The Non-Heart Beating donor, a new source of kidneys for transplantation : clinical and experimental studies

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THE NON-HEART BEATING DONOR, A NEW SOURCE OF KIDNEYS FOR TRANSPLANTATION

 

THE NON-HEART BEATING DONOR, A NEW SOURCE OF KIDNEYS FOR TRANSPLANTATION

Clinical and Experimental Studies

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op het gezag van de Rector Magnificus, Prof. mr. M.J. Cohen,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen
op vrijdag 31 maart 1995 om 14.00 uur

door

Maurits Harm Booster

geboren te 's Hertogenbosch op 14 juni 1963

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Aan mijn ouders
voor Ingrid

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GENERAL INTRODUCTION

With the advancement of kidney transplantation regarding quality of life and patient survival, the discrepancy between organ demand and supply is increasing. The Eurotransplant annual report indicates four times as many patients on the waiting list as patients transplanted with a kidney in 1992 (1). Transplantation is normally performed with kidneys from patients who are brain-dead or from family members. Brain-dead donors are commonly referred to as heart-beating (HB) cadaver donors since their heart is beating at the time of organ procurement. Nephrectomy in these donors can be performed with a minimum of ischemic damage to the organs. Unfortunately, HB donor procurement programs are faced with a high refusal rate by relatives (2). Organs from living related donors are often successfully transplanted. Excellent long-term results have been achieved with kidneys in particular. Though the availability of these organs is generally limited, there was in 1992 an increase from 3.9% to 5.5% of the total number of kidney transplants from living related donors in the Eurotransplant area (1). Still, the number of kidneys for transplantation available from these two donor categories is unlikely ever to meet the demand. It is therefore necessary to look for additional sources.

Non-Heart-Beating (NHB) donors are one such possible source of kidneys. NHB donors can be divided in so-called "controlled" NHB donors and "uncontrolled" NHB donors. Controlled NHB donors, are patients who are not yet fully brain dead, but have no chance for survival without artificial ventilation. These patients are taken to the operating room after which the ventilation is stopped and organ retrieval is not started until cardiac arrest. Since all preparations for cooling of the organs have already been made before the ventilation is stopped, the organs can be harvested without practically any warm ischemic damage. The other is the uncontrolled NHB donors. This category of kidney donors comprises patients who have sustained cardiac arrest either prior to arriving in the emergency room or in the emergency room and subsequently have not been able to be resuscitated successfully. These uncontrolled NHB donor kidneys are not utilized frequently, primarily because of the concern that the long warm ischemia times in these donors might have caused irreversible damage to the organs (3).

In 1980, a program to procure and transplant kidneys from uncontrolled NHB donors was started at the University Hospital Maastricht. Using an emergency in situ preservation technique it was possible to salvage the kidneys from these donors for transplantation. The initiator of this so-called Non-Heart-Beating

donor program was Prof. dr. G. Kootstra. With the implementation of this program, overall procurement effectiveness was increased by more than 20% (4). Although initially several transplant centres were reluctant to transplant these ischemically damaged kidneys, now over a hundred transplants of Non-Heart-Beating donor kidneys, harvested in the Maastricht region, have been performed.

Part I of this thesis describes the (clinical) Maastricht Non-Heart-Beating donor protocol and the results of a retrospective analysis of the outcome of Non-Heart-Beating donor kidneys, harvested in the Maastricht region between 1980 and 1992. In part II, five experimental studies concerning the optimal preservation method for ischemically damaged kidneys (chapters 2, 3, and 4) and two pharmacologic agents to limit the ischemia-reperfusion damage after transplantation (chapter 5 and 6), are reported.

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PART I

CLINICAL STUDY

1. Total duration of circulatory arrest less than 30 minutes.
2. Total duration of cerebral anasthesia less than 2 hours.
3. Age under 65 years.
4. No history of chronic disease, severe hypertension, or malignancies other than primary (non-invasive) central nervous system tumors.
5. No signs of systemic infection.
6. No signs of individual drug abuse.

Donor treatment

It is essential that up to the time that permission is granted and the permission contract is formed, cardiac massage and artificial ventilation of the lungs with 100% oxygen are continued. This will provide the organism with oxygenated

1

THE NON-HEART-BEATING DONOR, A NEW SOURCE OF KIDNEYS FOR TRANSPLANTATION

1.1 THE MAASTRICHT NON-HEART-BEATING DONOR PROTOCOL

Donor criteria

All patients who have sustained a permanent circulatory arrest have to be considered as potential kidney donors. In most cases this will concern patients who are admitted to an emergency service with cardiac arrest caused by myocardial infarction or with major trauma who succumb immediately before or upon arrival. When all attempts to restore spontaneous myocardial activity have failed, the physician in charge declares the patient dead and a check list of NHB donor criteria is evaluated (table 1). General kidney donor criteria can be checked after the donor nephrectomy. If the patient is considered suitable for kidney donation, the family is approached to ask permission to harvest the kidneys.

Table 1. Checklist Non-Heart-Beating donor criteria. Efficient resuscitation means sufficient cardiac output and artificial ventilation.

-
1. Total duration of circulatory arrest less than 30 minutes.
 2. Total duration of efficient resuscitation less than 2 hours.
 2. Age under 65 years.
 3. No history of kidney disease, severe hypertension, or malignancies other than primary (non-metastasizing) central nervous system tumours.
 4. No signs of systemic infection.
 5. No signs of intravenous drug abuse.
-

Donor treatment

It is essential that up to the time that permission is granted and the perfusion catheter is inserted, cardiac massage and artificial ventilation of the lungs with 100% oxygen are continued. This will provide the organism with oxygenated

blood and thus limit ischemic damage of the kidneys. To increase the effect of the latter in situ perfusion, we administer Phentolamine and Heparin intravenously to the patient during cardiac massage. Phentolamine is given in a 0.125 mg/kg dose. This alpha adrenergic blocker achieves maximal vasodilatation of renal blood vessels and thus allows better renal perfusion. Consequently, heat exchange will be facilitated and kidney temperature decrease will be faster. Heparin (20.000 IU) may prevent thrombosis of small vessels in the kidney which is likely to occur due to low blood flow during cardiac massage.

Double balloon triple lumen catheter

In 1975 Garcia-Rinaldi (1) introduced the concept of in situ perfusion of kidneys from NHB donors with a double balloon triple lumen (DBTL) catheter. This catheter was designed to block the aorta at his bifurcation and at the level of the diaphragm by means of two balloons. Selective perfusion of the part of the aorta where the renal arteries take their origin is hereby obtained (Fig.1). We use the Porgès DBTL catheter no. AJ 65. which is similar to the catheter introduced by Garcia-Rinaldi. The size of the catheter is 16 Charriere, its total length is 90 cm and the distance between the two balloons is 23 cm. Each balloon can contain 15 ml of fluid. The diameter of a fully inflated balloon is 40 mm. The catheter is made out of polyurethane. This material is flexible at all temperatures and does not induce trauma.

Figure 1. Diagnostic representation of the DBTL catheter when inserted into the abdominal aorta for selective preservation of the kidneys. Outflow is secured through a Foley urinary catheter in the femoral vein.

Insertion of the catheter

The procedure takes place directly in the emergency room where the patient is admitted. For a right-handed surgeon it is preferable to insert the DBTL catheter in the right groin. The insertion area is shaved and disinfected and sterile drapes are applied. A longitudinal incision is made over the femoral vessels from approximately 5 cm above to 10 cm below the inguinal ligament. This incision provides rapid access to and good exposure of the femoral vessels. The femoral artery is identified, blood samples for blood group typing, cross match serum, blood oxygen supply evaluation and biochemical (urea, creatinine,) and serological (hepatitis B surface antigen (Hbsag), hepatitis C virus (HCV), human immunodeficiency virus (HIV), cytomegalovirus (CMV), and *Treponema pallidum* hemoagglutination (TPHA)) diagnostic tests are obtained. After arteriotomy, the DBTL catheter is introduced as far as possible into the common iliac artery and aorta. First, the abdominal balloon is inflated with 10 ml of a sterile radiopaque solution. To hook the balloon on the bifurcation of the aorta, the catheter is retracted as far as possible, and an additional 5 ml of radiopaque solution is administered in order to obtain maximal expansion of the balloon. Now the thoracic balloon is inflated with 15 ml radiopaque solution. In all cases the position of the two balloons of the DBTL catheter is checked with simple X-ray investigation. The catheter is connected a perfusion circuit and care is taken that all air is removed in the perfusion system.

In situ perfusion

Emergency in situ preservation using a DBTL catheter offers the possibility to limit warm ischemia time by rapid and sufficient cooling of the organs. Experimental studies have shown that in situ perfusion results in kidney core temperatures varying from 12°C to 20°C centigrade (2). It is essential that perfusion volume, flow, and pressure can be measured and controlled during in situ perfusion. Low perfusion pressure can trigger a reactive vasoconstriction in perfused organs (3). Therefore, sufficient pressure must be maintained in order to provide uniform cooling of the kidneys and to flush out all blood cells pooling in the peripheral small arteries of the kidney. High perfusion pressures are of particular importance in case of procurement of kidneys from NHB donors, where the vascular resistance of the renal cortical microcirculation is high due to the prolonged warm ischemia (4). In order to guarantee adequate flow and pressure we use a small roller pump (Gambro BP-10, Gambro, Lund, Sweden) which produces an adjustable, pressure controlled, pulsatile flow.

