 g/L
Myo-Inositol	0.0001 g/L
Niacinamide	0.00005 g/L
Nicotinic Acid	0.00005 g/L
Para-Aminobenzoic Acid	0.0001 g/L
D-Pantothenic Acid Ca	0.00002 g/L
Polyoxyethylenesorbitan Monoolate	0.04 g/L
Pyridoxal HCI	0.00005 g/L
Pyridoxine HCI	0.00005 g/L
Retinol Acetate	0.00028 g/L
Riboflavin	0.00002 g/L
Ribose	0.001 g/L
Thiamine HCL	0.00002 g/L
Thymine	0.0006 g/L
Uracil	0.0006 g/L
Xanthine HCI	0.00069 g/L
Calcium Chloride $2H_2O$	0.265 g/L
Ferric Nitrate $9H_2O$	0.00144 g/L
Magnesium Sulfate (anhydrous)	1.2 g/L
Potasium Chloride	0.40 g/L
Sodium Acetate (anhydrous)	0.10 g/L
Sodium Chloride	6.8 g/L
Sodium Phosphate Monobasic (anh)	0.224 g/L
D-Glucose	2.0 g/L
Insulin	0.01 g/L
Bovine Serum Albumin	30 g/L
Sodium Bicarbonate	4.4 g/L
Pyruvate	0.22 g/L
Transferin	0.10 g/L
Serum	10 ml
B-cyclodextrin	0.50 g/L
Chondroitin sulfate B	0.004 g/L
Fibroblast growth factor	0.02 g/L
Heparin	0.18 g/L

MP, the pressure was readjusted to 50 mmHg, resulting in a mean flow rate of 20 ml/min, which remained virtually unchanged during the course of perfusion.

Group 3. All the warm perfused kidneys of group 3 demonstrated initially reduced flow rates that increased during the course of perfusion, together with a corresponding decrease in the vascular resistance Table 2. Oxygen consumption was lowest at the start of the ex vivo perfusion compared with the rates observed at subsequent time points.

Posttransplant Graft Function

Groups 1 and 2. After 120 min of WI, control kidneys were either immediately reimplanted or hypothermically MP for 18 hr. All control dogs were anuric posttransplant with resulting uremia. Because our results in the controls were consistent with the literature and because of the distress to the dogs, ethically the number of control dogs was limited to two in each group to demonstrate reproducibility. All control dogs (groups 1 and 2) autotransplanted with kidneys damaged by 120 min of WI demonstrated rising serum creatinine values that necessitated euthanasia (Fig. 1).

Group 3. Test kidneys subjected to 120 min of WI and 18 hr of warm perfusion reperfused well with evidence of restored and continual urine flow within minutes of reperfusion. The peak serum creatinine values ranged from 2.4 to 4.9 mg/dl. All five dogs survived with normalization of the serum chemistries on days 3, 7, 8, 11, and 10 (Fig. 1).

Histology

At autopsy the control kidneys that were immediately reimplanted after 2 hr of ischemic insult demonstrated moderate coagulative necrosis with marked interstitial and medullary hemorrhage. In control kidneys that were subsequently cold perfused, large luminal thrombi were observed in the intralobular arteries. Many glomerular capillaries were observed to contain thrombi. All control kidneys had evidence of tubular necrosis, with intermittent areas of active tubular necrosis. In contrast, the test kidneys that were warm perfused after 2 hr of warm ischemic insult demonstrated normal vessels and glomeruli. Tubule epithelium was essentially normal, although there were occasional, mild cystic dilation of tubules with mild multifocal peritubular mononuclear infiltrates.

The contralateral kidneys were subjected to 120 min of warm ischemia and then either MP (4°C) or warm perfused for 18 hr. Warm ischemia and subsequent MP resulted in widespread damage to the tubule system by hematoxylin and eosin staining consisting of cell swelling, individual cell death, loss of apical cytoplasm, and plugging of tubular lumina systems (Fig. 2). In contrast, warm perfusion resulted in preserved cellular integrity (Fig. 3). Widespread tubular damage was present as expected, because 18 hr of perfusion would be an inadequate period to observe tubule epithelial regeneration.

TABLE 2. Warm perfusion characteristics

	FR[a]	VR[b]	O2 cons[c]
Initial	37±25	1.53±1	0.08±0.02
90 min	79±20	0.64±0.1	0.11±0.01
12 hr	96±19	0.51±0.1	0.14±0.01
18 hr	101±16	0.45±0.1	0.14±0.01

All values expressed as mean with the standard deviation.
[a] FR, vascular flow rate (ml/min).
[b] VR, vascular resistance (MAP/FR).
[c] O_2, oxygen consumption (PaC_2 artery − PaO_2 vein) × FR/weight.