An initial flow rate of approximately 400 ml/min is necessary for rapid cooling of the kidneys and flushing out all blood pooling in the organs. Perfusion pressure is not allowed to rise above 100 mm Hg. Immediately after the perfu-

sion has started, a venectomy of the femoral vein is performed to facilitate venous outflow. A Charriere 18 Foley urinary catheter connected to a sterile urine bag is used for collecting the outflowing blood and preservation fluid. In order to avoid outflow obstruction care should be taken to empty the urine bag regularly. After infusion of 5000 ml of perfusion fluid the flow rate can be reduced. Low flow in situ perfusion (100 ml/min) is continued until donor nephrectomy. The average amount of perfusion fluid needed for the whole procedure is approximately 10-15 litres.

In the literature no limit concerning the admissible duration of the in situ preservation (the time interval between commencement of the in situ perfusion and subsequent donor nephrectomy) has been given. We have seen good transplantation results with kidneys which had been preserved in situ for 3 hours. However, we recommend to keep the time interval between commencement of the in situ perfusion and subsequent donor nephrectomy as short as possible. In our protocol in situ preservation times of up to 2 hours are tolerated.

Perfusion solution

In situ flushing of the kidneys requires a considerable amount of fluid (10 to 15 litres). At present there are three preservation solutions commonly used in the Eurotransplant area; Euro-Collins (EC) (5), University of Wisconsin (UW) (6), and Histidine-Tryptophan-Ketoglutarate (HTK) solution (7).

EC has the disadvantage that it has to be mixed with glucose (70 mL of glucose 50% with 930 mL of EC before use, which is inconvenient during an emergency procedure. Furthermore, the high Potassium concentration (115 mmol/L) of the EC solution leads to contraction of the glomerular capillaries (8,9). Therefore, perfusion with large volumes of EC can cause high vascular resistance and thus hamper optimal perfusion.

Uw solution also requires some additions before use (dexamethasone (16mg/L), insulin (40 IU/L), and penicillin ($2 \cdot 10^5$ IU). Moreover, UW is expensive.

The HTK preservation solution (Custodiol) was originally developed as a cardioplegic solution. It is available in 1 and 5 litre containers, is relatively cheap, and requires no additions before use. The crystalloid HTK solution is an excellent flush out solution due to its low viscosity. Moreover, HTK, unlike EC and UW, continues to have strong buffer capacities in temperatures above 10 centigrade, through a Histidine-HCL buffer of 180/18 mmols (10). This enables the cell to maintain stable and physiological pH during in situ perfusion. At present, HTK is first choice for the situ preservation of NHB donor kidneys in our hospital.

Effectiveness and difficulties

It is difficult to determine the effectiveness of the in situ cooling before nephrectomy. An impression can be obtained by evaluating surface temperature and skin colour in the flanks of the patient. The outflow of clear fluid indicates that blood is adequately flushed out of the organ. During nephrectomy special attention has to be paid to kidney temperature and colour. A kidney core temperature of less than 15°C is considered sufficient to reduce the metabolic demands in order to stop the decay of renal cells. The typical pale grey appearance of the kidney indicates it is adequately flushed.

Difficulties that may be responsible for the failure of the in situ kidney preservation in NHB donors can be divided into two categories. First, patient dependent factors such as anatomical disorders of iliac arteries, aorta or kidney arteries which are not uncommon in this group of patients. Malformation or occlusion of iliac arteries and aorta mostly due to severe arteriosclerosis may hinder insertion of the DBTL catheter. Also, renal artery stenosis may cause insufficient cooling of the kidneys even with high perfusion pressures. Perfusion fluid flow will be mainly through the mesenteric arteries in those cases. Second, procedure dependent difficulties we have encountered are: bursting of one of the balloons, obstruction of the venous outflow and inadequate positioning of the balloons.

Organization

In order to ensure excellent co-operation between the members of the emergency team, a transplant coordinator is in charge of the whole procedure. The minor surgical procedure required for in situ kidney preservation can be performed by the surgical resident on call. After each NHB donor, the procedure is evaluated and preservation kit and perfusion fluid are replenished. The protocol is yearly discussed and distributed amongst all those involved: emergency personnel, surgeons, anaesthesiologists, neurologists and cardiologists.

Legal aspects

Presently, Dutch law requires relatives consent for any medical procedure to be carried out on a cadaver even in the presence of a donor card. If relatives can not be located in time the procedure has to be cancelled. It is legally not permitted to start in situ preservation while awaiting consent for kidney donation. Due to these restrictions we lose approximately 15% of the total potential of Non-Heart-Beating donors. Fortunately, future legislation will provide the possibility to introduce the DBTL catheter without relatives consent and family approval for organ donation will only be necessary if the potential donor does not have a donor card. In case of an unnatural death (traffic accident) however,

permission of the legal authorities has to be obtained before the actual donor nephrectomy.

1.2 POST-TRANSPLANT OUTCOME OF NON-HEART-BEATING DONOR KIDNEYS, HARVESTED IN MAASTRICHT, BETWEEN 1980 AND 1992

Study group

To investigate the outcome of kidney transplantation using NHB donors, a retrospective multi-centre study was performed evaluating the post-transplant function of 57 NHB donor kidneys harvested in our procurement region (1 University Hospital and 4 district general hospitals, covering a population of 750,000) between 1980 and 1992 and transplanted in the Eurotransplant area (Austria, Belgium, Germany, Luxembourg and The Netherlands). Collaboration within the Eurotransplant exchange organisation implies a uniform centre policy of donor organ allocation according to restricted ABO blood group compatibility, HLA-A, -B, and -DR matching criteria, urgency code and time on the waiting list to centrally registered patients with end-stage renal disease. It also guarantees standardised techniques and reagents for donor and recipient HLA typing and cross-matching in a coordinated network of laboratories.

Matched control group

From the Eurotransplant files a matched group of kidneys from HB) donors was compiled to serve as a control group. For each NHB donor kidney recipient, two HB donor kidney recipients were selected. Matching was performed by stratification per recipient centre, thus preventing bias caused by post-transplant management (e.g., thromboembolic prophylaxis, hydration protocol, immunosuppressive therapy and treatment of acute rejection). Both groups were matched for the time period in which the transplantation took place (difference < 2 years), number of transplant (first, second or third), highest and latest level of Panel Reactive Antibodies ($PRA \leq 5\%$, $6-85\%$, $> 85\%$), immunosuppressive therapy given, donor age (difference < 10 years), recipient age (15-50 or > 50 years), and preservation solution used: Eurocollins (EC), University of Wisconsin solution (UW) or Histidine Tryptophan Ketoglutarate (HTK).

Data collection

All recipient centres were asked to collaborate in this retrospective multi-centre study. Data collection was performed by visiting all participating centres. The data collected included donor and recipient characteristics, and short- and long-term follow-up post-transplant.

Statistical analysis

All data are given as median values with 5 and 95 percentiles. Statistical analysis of the donor and recipient data was conducted in order to ascertain the adequacy of matching. Differences were evaluated using the Chi-square test with Yates correction or using the Fisher exact test for discrete variables and the Mann-Whitney U test for continuous variables. The post-transplant function of each kidney was classified as immediate, delayed, or primary non function. Immediate function (IF) was considered to be life-sustaining renal function without the need for post-transplant dialysis; delayed function (DF) was considered to be a kidney function that did ultimately support the patient, but required post-transplant dialysis; and primary non-function (PNF) was identified when renal function failed to reduce the recipient's serum creatinine and when the patient could not be maintained without dialysis. The rates for IF, DF and PNF for both groups were compared with the Chi-Square test. A multi-variate analysis using a logistic regression model, including a forward stepwise selection procedure, was performed to identify independent risk factors for DF, in addition to a possible donor type (HB or NHB) effect. The factors analyzed included: donor-age, preservation solution, cold ischemia time, anastomosis time, recipient-age, prior transplants, latest and highest panel reactive antibodies, number of HLA-A, -B and -DR mismatches and immunosuppressive therapy with or without Cyclosporin A. Serum creatinine and creatinine clearance were documented at 1, 3, 6 and 12 months after transplantation. A ANOVA test with repeated measurements was used to test for significant differences between the two groups.

To estimate graft survival, all causes of graft loss were included as determined by return to maintenance dialysis treatment, transplant nephrectomy, or patient death irrespective of graft function. Graft and patient survival were analyzed by Cox proportional hazard models (Cox regression analysis). Seven 'causal' factors were regarded relevant in predicting graft and patient survival (basic model). These factors include: donor age, recipients age over 50 years or not, latest and highest PRA, immunosuppressive therapy with or without cyclosporin A, preservation solution and number of transplantations. In addition to the seven factors for which a matching was performed (causal factors), the effect allowance for potential confounders on graft and patient survival was analyzed. These confounders include the donor demographic characteristics: gender, blood group, serum creatinine, left/right kidney, number of renal arteries, arterial patch (yes/no), aorta sclerosis (yes/no), multiple organ donor and cold ischemia time; and recipient demographic characteristics: gender, blood group, renal disease, dialysis type, months of dialysis, urine output pretransplant, HLA-A, -B and -DR mismatches, anastomosis time and prophylactic ATG or OKT3. Interaction for

each causal and confounding factor with type of kidney donor (NHB or HB) were analyzed. The differential risks for both groups of donor kidneys as to patient and graft survival were analyzed for statistical significance. Additional analysis was performed to determine the effect of delayed function on survival of the graft.

All data were analyzed by SPSS-PC Windows version 6.0 (1993). A p-value less than 0.05 was considered significant.

Procurement rate and loss of data

Over a period of 12 years (1980-1992) we harvested 112 kidneys from 56 NHB donors. Of these, 38 NHB kidneys were not used due to insufficient flushing and cooling of the kidneys during in situ preservation, physical damage to the kidney before or during harvesting, suspicion of bacterial contamination, or unavailability of a suitable recipient. Of the 74 transplanted kidneys, 14 were transplanted outside the Eurotransplant area and 60 within. The latter were distributed among 23 transplant centres, 21 of which collaborated in this study. As a result, 57 NHB kidneys were finally included in this study. The matched control group of HB donor kidneys has included 114 cases for comparison.

Demographic characteristics

All NHB donors died of irreversible cardiac arrest caused by trauma capitis (42%), myocardial infarction (37%), intracranial haemorrhage (18%), and other causes (3%). Causes of death in the HB group were intracranial haemorrhage (52%), trauma capitis (44%), and cerebral tumour (4%). Table 2 shows other donor characteristics for both groups. Only kidneys were harvested from the NHB donors, whereas 19% of the HB donors were multiple organ donors (kidneys, heart and liver). All kidneys were preserved by cold storage. Both groups show similar numbers of kidneys preserved with EC, 74% versus 78% respectively. The NHB group shows a higher number of HTK preserved kidneys. In the HB group the number of UW preserved kidneys is higher, due to the multiple organ donors in this group. Table 3 shows the recipient profile for both groups. No differences in demographic variables were seen.

Table 2. Relevant donor data (*NHB*, non-heart-beating donor; *HB*, heart-beating donor; *EC*, Euro-Collins cold storage solution; *UW*, University of Wisconsin cold storage solution; *HTK*, histidine tryptophane ketoglutarate cold storage solution).

		NHB ^a (n=57)		HB ^a (n=114)		P-value ^b
<u>Donor characteristics</u>						
Donor age (years)		49	(7-61)	43	(7-60)	0.68
Donor sex	- Male	37	(65%)	68	(60%)	0.62
	- Female	20	(35%)	46	(40%)	
Blood group	- O	15	(26%)	54	(47%)	0.05
	- A	35	(62%)	52	(46%)	
	- B	4	(7%)	6	(5%)	
	- AB	3	(5%)	2	(2%)	
Serum creatinine (μmol/L)		102	(49-181)	88	(48-155)	0.12
<u>Kidney characteristics</u>						
- Left kidney		28	(49%)	62	(54%)	0.63
- Right kidney		29	(51%)	52	(46%)	
1 renal artery		42	(74%)	85	(75%)	0.95
> 1 renal arteries		15	(26%)	29	(25%)	
Arterial patch	- Yes	55	(96%)	111	(97%)	1.00
	- No	2	(4%)	3	(3%)	
Aorta sclerosis	- Yes	11	(19%)	13	(11%)	0.24
	- No	46	(81%)	101	(89%)	
<u>Preservation characteristics</u>						
Multiple Organ Donor	- Yes	0	(0%)	22	(19%)	0.001
	- No	57	(100%)	92	(81%)	
First warm ischemia time (min)		30	(8-80)	0	(0-8)	0.001
Cold ischemia time (hours)		28	(15-44)	26	(16-40)	0.39
Kind of Perfusate	- EC	42	(74%)	89	(78%)	0.01
	- UW	1	(2%)	13	(11%)	
	- HTK	14	(24%)	12	(11%)	

a. Continuous variables are shown as median values (percentiles 5 and 95), discrete variables as number of patients (%).

b. Mann-Whitney test was used for continuous variables; Chi-square test with Yates correction or Fisher exact test for discrete variables. A P-value < 0.05 was considered significant.

Table 3. Relevant recipient data (*NHB*, non-heart-beating donor; *HB*, heart-beating donor; *PRA*, panel-reactive antibodies; *highest*, highest level pretransplantation; *latest*, last PRA prior to transplantation).

	NHB ^a (n=57)		HB ^a (n=114)		P-value ^b
Recipient age (yr)	44	(23-64)	45	(19-61)	0.62
Recipient sex					
- Male	41	(72%)	75	(66%)	0.52
- Female	16	(28%)	39	(34%)	
Blood group					
- O	13	(23%)	43	(38%)	0.14
- A	37	(65%)	56	(49%)	
- B	3	(5%)	10	(9%)	
- AB	4	(7%)	5	(4%)	
Recipient renal disease					
- Glomerulonephritis	17	(30%)	55	(48%)	0.24
- Pyelo/Interstitial nephritis	8	(14%)	13	(12%)	
- Cystic kidney disease	7	(12%)	9	(8%)	
- Diabetes Mellitus	4	(7%)	7	(6%)	
- Others	21	(37%)	30	(26%)	
Dialysis type					
- Hemodialysis	49	(86%)	92	(81%)	0.39
- CAPD	6	(11%)	20	(17%)	
- Not yet	2	(3%)	2	(2%)	
Months on dialysis	33	(0-129)	27	(4-125)	0.34
Urine output pretransplant (ml/24 hr)	275	(0-1870)	300	(1-1500)	0.79
Prior transplant					
- 0	51	(90%)	97	(85%)	0.58
- ≥1	6	(10%)	17	(15%)	
PRA latest					
- ≤ 5%	46	(81%)	93	(82%)	0.59
- 6 - 85%	10	(17%)	16	(14%)	
- > 85%	1	(2%)	5	(4%)	
PRA highest					
- ≤ 5%	39	(68%)	78	(68%)	0.90
- 6 - 85%	13	(23%)	28	(25%)	
- > 85%	5	(9%)	8	(7%)	
HLA A mismatches					
- 0	24	(42%)	50	(44%)	0.82
- 1	27	(47%)	49	(43%)	
- 2	6	(11%)	15	(13%)	
HLA B mismatches					
- 0	20	(35%)	54	(47%)	0.11
- 1	28	(49%)	52	(46%)	
- 2	9	(16%)	8	(7%)	
HLA Dr mismatches					
- 0	42	(74%)	79	(69%)	0.74
- 1	13	(23%)	32	(28%)	
- 2	2	(3%)	3	(3%)	
Anastomosis time (min)	32	(19-64)	35	(20-60)	0.47
Immunosuppressive protocol					
- CyA + steroids	25	(44%)	59	(52%)	0.57
- Imuran + steroids	20	(35%)	32	(28%)	
- CyA + Imuran + steroids	12	(21%)	23	(20%)	
Prophylactic ATG or OKT3					
- Yes	5	(9%)	17	(15%)	0.37
- No	52	(91%)	97	(85%)	

- Continuous variables are shown as median values (percentiles 5 and 95), discrete variables as number of patients (%).
- Mann-Whitney test was used for continuous variables; Chi-square test with Yates correction or Fisher exact test for discrete variables. A P-value < 0.05 was considered significant.
- PRA: Panel Reactive Antibodies (highest = highest level pretransplantation; latest = last PRA just before transplantation).

Short-term results

Data are shown in table 4. The number of kidneys with primary non function was higher in the NHB donor kidney group, although not significantly different from the HB kidney group (14% versus 8%, $p=0.32$). Eight NHB donor kidneys showed PNF after transplantation. Only one pair of kidneys from one single donor showed PNF in both cases. The outcome of the contralateral kidneys from the other six NHB donors suggested that the donor factor had only played a minor role; two IF, three DF, and one not transplanted). The contralateral kidneys from the nine PNF kidneys in the HB group also showed one 'pair' of kidneys with PNF.

Table 4. Short-term results (NHB, non-heart-beating donor; HB, heart-beating donor).