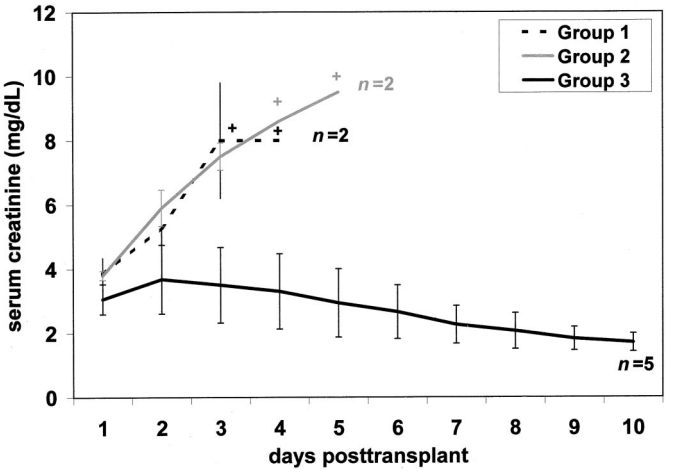

FIGURE 1. Serum creatinine concentration after renal transplantation of kidneys subjected to 120 min of WI. The two control groups consisted of transplantation immediately after 120 min of WI (group 1) and 120 min of warm ischemia followed by 18 hr of hypothermic MP (group 2). The test group (group 3) consisted of 120 min of warm ischemia followed by 18 hr of warm perfusion. An increase in serum-creatinine concentrations due to PNF was observed in groups 1 and 2. In group 3 serum creatinine levels increased slightly posttransplant and returned to normal values by day 9. Values are expressed as the mean with standard deviation for each experimental group. +euthanized due to primary nonfunction.

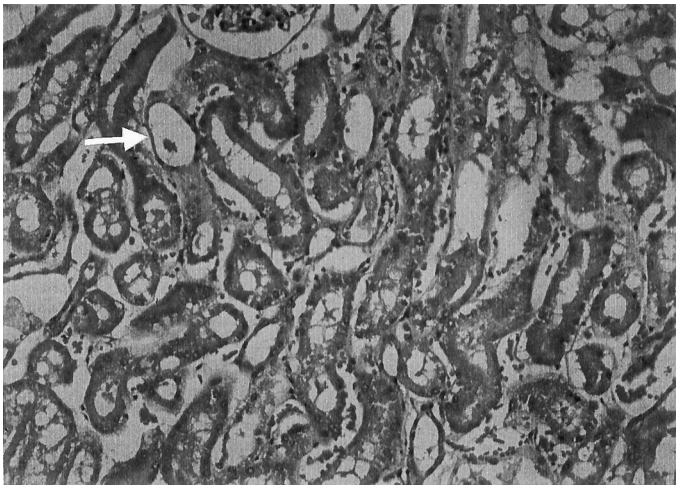

FIGURE 2. Canine kidney with 120 min of warm ischemic damage followed by 18 hr of hypothermic MP resulting in widespread damage to the tubule system consisting of individual cell death and loss of apical cytoplasm (arrow). (Hematoxylin and eosin staining ×20.)

DISCUSSION

The historic approach to organ preservation has involved using hypothermic techniques. Hypothermia exerts its ben-

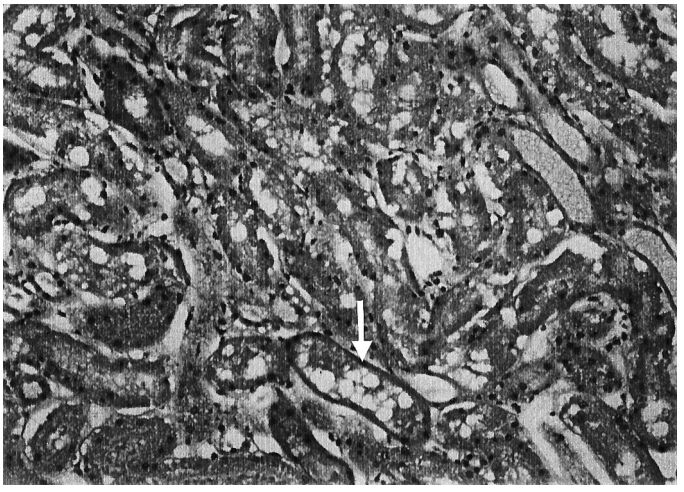

FIGURE 3. Canine kidney with 120 min of warm ischemia and subsequent 18 hr of warm perfusion resulted in preserved cellular integrity. Focal tubular damage was present (arrow). (Hematoxylin and eosin staining ×20.)

eficial effect by diminishing the oxygen demand of the organs and also by reducing the overall metabolic rate. However, hypothermic inhibition of metabolism is not benign (*5–7*). Hypothermia can lead to altered tissue integrity, which predisposes the development of reperfusion injury. The alterations in cellular integrity can include loss of the adenine compound pool, an accumulation of by-products such as free fatty acids, inhibition of the ion pumps, edema, and time-dependent structural changes in the vasculature. As the duration of cold ischemia becomes prolonged, there is a direct correlation with the severity of the inflammatory processes generally categorized as reperfusion injury. Most importantly, the inhibition of cellular metabolism that occurs during hypothermic preservations eliminates the possibility of substantial reparative processes occurring after warm ischemic injury.

The initial effects of ischemia, whether at warm or cold temperatures, are from the lack of molecular oxygen for oxidative phosphorylation; which leads to the depletion of cellular energy stores (*8*). Nucleotides are rapidly lost during ischemia, and this loss is an important factor in the failure of tissue subjected to prolonged ischemia to regenerate after restored blood circulation (*9*). The principal difference between ischemia at warm and cold temperatures is the rate at which the cell injury and death occur. The hypothermic time-dependent increase in the incidence of delayed function in kidneys is substantially increased with prior warm ischemic exposure, as is the case with kidneys procured from NHB donors. Therefore, the combination of warm and cold ischemic damage represents the major obstacle to substantially expanding the organ donor pool into the NHB cadaver population. Until the damaging effects of ischemia can be alleviated, the donor pool cannot be substantially expanded.

However, many of the ischemia-related problems would be eliminated if organs could be perfused at warmer temperatures. Warm perfusion at temperatures greater than 25°C would support membrane lipids remaining in a normal fluid state. Most enzyme systems functioning at 37°C also function at temperatures as low as 20°C but at a slower rate. Similarly, energy substrates can be readily resynthesized after ischemic insult once oxidative phosphorylation resumes at warm perfusion temperatures. Warm perfusion would support better oxygen utilization and raise the metabolic rate during the period an organ is maintained ex vivo.

The results of this study demonstrate that warm perfusion is feasible and presents the opportunity to reestablish metabolism ex vivo after a severe warm ischemic insult. Most importantly, warm perfusion after 2 hr of warm ischemic insult recovered metabolism sufficiently to support the eventual recovery of normal renal function. In contrast, primary nonfunction occurred in all control dogs. Hypothermic preservation did not alter this outcome.

Previous attempts at isolated organ perfusion at near normothermic temperatures using acellular solutions have resulted in deteriorating perfusion characteristics and metabolic function after relatively short periods (*10*). In reviewing the literature, it becomes apparent that the previous attempts at mimicking a physiologic preservation failed for two major reasons (*11*). First, the failure to maintain the integrity and normal barrier functions of the vasculature leads to a rapid deterioration in vascular flow and the concurrent development of edema. Second, the inability to support adequate delivery of nutrients and oxygen lead to a deteriorating metabolic state.

The perfusate we used consists of a highly enriched tissue culture-like media. Certain components are distinct from traditional tissue culture media formulations and provide unique functions:

- Oxygen carrier. An adequate supply of oxygen cannot be dissolved by an aqueous solution. A major reason why previous studies attempting warm perfusion failed can be attributed to the inability to supply adequate oxygen tension. The use of red blood cells has been problematic because of the mechanical damage that occurs to the circulating red cells over time. We used chemically modified hemoglobin as the source of an oxygen carrier.
- Albumin. Aside from providing colloid osmotic pressure, by far the most important function of albumin is its carrier functions. In the same manner that an oxygen carrier delivers oxygen and removes carbon dioxide, albumin delivers nutrients to the cells within the nephron and removes the by-products of the ongoing metabolism.

Metabolism is directly correlated to the temperature at which tissue is maintained ex vivo. It was determined from previous studies that warm perfusion could adequately support organ metabolism at temperatures ranging from 30°C to 34°C (*12–15*). In current studies, warm perfusion is conducted at a temperature of 32°C.

The recovery processes during warm perfusion should not to be confused with the physiologic processes involved in normal wound repair, because wound repair consists of the primary steps of coagulation and inflammation, along with a migratory/adhesion phase. Because EMS is acellular and ex vivo, the recovery is more analogous to the cellular recovery that occurs with restoration of metabolism during cell culture. Nonetheless, the ex vivo recovery entails these distinct features: reestablishment of oxidative metabolism, restoration of cell volume regulation, and recovery of vascular functions, including autoregulation, barrier functions, normalized vasculature resistance, and resumed urine flow. Most

importantly, it is sufficient to render an organ damaged by 120 min of warm ischemic damage capable of providing life-sustaining function with serum chemistries within a normal range, normal urine, and essentially normal histologic evaluation after reimplantation.

Ex vivo recovery of organs could provide the foundation for changing the current time frame for procuring organs from within minutes of death to hours. These results provide the first experimental evidence to recover organs after an ischemic insult of as much as 120 min and represent a potential solution to the persistent organ shortage.