	NHB ^a	(n=57)	HB ^a	(n=114)	P-value ^b
Kidney function post-Tx					
- Immediate function	15	(26%)	65	(57%)	0.0003
- Delayed function	34	(60%)	40	(35%)	0.004
- Primary non-function	8	(14%)	9	(8%)	0.32
Diuresis first 24 hrs post-Tx (ml)	130	(0-3424)	1000	(0-7120)	0.015
Hospital stay (days)	32	(14-68)	23	(13-66)	0.005
Number of Dialysis post-Tx	5	(2-17)	4	(1-11)	0.085
Period of Dialysis post-Tx (days)	14	(4-37)	12	(1-30)	0.083
Number of acute rejections ^c					
- 0	27	(47%)	57	(50%)	0.75
- 1	22	(39%)	40	(35%)	
- 2 or more	5	(9%)	14	(12%)	
- not recorded ^d	3	(5%)	3	(3%)	

a. Continuous variables are shown as median values (percentiles 5 and 95), discrete variables as number of patients (%).

b. Mann-Whitney test was used for continuous variables; Chi-square test with Yates correction or Fisher exact test for discrete variables. A P-value < 0.05 was considered significant.

c. Number of rejection treatments during the first three months post-transplantation.

d. The not recorded data are not included in the analysis.

Kidneys from NHB donors showed a higher percentage of DF after transplantation (60% versus 35%, $p=0.004$). The higher percentage of DF in the NHB donor kidney group was reflected in a lower first 24-h diuresis and resulted in a longer hospital stay. In such cases, there were no differences between the NHB and HB group in the number of dialysis treatments needed (five and four times, respectively, $p=0.085$) or the time period in which dialysis was necessary (14 and 12 days, respectively, $p=0.083$).

The number of acute rejections, defined as the number of rejection treatments within the first 3 months after transplantation, did not differ between the NHB and HB donor grafts ($p=0.74$). Delayed graft function was significantly associated with a higher number of acute rejections after transplantation in both groups ($p=0.04$, Chi-square test with Yate's correction).

A multivariate logistic regression model, including a forward stepwise selection procedure, was used to investigate independent risk factors for DF of the graft (table 5). Besides the type of donor (NHB or HB), one other factor (having 1 or 2 HLA-DR mismatches) remained statistical significant with respect to the occurrence of DF ($p=0.0009$). With this factor taken into account, the difference in incidence of DF after transplantation in recipients of a NHB donor kidney compared to those who received a HB donor kidney was still significant ($p=0.0008$).

Serum creatinine values and creatinine clearance at 1, 3, 6, and 12 months after transplantation for both groups are shown in table 6. NHB donor kidneys showed significantly higher serum creatinine values at 1 month post-transplantation ($p=0.003$). At 3, 6 and 12 months, the function of the graft did not differ between both groups.

Table 5. Potential risk factors for delayed graft function (*NHB*, non-heart-beating donor; *HB*, heart-beating donor; *DF*, delayed function; *IF*, immediate function; *EC*, Euro-Collins cold storage solution; *UW*, University of Wisconsin cold storage solution; *HTK*, histidine tryptophane ketoglutarate cold storage solution; *CyA*, cyclosporin A).

		DF ^a (n=74)		IF ^a (n=80)		P-value ^b
Donor and preservation factors						
Donor type	- NHB	34	(46%)	15	(19%)	0.001
	- HB	40	(54%)	65	(81%)	
Donor age	- ≤50 years	48	(65%)	50	(63%)	0.77
	- >50 years	26	(35%)	30	(37%)	
Preservation solution	- EC	59	(80%)	57	(71%)	0.23
	- HTK	5	(7%)	9	(11%)	
	- UW	10	(13%)	14	(18%)	
Cold ischemia time	- ≤24 hrs	24	(33%)	26	(33%)	0.67
	- 25-36 hrs	35	(47%)	40	(50%)	
	- >36 hrs	15	(20%)	14	(17%)	
Anastomosis time	- ≤45 min	54	(73%)	60	(75%)	0.81
	- >45 min	13	(18%)	16	(20%)	
	- not recorded	7	(9%)	4	(5%)	
Recipient factors						
Recipient age	- ≤50 years	42	(57%)	51	(64%)	0.55
	- >50 years	32	(43%)	29	(36%)	
Prior transplantation	- 0	66	(89%)	67	(84%)	0.25
	- ≥1	8	(11%)	13	(16%)	
Highest PRA	- ≤5%	51	(69%)	54	(67%)	0.54
	- 6-85%	15	(20%)	24	(30%)	
	- >85%	8	(11%)	2	(3%)	
Latest PRA	- ≤5%	59	(80%)	65	(81%)	0.61
	- 6-85%	11	(15%)	13	(16%)	
	- >85%	4	(5%)	2	(3%)	
HLA-A mismatches	- 0	30	(41%)	38	(47%)	0.50
	- 1, 2	44	(59%)	42	(53%)	
HLA-B mismatches	- 0	32	(43%)	38	(47%)	0.62
	- 1, 2	42	(57%)	42	(53%)	
HLA-DR mismatches	- 0	45	(61%)	64	(80%)	0.002
	- 1, 2	29	(39%)	16	(20%)	
Immunosuppression	- with CyA	53	(72%)	57	(75%)	0.94
	- without CyA	21	(28%)	23	(25%)	

a. Discrete variables are shown as number of patients (%).

b. Multivariate analysis using a logistic regression model. A P-value < 0.05 was considered significant.

Table 6. Median serum creatinine values and median creatinine clearance of the functioning kidneys up to 12 months post-transplantation (NHB, non-heart-beating donor; HB, heart-beating donor).

Months post-Tx	S-creatinine (μmol/l)			Creatinine clearance (ml/min)		
	NHB	HB	P-value ^a	NHB	HB	P-value ^a
1	207 (104-981)	160 (87-868)	0.003	38 (6-83)	41 (7-96)	0.21
3	160 (106-423)	153 (87-440)	0.19	41 (14-101)	54 (12-122)	0.21
6	163 (99-388)	153 (88-413)	0.37	56 (15-126)	52 (22-113)	0.74
12	160 (106-284)	141 (80-422)	0.28	45 (16-96)	50 (16-115)	0.79

a. The median levels of all patients were computed since the data obtained were not equally distributed. A ANOVA test with repeated measurements was used to test for significant differences between the two groups. A P-value < 0.05 was considered significant.

Long-term results

The mean follow-up was 85 months (range 26-165 months). Patient survival rates calculated at 1, 3 and 5 years post-transplantation were 91%, 82% and 75%, respectively, in patients with a NHB donor kidney versus 87%, 79%, and 77% in patients who received a HB donor kidney ($p=0.344$; figure 2). The 1, 3 and 5-year graft survival was 73%, 61%, and 54% for NHB kidneys versus 73%, 60%, and 55% in the matched control group ($p=0.873$; figure 3). The prognostic value of DF on graft survival was also analyzed. Delayed graft function was not associated with an inferior survival of the graft ($p=0.630$; figure 4).

Patient survival

Donor type (HB or NHB) had no effect on patient survival ($p=0.873$). None of the interactions between donor type and any of the causal factors of the basic model was significant. P-values ranged between 0.414 and 0.976. None of the 21 confounding factors with donor type had p-values lower than 0.05. Interactions of the 21 confounding factors with donor type were analyzed. The lowest

p-value in its interaction with donor type was donor serum creatinine ($p=0.03-22$). Deviding our data into NHB and HB donor kidneys to analyze the differential effects of donor serum creatinine, it turned out that a high donor serum creatinine was a 'marginal significant' risk factor in NHB donor kidney group ($p=0.076$).

Graft survival

Donor type had no effect on graft survival ($p=0.344$). One of the interactions between donor type and a causal factor of the basic model was significant; donor age ($p=0.039$). If we split our data into NHB and HB groups, we find that a high donor age is a clear risk factor in the NHB group ($p=0.019$), but not in the HB group ($p=0.800$). None of the 21 confounding factors with donor type had p-values lower than 0.05. The analysis of interactive effects of donor type and potential confounding variables, showed one confounding factor with a p-value lower than 0.05; the cold ischemia time ($p=0.032$). If one categorizes the duration of cold ischemia time into three groups (≤ 24 hours, 25 to 36 hours, and > 36 hours) only the group above 36 hours of cold ischemia time appeared to be a higher risk factor in the NHB compared to the HB group.

Figure 2. Graft survival in the NHB and HB donor group ($p=0.344$, by Cox regression analysis).

Figure 3. Patient survival in the NHB and HB donor group ($p=0.873$, by Cox regression analysis).

1.3 DISCUSSION

The growing success in renal transplantation has resulted in an increase in the need of donor organs. Procurement of kidneys from HB cadaver donors or living related donors seems to have reached its maximum potential. Although a more liberal legislation and a more positive public attitude towards organ donation and transplantation - which could reduce the number of refusals - might increase the effectiveness of these conventional donor programs, using these sources exclusively, the number of suitable donor organs is unlikely ever to meet the demand. NHB donors offer a yet untapped source of renal grafts.

The extent of the warm ischemic damage to the kidneys from asystolic donors is a major concern. In order to slow down metabolism and to prevent the kidneys from further decay, immediate cooling of the kidneys is mandatory. The emergency in situ preservation prior to nephrectomy, using a DBTL catheter, is simple and can be performed by the surgical resident on call. Other methods to salvage the kidneys of NHB donors such as extracorporeal cardiopulmonary bypass and total body cooling have been described (11,12). These methods are, however, time-consuming and require more qualified personnel. Theoretically, it would also be possible to rapidly transfer the body to the operating room, after declaration of death, for immediate nephrectomy. In most smaller hospitals

this would, however, cause logistic problems (no immediate availability of a standby operating room and personnel).

The results of our retrospective study comparing the outcome of NHB and HB donor kidneys, respectively, show that long-term patient and graft survival are similar for both groups. This important finding justifies the use of kidneys from donors who have suffered irreversible cardiac arrest for transplantation. Special risk factors which have a negative predictive value on graft or patient survival are, however, a high donor serum creatinine, a high donor age and a long cold ischemia time.

One negative aspect related to the use of NHB donor kidneys is the high incidence of DF of the graft. In our study, the incidence of DF was found to be nearly twice as high in the NHB group (60%) as in the HB group (35%). This high incidence of DF after transplantation of kidneys which are subjected to prolonged warm ischemia is confirmed by data from other centres (13,14,15). Analyzing all donor and recipient factors that could influence occurrence of DF using multi-variance analysis, besides the type of donor (NHB or HB), the number of HLA-DR mismatches was significantly associated with a higher incidence of DF of the graft. This finding confirms reports in literature, suggesting a possible immunologic factor in the onset of DF and stressing the importance of adequate HLA matching (16,17). In contrast to two recently published studies performed by other groups (18,19), our results did not identify the type of preservation solution used as a risk factor for DF. The reason for this discrepancy might be the fact that, in our study the EC solution was predominantly used to preserve the kidneys in both groups, thus obscuring any possible influence of other solutions.

Delayed function interferes with optimal patient treatment and has been said to both increase the number of rejection episodes and reduce graft survival (20,18). Our study confirms the higher number of rejection episodes in kidneys with DF. Delayed function is thought to affect the number of acute rejections of the graft by a more pronounced presentation of adhesion molecules and HLA class II antigens, as well increased phagocytic/inflammatory activity in the damaged organ. It is suggested that adhesion molecules may promote rather 'unspecific' adhesion of leukocytes, thus contributing to the process of cellular allograft infiltration (21,22,23). In contrast to the study by Ploeg et al (18), DF was not significantly associated with inferior graft survival in our study.

An important issue is the cost-effectivity of a NHB donor program. Patients transplanted with NHB donor kidneys had a longer hospital stay caused by the

higher percentage of DF. We assume that the overall costs of transplantation, as a result, be higher in NHB donor kidney recipients. The long-term graft survival, however, accounts for considerable savings on health care costs compared to the long-term dialysis treatment. Without our NHB donor program fewer patients would have been transplanted, so the use of NHB donor kidneys for transplantation is also justified from a financial point of view.

It is concluded that when the high incidence of delayed function of the graft can be diminished, the Non-Heart-Beating donor can be a valuable asset to reduce the current shortage of kidneys for transplantation.

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PART II

EXPERIMENTAL STUDIES

UNIVERSITY OF WISCONSIN SOLUTION IS SUPERIOR TO HISTIDINE TRYPTOPHAN KETOGLUTARATE FOR PRESERVATION OF ISCHEMICALLY DAMAGED KIDNEYS

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ABSTRACT

The current shortage of transplantable organs has renewed interest in kidneys obtained from Non-Heart-Beating (NHB) donors. Kidneys from these donors have suffered warm ischemia (WI). The effectiveness of two preservation solutions, i.e. the University of Wisconsin (UW) and the Histidine Tryptophan Ketoglutarate (HTK) solutions, for preservation of kidneys which have been subjected to WI was tested in dogs. The left kidney was auto-transplanted after 30 min of WI, and subsequent 24 hours cold storage (CS) in either UW (n=6) or HTK (n=6), with immediate contralateral nephrectomy. Surgical biopsies from the cortex were taken before WI, after 30 min of WI, after 24 hours of CS, and following 1 hour of reperfusion for electron microscopy and for analysis of energy metabolites. *Survival*: at two weeks after transplantation in the UW group 4 out of 6, and in the HTK group 1 out of 6 dogs survived. *Renal function*: as from day 2, serum creatinine was lower in the UW group as compared to the HTK group ($p < 0.05$). *Ultrastructural damage*: after 24 hours of cold storage, in the HTK group the luminal membranes of proximal tubular cells were partly denuded of microvilli. Moreover, the tubular lumen was filled with blebs and debris. In the UW group, the brush borders remained intact, although microvilli were swollen. *Energy metabolism*: energy metabolites were analyzed with HPLC. Thirty minutes of WI resulted in a $\pm 45\%$ reduction of total adenine nucleotide (TAN) content. During CS, TAN levels further decreased in both groups, however after 24 hours of CS the levels of adenosine, inosine, hypoxan-

thine and xanthine were significantly higher in the UW group as compared to the HTK group ($p<0.05$, $p<0.01$, $p<0.01$, $p<0.01$). At 1 hour of reperfusion TAN levels were higher in the UW group as compared to the HTK group (median; 4.74, interquartile ranges; 4.22-4.98 versus median; 4.06, interquartile ranges 3.40-4.65, $p<0.05$). *Conclusion:* our results show that UW is a superior solution compared with HTK in the preservation of ischemically damaged kidneys, demonstrating better survival, better recovery of kidney function, better protection against ischemia-induced ultrastructural damage and better preservation of energy metabolism indicated by (a faster) regeneration of TAN levels after reperfusion.

INTRODUCTION

The current shortage of transplantable organs has renewed interest in kidneys obtained from Non-Heart-Beating (NHB) donors. Effective use of NHB donor kidneys is hampered by the fact that these kidneys have suffered warm ischemia resulting in a high incidence of delayed function of the graft (1,2,3). One of the important determinants of post-transplant outcome is the efficacy of the preservation of the donor organ.

In this study we compared the two preservation solutions most frequently used in the Euro-Transplant exchange organisation (University of Wisconsin (UW) and Histidine Tryptophan Ketoglutarate (HTK) solution) to determine which one is the most effective in preservation of kidneys that have been subjected to prolonged warm ischemia.

Introduction of the UW solution in 1986 has had a major impact on solid organ transplantation. The solution has extended the safe preservation time from 6 to 72 hr for the pancreas and from 6 to 48 hr for the liver, and is effective for up to 72 hr cold storage of the kidney (4,5,6). Currently, UW solution is the standard in clinical preservation of all abdominal organs during multiorgan procurement and is preferable for longer-term (> 24 hr) preservation of kidneys from Heart-Beating (HB) donors (7). Theoretically, UW solution is also an ideal solution for the preservation of kidneys that have been subjected to prolonged warm ischemia. Warm ischemia results in a rapid breakdown of cellular adenine nucleotides (8). During hypothermic preservation, the nucleotide catabolism is considerably reduced, although still present (9). UW solution contains adenosine, a nucleotide precursor, which appears to improve the ATP resynthesis in isolated tubules (10). Since adenine nucleotides are a critical link between energy production and energy utilization, it seems logical that rapid restoration of these

metabolites upon reperfusion of the graft will be protective against the deleterious effects of warm ischemia on posttransplant function.

HTK solution, originally designed for cardio-protection and in clinical use for many years in open heart surgery, was introduced for human kidney preservation in 1987 (11). The first reports claim HTK to be an effective preservation solution for short-term (up to 24 hr) preservation of kidneys from HB donors (12). Compared with UW solution, HTK has a low viscosity and a high buffering capacity. Both characteristics may be beneficial in case of kidneys from NHB donors. Kidneys that suffered a considerable period of warm ischemia exhibit high intrarenal resistances, which may hinder adequate flushing (13). Furthermore, the pH of the renal tissue usually is very low after warm ischemia (14).

The two preservation solutions were tested in canine model for auto-transplantation of kidneys which were subjected to 30 min of warm ischemia in situ, ex vivo flushing and subsequent 24-hr cold storage. Besides a survival protocol, efficacy of preservation was evaluated by assessing the morphological changes in the proximal tubular cell and the ability of the cell to regenerate high energy nucleotides at reperfusion.

MATERIALS AND METHODS

Animals and anesthesia

Experiments were performed on 12 female Beagle dogs age 2-4 years, and weighing 10-12 kg. The animals were fasted during the 12 hr before surgery with free access to water. Anesthesia was induced with Thiopental (20 mg per kg body weight) and maintained with a nitrous oxide-oxygen gas mixture delivered continuously by a positive pressure respirator via an endotracheal tube. An antibiotic prophylaxis of 500 mg ampicillin was given before each operation. During surgery the dogs were hydrated by intravenous infusion of 500 mL Ringer's lactate solution and body temperature was kept constant (37°C) using a thermostatic watermattress. All experiments were performed after approval by the responsible ethics committee, and principles of laboratory animal care according to the NIH standards were followed.

Donor operation

The kidneys were exposed through a midline abdominal incision. Heparin (1000 IU) was given 10 min before induction of warm ischemia in order to prevent thrombosis. The left kidney was carefully dissected from the surrounding tissue.