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CARDIOVASCULAR MORBIDITY AND MORTALITY AFTER ORTHOTOPIC LIVER TRANSPLANTATION

SIMON D. JOHNSTON,[1,4] JOAN K. MORRIS,[2] ROB CRAMB,[3] BRIDGET K. GUNSON,[1] AND JAMES NEUBERGER[1]

Liver Unit and Department of Clinical Biochemistry, Queen Elizabeth Hospital, Birmingham, B15 2TH, and Department of Environmental and Preventive Medicine, Wolfson Institute of Preventive Medicine, St. Bartholomew's and the Royal London School of Medicine and Dentistry, London, EC1M 6BQ2

Background. **Hyperlipidemia and hypertension have been reported in liver allograft recipients and contribute to an increased risk of ischemic heart disease (IHD) after orthotopic liver transplantation (OLT). The aims of the study were (1) to determine the prevalence of risk factors for IHD in these patients and (2) to compare the observed incidence of cardiovascular events and related mortality in allograft recipients with a matched population.**

Methods. **One hundred ten consecutive adults (50 male) who attended for review after OLT (median follow-up 3.9 years; range 0.1–17.9) were assessed for cardiovascular risk factors using current blood pressure, diabetic status, and smoking history and measurements of total cholesterol, high-density lipoprotein cholesterol, and triglyceride concentrations. Cardiovascular events and cardiovascular mortality data were collected from the prospective database of all adult liver allograft recipients and compared to**

[1] Liver Unit, Queen Elizabeth Hospital, Birmingham, B15 2TH.
[2] Department of Environmental and Preventive Medicine, Wolfson Institute of Preventive Medicine, St. Bartholomew's and the Royal London School of Medicine and Dentistry, London.
[3] Department of Clinical Biochemistry, Queen Elizabeth Hospital, Birmingham, B15 2TH.
[4] Address for correspondence: Dr. Simon D. Johnston, MD, MRCP, Consultant Gastroenterologist, Department of Gastroenterology (Level 6), Belfast City Hospital, Lisburn Road, Belfast BT9 7AB, Northern Ireland, UK. E-mail: sdjohnston@utvinternet.com.

matched data from myocardial infarction registries and Office for National Statistics data, respectively.

Results. **Raised serum cholesterol (>5.0 mmol/L) was found in 48 (44%) patients (18 male), and systolic hypertension (>140 mmHg) was found in 69 (63%) patients (27 male). The relative risk of ischemic cardiac events was 3.07 (95% [confidence interval] CI, 1.98–4.53) and the relative risk for cardiovascular deaths was 2.56 (95% CI, 1.52–4.05) in allograft recipients compared to an age-matched population without transplants.**

Conclusions. **Liver allograft recipients have a greater risk of cardiovascular deaths and ischemic events than an age- and sex-matched population. The prevalence of raised cholesterol concentrations in patients after OLT is similar to those in previous reports. Moderate hypertension and hyperlipidemia may be more detrimental in patients after OLT compared to non-transplant patients without these risk factors.**

Liver allograft recipients are at increased risk of cardiovascular disease because of hypercholesterolemia, hypertension, and diabetes. Hypercholesterolemia has been reported in 31% of patients at 1 year (*1*), 46% after 2 years (*2*), and 37% at 3.5 years (*3*) post-orthotopic liver transplantation (OLT). Corticosteroids, rather than cyclosporine or tacrolimus (*4*), seem to be the major determinant of increased cholesterol levels, and early withdrawal of prednisolone has been reported to decrease the incidence and severity of hypercholesterolemia (*5*, *6*), hypertension (*7*), and diabetes (*8*) after OLT. A modest reduction in prednisolone dose from 10 to 5 mg daily can lead to a reduction in hypercholesterolemia with no increase in graft loss (*5*).

The incidence of hypercholesterolemia is lower in patients who receive tacrolimus than cyclosporine (*9*, *10*). Cyclosporine may contribute to more hypertension and obesity compared to tacrolimus within the first year post-OLT (*11*), and some authors have suggested that serum cholesterol levels can be reduced by changing from cyclosporine to tacrolimus (*12*, *13*). Both systolic hypertension and hyperlipidemia post-OLT contribute to increased morbidity and mortality. Tacrolimus is also associated with hypertrophic obstructive cardiomyopathy, which may resolve or improve on changing to cyclosporine but with the potential complications of cardiac failure and arrhythmias (*14*).

Other possible factors that may contribute to an increased risk of cardiovascular disease include postoperative complications; for example, hypotension or reperfusion injury. Therefore, Dec et al. (*14*) reported that 23% of patients had a major cardiac event (defined as myocardial infarction, reversible myocardial ischemia, pulmonary embolism, pulmonary edema, cardiogenic shock, or symptomatic rhythm disturbances). Although 2.7% of patients died of cardiac causes and although there was no effect on survival at 6 months, 5-year survival was reduced in patients who had cardiac events (*14*). Other risk factors for cardiac events in the posttransplant period include older age (*14*, *15*), a long QT interval (*16*), unrecognised hemochromatosis (*17*, *18*), and undetected coronary artery disease (*19*). Excluding those patients with a previous history of ischemic heart disease (IHD), the incidence of angiographically proven moderate or severe coronary artery disease may be as high as 13% in transplant recipients over 50 years of age (*19*). Cardiovascular disease may also account for up to 14% of late deaths (>1 year) post-OLT (*20*). However, cardiac surgery has been safely performed post-OLT with significant short-term morbidity but low mortality (*21*, *22*). In one study, 15 (1.25%) of 1200 patients who had undergone OLT required cardiac surgery at a mean time interval of 30 months (range 9 days to 62 months) posttransplant (*21*).

The aims of this study were to investigate the prevalence of conventional risk factors for IHD in consecutive adult patients who attended for review post-OLT and to calculate their risk for IHD. In addition, we have reviewed our database of all adult patients who have undergone OLT in our center to determine if morbidity and mortality rates from cardiac events are higher than a matched population.

MATERIALS AND METHODS

Cardiovascular Risk Factors

One hundred ten consecutive adult patients who attended for outpatient review after OLT were assessed between June and August 1999. There were no exclusion criteria. Of these, 50 were male; the ages ranged from 24 to 73 years (median 54 years). The median time from OLT was 3.9 years (range 0.1–17.9 years). The indications for liver transplantation in these patients are shown in Table 1, and the immunosuppressive therapy at the time of their assessment is listed in Table 2. After liver transplantation, the standard immunosuppression protocol was based on prednisolone, azathioprine, and cyclosporine. In the event of recurrent rejection, mycophenolate and tacrolimus were used as alternatives. Prednisolone was withdrawn by 3 months, except in patients with autoimmune hepatitis, recurrent rejection, or concurrent medical problems; for example, ulcerative colitis. The assessment of all patients being considered for liver transplantation includes chest x-ray, electrocardiograph, pulmonary function tests, and echocardiography or cardiac catheterization if indicated.

The assessment of risk for coronary heart disease was calculated using the Framingham risk score (*23*). Calculation of risk is based on age, gender, smoking habit, presence or absence of diabetes, knowledge of left ventricular hypertrophy (assessed by electrocardiograph and echocardiography), and the measurement of cholesterol and high-density lipoprotein (HDL) cholesterol. Five patients under 30 years of age were assumed to be 30 years of age for the purposes of the Framingham equation. Blood samples were taken and serum was assayed for liver tests, total cholesterol, HDL cholesterol, and triglyceride concentrations. Total cholesterol was measured using standard laboratory techniques, and HDL cholesterol was measured using the N-Geneous method (Biostat UK). Data were calculated for the risk of coronary disease over a period of 10 years and for the relative risk compared to a baseline population (*24*). Diabetes mel-

TABLE 1. Indications for liver transplantation in 110 patients

Diagnosis	Number (n)
Primary biliary cirrhosis	40
Primary sclerosing cholangitis	14
Cryptogenic cirrhosis	13
Alcoholic liver disease	10
Chronic hepatitis C	7
Chronic hepatitis B	4
Autoimmune hepatitis	3
Fulminant liver failure	2
Non-A, non-B fulminant liver failure	6
Drug-induced fulminant liver failure	4
Miscellaneous	7
Total	110

TABLE 2. Immunosuppressive therapy in 110 patients

Therapy[a]	Number (n)
Cyclosporine	18
Cyclosporine, azathioprine	33
Cyclosporine, prednisolone	4
Cyclosporine, azathioprine, prednisolone	11
Cyclosporine, prednisolone, mycophenolate	4
Cyclosporine, mycophenolate	1
Tacrolimus	11
Tacrolimus, azathioprine	8
Tacrolimus, azathioprine, prednisolone	3
Tacrolimus, prednisolone, mycophenolate	6
Tacrolimus, mycophenolate	2
Prednisolone, mycophenolate	5
Prednisolone, azathioprine	3
Mycophenolate	1
Total	110

[a] The proportion of patients on azathioprine (53%), mycophenolate (17%), and prednisiolone (33%).

litus was defined according to the World Health Organization (WHO) criteria (*25*). Blood pressure was recorded using a dedicated automated sphygmomanometer. Systolic hypertension was defined as systolic blood pressure (SBP) greater than 140 mmHg and diastolic hypertension as diastolic blood pressure (DBP) greater than 90 mmHg.