Ischemia was induced by cross-clamping the left renal vessel pedicle. During the ischemic interval the abdomen was kept closed to maintain the kidney at body temperature.

Preservation and experimental groups

After 30 min of warm ischemia the kidney was removed and flushed with 200 mL 4°C preservation solution. Flushing was performed by gravity from a bottle at 100 cm height. Subsequently, the kidney was wrapped in a sterile plastic bag containing 500 mL preservation solution, and stored for 24 hr on melting ice. The animals were allocated to two experimental groups. In six dogs the UW solution was employed for flushing and cold storage of the kidney; in the other six dogs, the HTK solution was used.

Recipient operation

At the start of the recipient operation, morphine (0.5 mg/kg) was given intravenously in order to prevent intestinal invagination. Surgery started with removal of the right kidney. The preserved (left) kidney was then re-implanted autologously onto the iliac vessels in the right lower abdomen. During vascular anastomosis rewarming was prevented by maintaining the kidney at 4°C with sterile saline. Sorbitol 20%, 150 mL, was administered intravenously after completion of the anastomoses in order to induce osmotic diuresis after implantation. The ureter was implanted into the bladder using a technique described by Politano (15). A catheter for blood sampling was placed in the right jugular vein. After recovery from surgery the dogs were allowed free access to food and water.

Cortical biopsies; electron microscopy, energy metabolites

Wedge-shaped cortical biopsies were taken from the left kidney at the following time points: upon exposure, after 30 min of warm ischemia, after 24 hr of preservation, and after 1 hr of reperfusion. Half of each biopsy was fixed in glutaraldehyde 3%, and stored at 4°C. These samples were used for electron microscopic evaluation. After immersion fixation in 3% glutaraldehyde (pH 7.3), the tissue blocks were washed in 0.1 M sodium cacodylate buffer (pH 7.3), and postfixed for 1 hr in 1% osmium tetroxide. The blocks were then dehydrated through a graded series of alcohols, passed through propylene oxide, and embedded in Taab-resin. Of each block four or five sections ($\pm 600 \text{ \AA}$) were cut, mounted on copper grids and double stained with uranyl acetate and lead citrate. A Hitachi H600-A transmission electron microscope was used to examine each section at an acceleration voltage of 60 kV. The other half of the cortical biopsy was immediately frozen in liquid nitrogen, stored at -80°C, and used for analysis of energy metabolites. The concentration of energy metabolites in the cortical

biopsies was determined using a HPLC method as described by Van der Vusse et al (16). Before analysis the tissue samples were lyophilized at -30°C . Adherent blood was removed. The samples were weighed and extracted in 25-50 $\mu\text{L}/\text{mg}$ tissue HClO_4 (2 mol/L), containing 5 mmol/L dithiothreitol. The tissue HClO_4 mixture was centrifuged at 1200 G for 5 min at 4°C and the supernatant was frozen in liquid nitrogen. Following addition of 40-80 $\mu\text{L}/\text{mg}$ tissue KHCO_3 (2 mol/L) to the supernatant, this mixture was allowed to thaw during centrifugation at 1200 G at 4°C for 1 hr, during which neutralization took place. A Varian Vista 5000 (Varian, Walnut Creek, California, USA) equipped with a narrow bore, stainless steel column filled with spherical liChrosorb RP-18 particles of 5 μm (Merck, Darmstadt, Germany) was used for a gradient HPLC analysis. Injection volume of standard or sample extract was 10 μL . After each single run which took 30 min the column was re-equilibrated for 15 min. All chemicals were obtained from Merck. Peaks were quantitated at 254 nm with a Varian 604 data-system using a conversion factor of peak area per concentration of a known external standard. Standards were of the highest purity available. ADP, AMP, and IMP were purchased from Boehringer (Mannheim, Germany), hypoxanthine (HX) and adenosine (ADO) from Merck and ATP, inosine (INO) and xanthine (X) from Sigma (St. Louis, USA).

Posttransplant function and survival

Renal function was determined by daily serum creatinine measurements (in $\mu\text{mol}/\text{L}$) using a Dimension creatinine kit (DuPont de Nemours, Wilmington, DE, USA). Animals with life-sustaining function up to 14 days, were considered to be survivors.

Data analysis

All values reported are medians with interquartile ranges (25th and 75th percentiles). Energy metabolite measurements are given in $\mu\text{mol}/\text{g}$ dry weight of tissue. Total adenine nucleotide (TAN) levels were defined as the sum of ATP, ADP, and AMP. The total level of degradation products (TDP) were determined by summing IMP, ADO, INO, HX, and X. For statistical analysis of differences in daily serum creatinine values and energy metabolites between the two experimental groups the nonparametric Kruskal-Wallis test was used. The level of significance was set at 5%.

RESULTS

Posttransplant renal function and survival

In the HTK group, one of six dogs survived the study period of two weeks. Of the six dogs that received a UW-preserved kidney four survived. As from day two, mean serum creatinine values were significantly lower in the UW group compared with the HTK group (Figure 1).

Figure 1. Daily median serum creatinine values ($\mu\text{mol/L}$) with 25th and 75th percentiles measured before nephrectomy and subsequently for the first 14 days after implantation of the graft. As from day two there is a significant difference in serum creatinine values between the two groups. († refers to death of an animal)

Electron microscopic evaluation

Tissue samples taken before induction of ischemia and after 30 min of warm ischemia showed no signs of proximal tubular damage. After 24-hr cold storage preservation, damage was apparent in both experimental groups. Condensation and margination of chromatin was seen in the nuclei, and mitochondria showed some swelling. The apical cytoplasm contained vacuoles of small size. In the HTK group, the luminal membranes of proximal tubular cells were partly

denuded of microvilli (Figure 2). Moreover, the tubular lumen was filled with blebs and debris from protruding cell cytoplasm together with disintegrated microvilli. In the UW group, the brush borders remained intact, although microvilli were swollen (Figure 3). Tissue samples taken one hour after reperfusion showed reversal of the mitochondrial swelling in both experimental groups. In both the HTK and UW group, loss of the brush border and a considerable quantity of luminal debris was observed. However, damage was more profound in the HTK group.

Figure 2. Electron micrograph (x 20.000) of a proximal tubular cell after 30 min of warm ischemia in situ, ex vivo flushing, and subsequently 24 hours cold storage in HTK showing loss of brush border (see arrow).

Figure 3. Electron micrograph (x 15.000) of a proximal tubular cell after 30 min of warm ischemia in situ, ex vivo flushing, and subsequently 24 hours cold storage in UW. The brush border is still intact, although the microvilli are swollen (see arrow).

Table 1. Adenine nucleotides and their degradation products ($\mu\text{mol/g}$ dry weight) in renal cortical tissue before warm ischemia (0 min WI), after 30 minutes of warm ischemia (30 min WI), after 24 hours of cold storage (24 h CS), and one hour after reperfusion (60 min REP) in the HTK group. Median values with interquartile ranges (25th and 75th percentiles) are given.

	0 MIN WI	30 MIN WI	24 H CS	60 MIN REP
TAN	9.53 (7.63-11.96)	5.74 (4.98-6.59)	4.07 (3.59-5.39)	4.06 (3.40-4.65)
ATP	3.34 (2.40- 5.58)	1.50 (1.26-1.81)	0.57 (0.49-1.23)	1.45 (1.13-1.78)
ADP	3.97 (2.88- 4.43)	1.73 (1.45-2.10)	1.34 (0.94-1.62)	1.63 (1.37-1.73)
AMP	2.18 (2.00- 2.45)	2.31 (2.23-2.68)	2.54 (1.38-2.72)	0.98 (0.79-1.23)
TDP	3.33 (2.76- 4.37)	6.90 (2.23-7.31)	4.25 (1.23-4.41)	0.54 (0.36-1.22)
IMP	1.26 (1.15- 1.53)	1.22 (0.78-1.65)	1.15 (0.93-1.57)	0.00 (0.00-0.27)
ADO	0.26 (0.00- 0.82)	0.21 (0.00-0.69)	0.00 (0.00-0.16)	0.06 (0.00-0.23)
INO	0.53 (0.42- 0.79)	0.84 (0.61-1.24)	0.18 (0.08-0.48)	0.09 (0.00-0.29)
HX	0.74 (0.00- 0.83)	2.51 (1.12-2.78)	1.51 (0.00-1.70)	0.00 (0.00-0.26)
X	0.70 (0.00- 0.79)	1.47 (0.00-2.06)	1.06 (0.00-1.30)	0.00 (0.00-0.63)

Abbreviations (TAN, total adenine nucleotides; TDP, total level of degradation products; ADO, adenosine;; INO, inosine, HX, hypoxanthine; X, xanthine).

Table 2. Adenine nucleotides and their degradation products ($\mu\text{mol/g}$ dry weight) in renal cortical tissue before warm ischemia (0 min WI), after 30 minutes of warm ischemia (30 min WI), after 24 hours of cold storage (24 h CS), and one hour after reperfusion (60 min REP) in the UW group. Median values with interquartile ranges (25th and 75th percentiles) are given.