Group comparisons were performed with the Mann-Whitney *U* test for continuous data and with the chi-square test for categoric data. Values, where appropriate, are given as median and range. The number of patients with elevated serum cholesterol was expressed as a percentage of the total number of patients who were alive 1 and 3 years after OLT. $P<0.05$ was considered significant.

Cardiovascular Events and Related Mortality

A prospective database of all adult liver allograft recipients (n=1312) has been collected in the Queen Elizabeth Hospital, Birmingham, since the commencement of the program in January 1982. All significant events related to these patients, including cardiovascular disease, have been recorded during their follow-up. To estimate the relative risk of cardiovascular mortality, the following cardiovascular events were recorded as ischemic in origin: myocardial infarction, angioplasty, coronary artery bypass grafting, and cardiac arrest. Cardiac arrests in patients with sepsis or severe hemorrhage were excluded. Other cardiac problems, mainly atrial fibrillation, cerebrovascular events, or valve replacement, were regarded as nonischemic in origin. The length of follow-up was the time from transplantation to time of death if they had died or to date of last follow-up if they were still alive. Patients who died or had their first cardiovascular event within the first 3 months posttransplant were excluded because of the multiple factors that may contribute to a cardiac event during this period. The length of follow-up of all remaining patients was reduced by 3 months to take account of this.

The number of cardiovascular deaths that occurred in the liver transplant patients was compared with the Office for National Statistics (ONS) death rates for ischemic heart disease in 5-year age bands from 1982 to 1998 in England and Wales. Nineteen ninety-eight was the latest year of data available and was used to approximate the death rates that occurred in 1999 and 2000. For male and female patients separately the length of time of follow-up in each 5-year age band for each calendar year was calculated, and this was multiplied by the ONS death rates to obtain the expected number of deaths.

For the purpose of comparing the rate of cardiovascular events in transplant recipients, the first event attributed to IHD was retained for each patient if they had two or more cardiovascular events recorded. The length of follow-up was the time from transplantation to first event, if appropriate; the time from transplantation to death; or to last follow-up if they were still alive. Unlike cancer, regional or national statistics are not maintained on the incidence of IHD because the data are not routinely collected. Estimates of the incidence of IHD are best derived from registries that have been maintained for research purposes in certain localities for periods of 1 to 3 years. We have used estimates of IHD incidence on the basis of a meta-analysis of data from eight myocardial infarction registries in England and Wales (*26*). For all the patients, the length of time of follow-up in each 5-year age band was calculated, and this was multiplied by the estimated incidence rates to obtain the expected number of events. The observed and expected numbers of deaths or cardiovascular events were compared, and the confidence interval (CI) of this rate was obtained by assuming the observed numbers of events came from a Poisson distribution.

RESULTS

Cardiovascular Risk Factors

There were 10 subjects with diabetes (6 male, median age 54 years), of whom 7 were diabetic pretransplant and 3 developed diabetes posttransplant. Of these, one in each group was treated with insulin. There were 21 known hypertensive patients on treatment (4 male, median age 58 years), and there was one patient with known IHD. One patient was being treated with a statin. There were no smokers in the group.

Raised serum cholesterol (>5.0 mmol/L) was present in 48 patients (44%) (18 male, median age 55 years), low-serum HDL cholesterol (<1.2 mmol/L) was present in 57 patients (52%) (32 male, median age 56 years), and high-serum triglyceride concentration (>3.0 mmol/L) was present in 9 patients (8.2%) (7 male, median age 54 years). The lipid parameters are given in Figure 1. Elevated serum cholesterol levels were present in 43% of patients (39 of 91) 1-year post-OLT, and in 42% of those patients (28 of 66), elevated serum cholesterol levels were present 3 years post-OLT.

There was no difference between the lipid parameters, SBP, or 10-year risk score for IHD in those on prednisolone and those who were not (Table 3). Patients on prednisolone were younger (51.3 vs. 55.4, $P=0.014$) than those who were not receiving steroids and, as expected, were followed for a shorter period post-OLT (median 3.2 vs. 4.1 years). There was no difference between the lipid parameters, SBP, or 10-year risk of IHD in those on cyclosporine compared to tacrolimus (Table 3). Patients on cyclosporine were at a similar duration post-OLT compared to those on tacrolimus (4.5 vs. 4.3 years, $P=0.96$).

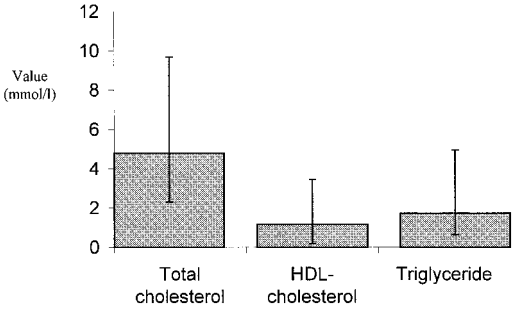

Figure 1. Comparison of cholesterol levels. HDL-cholesterol, high-density lipoprotein cholesterol.

TABLE 3. A comparison of risk factors for IHD in different immunosuppressive therapy groups

	Prednisolone (n = 36)	No prednisolone (n = 74)	*P* value	Cyclosporine (n = 71)	Tacrolimus (n = 30)	*P* value
Median age (years)	51.3	55.4	0.014	53.7	55.4	0.33
Male:female	17:19	32:46	0.59	37:34	10:20	0.08
Total cholesterol	5.35	4.83	0.24	5.03	4.78	0.51
HDL cholesterol	1.22	1.24	0.56	1.22	1.27	0.44
Triglyceride	2.15	1.85	0.18	2.05	1.71	0.19
SBP	145	149	0.4	150	145	0.3
10-year risk score	9.9	9.1	0.9	10.3	7.9	0.29

Using multiple regression analysis for the lipid parameters and the independent variables of age, weight, and use of azathioprine, cyclosporine, tacrolimus, mycophenolate, and prednisolone, only age ($P=0.03$) and prednisolone usage ($P=0.048$) were independent risk factors for high cholesterol. There were no independent risk factors for high triglyceride or low-HDL cholesterol.

Median SBP was 146 mmHg (SD 23.7, range 104–212) and DBP was 79.0 mmHg (SD 13.9, range 42–112). Systolic hypertension (>140 mmHg) was present in 69 patients (63%, 27 male, median age 55.3 years) and diastolic hypertension (>90 mmHg) was present in 26 patients (24%, 9 male, median age 57.1 years) who attended the outpatient clinic.

Estimated Risk of IHD in Clinic Patients (n=110)

The median calculated 10-year risk of IHD in the 110 patients was 7.9% (SD 7.45, range 0.1–37.5%), and the median calculated relative risk for IHD was 10% lower compared to a standard Framingham population.

Observed Incidence of Cardiovascular Events in all Transplant Recipients (n=1312)

Eleven patients had their first event on the day of transplant, and 48 patients had their first event within 3 months of transplant. Both of these groups of patients were excluded. Twenty-five patients who had an event more than 3 months posttransplant were included.

The observed number of ischemic cardiovascular events was 25 in the transplant recipients compared to an expected number of events of 8.15 in men and women of the same age from England and Wales, given a relative risk of IHD of 3.07 (95% CI, 1.98–4.53). When comparing patients with cardiovascular events as a result of IHD (n=25, 9 male, median age 61 years, range 27–71 years) and with cardiovascular events as a result of other cardiovascular causes (n=32, 13 male, median age 66 years, range 32–78 years), the median time to event was 30 months posttransplant (range 3–121 months) in the former compared to 28 months (range 3–199 months) in the latter. The cardiovascular events in each group are listed in Table 4. The observed incidence of IHD events was 5% per 10 years (25 ischemic cardiovascular events in 4962 person-years of observation).

Observed Cardiovascular-Related Mortality in Transplant Recipients

The observed number of deaths from cardiovascular disease in the transplant recipients was 18 compared to an expected number of deaths of 7.03 estimated from myocardial infarction registries in England and Wales, given a 2.56 relative risk of death (95% CI, 1.52–4.05). When comparing the cardiovascular deaths in those as a result of IHD (n=18, 7 male, median age 56 years, range 46–71 years) and those that were unrelated (n=19, 11 male, median age 63 years, range 33–77 years), the median time to death was 27 months posttransplant (range 5–101 months) in the former compared to 44 months posttransplant (range 5–109 months) in the latter. The causes of death in each group are compared in Table 5.

TABLE 4. Ischemic and non-ischemic cardiovascular events in liver allograft recipients

Event	Number	Time to event (median, months)	Time to event (range, months)
Ischemic cardiovascular events (n = 25)			
Angioplasty	8	52	5–84
Cardiac arrest	9	27	3–87
Myocardial infarction	4	32	12–121
Coronary artery bypass graft	4	41	12–92
Nonischemic cardiovascular events (n = 32)			
Cerebrovascular accident	18	34	13–163
Atrial fibrillation	13	26	3–199
Valve replacement	1		

DISCUSSION

This is the first study to report on cardiovascular risk scores after liver transplantation. The calculated risk of IHD in our clinic patients was 7.9% over 10 years compared to the Framingham population, which represented a slight increase in risk. Those patients who are listed for transplantation are selected and those with evidence of heart disease are ex-

TABLE 5. Causes of death in liver allograft recipients after ischemic and nonischemic cardiovascular events

	Ischemic cardiovascular events (n = 18)	Nonischemic cardiovascular events (n = 19)
Cardiac	4	2
Cerebrovascular accident	2	8
Metastatic disease	1	1
Sepsis	4	2
Recurrent disease	2	1
Renal failure	1	2
Pulmonary	2	2
Multi-organ failure	2	1

cluded. Those who smoke are advised to stop in view of both the increased morbidity in the perioperative period and the other associated medical sequelae. This inevitably leads to a preselection of patients and accounts for why there were no smokers in our study. Thus, despite a full pretransplant assessment and that the patients studied had survived the operative stress, it is surprising that there were so many cardiovascular events in our patients.