	0 MIN WI	30 MIN WI	24 H CS	60 MIN REP
TAN	9.45 (8.86-10.08)	4.86 (4.30-5.99)	3.56 (3.42-3.99)	4.74 (4.22-4.98)*
ATP	3.02 (2.55- 3.43)	1.26 (0.74-1.53)	0.79 (0.61-0.83)	1.82 (1.69-1.99)*
ADP	3.87 (3.29- 4.26)	1.49 (1.22-2.08)	1.19 (1.05-1.45)	1.85 (1.70-2.18)*
AM	2.63 (2.18- 2.99)	2.13 (1.91-2.88)	1.76 (1.54-1.85)	0.92 (0.72-0.95)
TDP	2.93 (2.47- 3.83)	5.16 (3.42-7.25)	11.48 (9.66-13.87)**	1.26 (0.51-1.74)
IMP	1.05 (0.72- 1.31)	1.06 (0.86-1.56)	1.48 (1.00- 1.56)	0.30 (0.19-0.40)*
ADO	0.21 (0.14- 0.32)	0.26 (0.00-0.34)	0.46 (0.11- 1.03)*	0.06 (0.00-0.17)
INO	0.33 (0.28- 0.36)**	0.77 (0.63-1.65)	2.02 (1.40- 2.37)**	0.18 (0.15-0.23)
HX	0.62 (0.49- 0.88)	1.78 (1.07-3.64)	6.05 (5.01- 8.64)**	0.00 (0.00-0.54)
X	0.89 (0.54- 1.08)	0.80 (0.00-1.66)	1.40 (0.46- 2.01)**	0.61 (0.00-0.74)

Abbreviations (TAN, total adenine nucleotides; TDP, total level of degradation products; ADO, adenosine; INO, inosine, HX, hypoxanthine; X, xanthine).

*, **, significantly ($p < 0.05$, $p < 0.01$) different from corresponding values in the HTK group.

Energy metabolites

Data are shown in Tables 1 and 2. Thirty minutes of warm ischemia resulted in a $\pm 45\%$ (48% in the HTK group and 40% in the UW group) reduction of the pre-ischemic total adenine nucleotide levels (TAN). Hypoxanthine and Inosine were the main degradation products (TDP). The increase of TDP was not proportional to the decrease of TAN. During 24-hr cold storage TAN levels further decreased in both experimental groups. In contrast to the HTK group, an increase of adenosine, inosine, hypoxanthine and xanthine levels was observed in the UW group after preservation. Regeneration of TAN after reperfusion was observed in the UW group but not in the HTK group. Both groups showed a marked decrease of TDP in the same time interval. Measured at one hour after reperfusion, TAN levels were significantly higher in the UW group as compared with the HTK group, although still less than 50% of preischemic values.

In figure 4 the correlation between the total level of adenine nucleotides measured one hour after reperfusion and the highest serum creatinine concentration during the 14-day study period was assessed. The correlation between the two parameters did not reach the level of significance ($r=0.06$, $p=0.85$).

Figure 4. Correlation between total level of adenine nucleotides (TAN) ($\mu\text{mol/g}$ tissue dry weight (dw)) measured one hour after reperfusion of the graft and the highest serum creatinine ($\mu\text{mol/L}$) during the follow-up period of 14 days. No correlation between both parameters was found ($r=0.06$, $p=0.85$). ($\square$ refers to the UW group, $\blacktriangle$ to the HTK group)

DISCUSSION

Hypothermic preservation has been successfully used to decrease the susceptibility to ischemia in cadaveric donor kidneys. However, if normothermic ischemia precedes hypothermic preservation, its efficacy is markedly reduced, indicating that hypothermia itself is not sufficient to preserve organ viability (17). Successful preservation of kidneys which have been subjected to prolonged warm ischemia, therefore, largely depends on the quality of the solution used. Our results show that the UW solution is superior to HTK for cold storage preservation of ischemically damaged kidneys. Posttransplant function of the graft,

measured by the serum creatinine concentration, was significantly better in the UW group compared with the HTK group. Moreover, the survival rate was higher in the UW group as compared with the HTK group.

A clear difference in the severity of the ultrastructural damage was observed after 30 min of warm ischemia and subsequent 24-hr cold storage in either solution. In the UW group, the brush borders of the proximal tubular cells were remarkably well preserved in comparison to the HTK group. The proximal tubule is particularly vulnerable to ischemia and loss of brush border is considered one of the early signs of deterioration of cellular integrity (18). Moreover, extensive literature exists regarding the pathophysiological consequences of tubular debris on kidney function. Impacted brush border is considered to cause tubular obstruction downstream resulting in high intra-tubular pressure, tubular leakage, and impairment of glomerular filtration (19,20).

Warm ischemia rapidly exhausted the pool of high energy phosphates (ATP, ADP, AMP) resulting in an accumulation of degradation products, mainly hypoxanthine and inosine. The adenine nucleotides and their degradation products might be considered as a pool with a fixed content since no further breakdown beyond xanthine is observed during ischemia (21). Therefore, the fact that the increase of the total level of degradation products (IMP, adenosine, inosine, hypoxanthine, and xanthine) was not proportional to the reduction of TAN suggests that other metabolites are formed in the degradation process of adenine nucleotides which were not detected by our HPLC analytical procedure.

During 24 hr of cold storage, the TAN content further decreased in both experimental groups. This confirms earlier observations from our laboratory showing the inability of cold storage preservation to maintain adenine nucleotide levels (9). In the HTK group, the TDP level was lower after preservation as compared with the level of TDP measured after 30 min of warm ischemia. Apparently, degradation products were washed out of the kidney during *ex vivo* flushing preceding storage. Although the washout of degradation products must also have occurred to the same extent in the UW group, the levels of adenosine, inosine, hypoxanthine, and xanthine measured after 24 hours preservation were significantly higher in the UW group as compared with the HTK group. This phenomenon can be explained by the fact that UW solution, in contrast to HTK solution, contains adenosine (5 mmol/L). During cold storage, the adenosine is further metabolized, resulting in high levels of inosine, hypoxanthine and xanthine after preservation with UW solution. Reports in literature are ambivalent concerning the consequences of high amounts of degradation products in ischemic tissue prior to reperfusion. Some degradation products can potential-

ly act as precursors in the regeneration of adenine nucleotides (22,23). The actual importance of these so-called salvage pathways is not yet known. A direct conversion of degradation products to AMP after renal ischemia and reperfusion has not yet been demonstrated. On the other hand, degradation products, especially hypoxanthine, might be responsible for part of the damaging effect of renal ischemia-reperfusion-associated injury. Quantitatively, hypoxanthine is by far the most important degradation product in ischemic kidneys. In undamaged tissue, hypoxanthine is metabolized to xanthine by xanthine dehydrogenase. During ischemia however, xanthine dehydrogenase is converted to xanthine oxidase (24). When oxygen is reintroduced after a period of ischemia during reperfusion, xanthine oxidase converts hypoxanthine to xanthine with a concomitant production of superoxide anions. This process is followed by a cascade of reactions that release other toxic oxygen radicals and H_2O_2 with consequent damage (25). For this reason, the (xanthine oxidase) scavenger allopurinol and antioxidant glutathione are included in the UW solution (26). Reperfusion of the graft resulted in an increase of TAN content in the UW group. The regeneration of TAN was, however, not proportional to the decrease in TDP content in the same interval. In the HTK group, no recovery of TAN levels could be observed within 1-hr reperfusion, although there was a marked decrease in TDP. These results confirm observations made in isolated perfusion models that suggest that most degradation products are rapidly and quantitatively washed out of the ischemic tissue during reperfusion (27).

One hour after reperfusion of the graft, TAN levels were significantly higher in the UW group compared with the HTK group. Although a higher TAN content early after reperfusion was not correlated with a lower serum creatinine during the first two weeks after transplantation in our study, the faster regeneration of TAN after reperfusion indicates a better preservation of energy metabolism and thus more cellular viability in the UW group.

In conclusion, our results show that UW is a superior solution compared with HTK in the preservation of ischemically damaged kidneys, demonstrating better survival, better recovery of kidney function, better protection against ischemia-induced ultrastructural damage, and better preservation of energy metabolism, indicated by (a faster) regeneration of TAN levels after reperfusion. Therefore, UW is to be preferred as cold storage solution for NHB donor kidneys.