Raised cholesterol concentrations are similar to those reported elsewhere (*1–3*) and were found in approximately two-fifths of patients at 3 years post-OLT. Using multiple regression analysis, we found that age and prednisolone use were independent risk factors for elevated cholesterol levels. Patients on cyclosporine had a trend for higher levels of total cholesterol and triglyceride, systolic blood pressure, and 10-year risk of IHD compared to patients on tacrolimus, although none of these reached statistical significance in contrast with earlier studies (*9*, *10*).

Because the individual risk factors for IHD, in particular, systolic hypertension, are so common in transplant recipients, we assessed whether this translates into an increased number of cardiovascular events. Although the Framingham equation only predicts a slightly increased risk of IHD in our 110 clinic patients (7.9% over 10 years), there is a high actual incidence of IHD events and mortality compared to meta-analysis data for England and Wales. It has not been previously reported that liver transplant recipients have two and a half times the cardiovascular mortality and three times the ischemic events of a matched population. Both ischemic cardiovascular events and nonischemic cardiovascular events occurred at a similar time posttransplant. Deaths after ischemic cardiovascular events occurred earlier posttransplantation than deaths after nonischemic cardiovascular events, indicating that ischemic cardiovascular events are associated with a higher mortality.

Although we found that there was an increased incidence of cardiovascular mortality and ischemic events, the calculations provided by the Framingham risk program did not identify that this group was necessarily at such a high risk from IHD. This may indicate that there are other factors, which have an influence on the risk of coronary heart disease, that are not taken into account in the Framingham equation.

There are several other reasons that may be of some importance in assessing whether these patients are at risk of coronary disease. Cholesterol is carried principally in low-density lipoproteins (LDL), but LDL particles vary in size and density (*27*). Small dense LDL particles are known to be more atherogenic and are related to the risk of coronary disease (*28*) and are typically found in patients who are diabetic or have impaired glucose tolerance (*29*). The patients in our group are given drugs that can precipitate glucose intolerance or frank diabetes, and these include prednisolone, cyclosporine (*30*), and tacrolimus (*31*). The majority of patients in our group had prednisolone discontinued at 3 months posttransplant (*32*), but the continued use of these other drugs with these known effects may influence the risk of coronary heart disease.

Other risk factors for coronary heart disease, which are now appreciated as contributing to coronary heart disease, include hyperhomocystinemia and underlying inflammatory risks, which are monitored by highly sensitive C-reactive protein (CRP) measurements (*33*). There are no data on homocysteine concentrations or metabolism found in post-liver transplant patients. The use of highly sensitive CRP measurements in post-liver transplant may not be appropriate because of the intercurrent problems of immunosuppression, but there are no data to support this view. Nonetheless, the prevalence of hypertension in our patients, with 69 patients having a SBP greater than 140 mmHg, demonstrated that conventional modification of hypertension was an area in which more aggressive therapy could be undertaken.

We have attempted to assess the prevalence of cardiovascular risk factors in liver transplant recipients and to estimate their predicted risk of IHD over 10 years. While the Framingham population differs from our clinic patients in age range, it is not an ideal control population, but it is as good an estimate as can realistically be achieved. Similarly, comparison of the incidence of cardiovascular events and mortality in liver transplant recipients to estimates of IHD incidence obtained from a meta-analysis of data from eight myocardial infarction registries in England and Wales and with the ONS death rates for IHD, respectively, also has its limitations.

Regular measures of serum cholesterol and triglyceride were not part of our standard protocol for following patients post-OLT. Clearly, frequent blood pressure measurement is mandatory and is the most significant modifiable risk factor for IHD in our patients. Raised blood pressure at outpatient review may often be attributed to multiple factors (for example, the stress of coming to the clinic or the "white coat" effect), but it is clear that hypertension in the outpatient setting should be treated more aggressively. All patients who have a SBP consistently greater than 160 mmHg should have their blood pressure lowered according to the British Hypertension Society guidelines (*34*). In addition, those patients with an estimated 10-year cardiac risk greater than 15% and with a SBP above 140 mmHg should also have their blood pressure lowered.

Because the increased incidence of cardiovascular events is not accurately predicted by an increase in the Framingham risk score calculation, moderate hypertension and hyperlipidemia may be more detrimental in patients after OLT compared to patients without these risk factors. In addition, there may be other risk factors for IHD after OLT that are not represented in the risk calculation based on the Framingham risk score.

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