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ENHANCED RESISTANCE TO THE EFFECTS OF NORMOTHERMIC ISCHEMIA ON KIDNEYS USING PULSATILE MACHINE PERFUSION

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ABSTRACT

The aim of this study was to compare the effectiveness of cold storage (CS) in Histidine Tryptophan Ketoglutarate (HTK) solution versus machine perfusion (MP) with the new Belzer solution in the case of ischemically injured kidneys. *Transplant model*: 12 dogs underwent auto-transplantation of the left kidney after 30 min of warm ischemia and subsequent 24 hrs of CS (group I, n=6) or 2 hrs of CS followed by 22 hrs of MP (group II, n=6). Contralateral nephrectomy was performed before re-anastomosis was established. Post-transplant function was determined by serum creatine (SC) measurement up to 14 days after implantation. *Contralateral kidneys*: Contralateral kidneys were treated identically. Following 24 hrs of CS (group III, n=6) or 2 hrs of CS and 22 hrs of MP (group IV, n=6) respectively, all contralateral kidneys were perfused for 20 min on the machine with a constant systolic pressure of 60 mm Hg. After measurement of the intrarenal resistance (IRR), these kidneys were used for light microscopic analysis. *Survival*: At two weeks post-transplantation in group II 5 out of 6, and in group I 1 out of 6 dogs survived. *Renal function*: Group II, in contrast to group I, showed a rapid return of the SC to normal values. *Contralateral kidneys*: Median IRR after preservation was lower in group IV (0.4) as compared to group III (0.8). Light microscopic analysis revealed red blood cell trapping and extensive tubular cast formation in group III. Kidneys in group IV showed less tubular degeneration. *Conclusion*: MP is superior to CS for preservation of ischemically damaged kidneys, demonstrating better survival, better recovery of kidney function, better protection against the high vascular resis-

tance that is induced by warm ischemia and prevention of red blood cell trapping and tubular obstruction by cast.

INTRODUCTION

Due to the persistent organ shortage, the Non-Heart-Beating (NHB) donor might gain importance as a source of kidneys for transplantation. Still, there is no consensus as how to preserve these ischemically damaged kidneys (1,2). The two major modalities of preservation available are Cold Storage (CS) and pulsatile Machine Perfusion (MP).

The CS method is based on minimization of cell metabolism by hypothermia and prevention of edema by use of a hyperosmolar preservation solution (3). The preservation solutions commonly used in Europe are Euro-Collins (EC) (4), University of Wisconsin (UW) (5), and Histidine Tryptophan Ketoglutarate (HTK) (6). The method is simple and inexpensive. Preservation by continuous pulsatile MP was first described by Humphries et al in 1963 (7). The principle of MP is that even in hypothermia, where metabolism is very low, the kidney must be provided with nutrients and waste products should be removed (8). Originally, all MP solutions contained human blood products (9,10,11,12). In 1989, Belzer's group reported encouraging results with a new synthetic perfusate (13).

In a previous article we retrospectively analyzed the post- transplant function of a group of NHB donor kidneys versus a matched control group of Heart-Beating (HB) donor kidneys (14). All kidneys were preserved by the CS method. A statistically significant higher percentage of delayed function was observed in the NHB donor kidney group.

The aim of this study was to compare the effectiveness of CS in HTK versus MP with the new Belzer solution in the case of ischemically injured kidneys. It is concluded that machine preservation, using Belzer solution, has the potential to decrease the high percentage of delayed function after transplantation of kidneys from NHB donors.

MATERIALS AND METHODS

Animals and anesthesia

Experiments were performed on 12 female Beagle dogs age 2-4 years and weighing 10-12 kg. The animals were fasted during the 12 hours before surgery with free access to water. Anesthesia was induced with Thiopental (20 mg per kg body weight) and maintained with a nitrous oxide-oxygen gas mixture. An antibiotic prophylaxis of 500 mg ampicillin was given before each operation. During surgery the dogs were hydrated by intravenous infusion of 500 mL Ringer's lactate solution and body temperature was kept constant (37°C) using a thermostatic watermattress. All experiments were performed after approval by the responsible ethics committee, and principles of laboratory animal care according to the NIH standards were followed.

Donor operation

The kidneys were exposed through a midline abdominal incision. Heparin (1000 IU) was given 10 minutes before induction of warm ischemia in order to prevent thrombosis. Ischemia was induced by cross-clamping the renal vessel pedicle of the left kidney. During the ischemic interval the abdomen was kept closed to preserve the kidney at body temperature. After 30 minutes the kidney was removed, flushed with 200 mL of cold (4°C) HTK solution, and subsequently weighed. Flushing was performed by gravity from a bottle at 100 cm height.

Preservation

Six kidneys were stored for 24 hours at 4°C in a plastic bag containing 500 mL HTK solution (group I). The other 6 kidneys were stored on melting ice for 2 hours and were then perfused with 500 ml of Belzer solution for 22 hours in a preservation machine (PF 200, Gambro, Lund, Sweden) at 4°C (group II). Perfusion pressure (mm Hg) (systolic, diastolic and mean) and flow (mL/min per 100 g of tissue) were recorded. Intra-renal resistance (IRR) {mm Hg/(mL/min per 100 g of tissue)} was calculated by dividing the mean pressure by the measured flow. The initial systolic pressure was set at 60 mm Hg and this pressure was reinstalled only once (after one hour of perfusion). During MP, 100% filtered oxygen (200 mL/min) was blown over the surface of the preservation fluid. Perfusate samples for Lactate dehydrogenase (LDH) and pH evaluation were taken at T = 0; 1; 2; 4; 6; 8; and 22 hours of perfusion and determined at 37°C using a Dimension LDH kit (DuPont de Nemours, Wilmington, USA), and an ABL blood gas analyzer (Radiometer, Copenhagen, Denmark) respectively. During perfusion the pH was kept within a 7.0-7.2 range. A pH lower than 7.0 was adjusted by addition of NaHCO₃ 8.4%.

Recipient operation

At the start of the recipient operation morphine, in a 0.5 mg/kg dose, was given intravenously in order to prevent intestinal invagination. Surgery started with removal of the contralateral (right) kidney. The kidney was reimplanted autologously onto the iliac vessels in the right lower abdomen. Rewarming during vascular anastomosis was prevented by maintaining the kidney at 4°C with sterile saline. Sorbitol 20%, 150 mL, was administered intravenously after completion of the anastomoses to induce osmotic diuresis after implantation. The ureter was implanted into the bladder using a technique described by Politano (15). A catheter for blood sampling was placed in the right jugular vein. Daily serum creatinine values were determined upto 14 days after implantation using a Dimension creatinine kit (DuPont de Nemours, Wilmington, USA). Animals that survived were sacrificed 2 weeks after transplantation.

Contralateral kidneys

Contralateral kidneys were subjected to 30 minutes of warm ischemia by cross-clamping the renal artery and vein. Subsequently, they were flushed and stored in the same experimental conditions using fresh preservation solutions. The weight of the contralateral kidneys was measured before and after preservation. After 24 hours of CS (group III), and 2 hours of CS followed by 22 hours of MP (group IV) respectively, all contralateral kidneys were perfused for 20 minutes with cold (4°C) Belzer solution on the Gambro perfusion machine using a constant systolic pressure of 60 mm Hg. IRR during perfusion was calculated and a comparison was made between the two groups.

Composition of the preservation solutions

HTK was used for the initial flushing of all kidneys and for the CS preservation. Belzer solution was used for MP. In Table 1 the composition of both solutions is shown. HTK, a crystalloid solution, has a low sodium and an elevated potassium concentration in comparison to the extracellular space. It contains a strong buffer capacity by the histidine/ histidine-Chloride buffer of 180/18 mmol/L. The calculated osmolality of HTK is 310 mosmol/L. The MP solution of Belzer contains a non-toxic colloid hydroxyethyl starch (HES) (Dupont Critical Care, Waukegan, USA) that prevents expansion of the interstitial space and gluconate as an impairment anion to prevent cell swelling. Sodium is the major cation. Mannitol is added for additional osmotic support. The calculated osmolality of Belzer's solution is 320 mosmol/L. Prior to use, 200.000 IU of penicillin G, 40 IU of Insulin, and 16 mg dexamethasone per liter is added.

Table 1. Composition of the preservation solutions.

Content	HTK solution		Belzer's solution	
Na ⁺		15 mmol/l		100 mmol/l
K ⁺		10 mmol/l		25 mmol/l
Ca ⁺⁺		—		0.5 mmol/l
Mg ⁺⁺		4 mmol/l		5 mmol/l
Cl ⁻		50 mmol/l		20 mmol/l
Substrates	Tryptophan	2 mmol/l	Gluconate	85 mmol/l
	α-Ketoglutarate	1 mmol/l	Adenine	5 mmol/l
	Mannitol	30 mmol/l	Ribose	5 mmol/l
			Glucose	10 mmol/l
			Glutathione	3 mmol/l
			Mannitol	30 mmol/l
Buffer	Histidine	180 mmol/l	H ₂ PO ₄ ⁻	25 mmol/l
	Histidine-HCl	18 mmol/l	HEPES *	10 mmol/l
Pharmaca	—		Insulin	40 IU/l
			Dexamethasone	16 mg/l
			Penicillin	2 · 10 ⁵ IU
Colloid	—		HES **	50 g/l
Osmolarity		310 mosmol/l		320 mosmol/l

* (HEPES = N-2- hydroxyethylpiperazine-N'-2-ethanesulfonic acid)

**(HES = Hydroxyethyl starch)

Histological examination

At autopsy the implanted kidneys were bisected for gross macroscopic examination. Wedge-shaped biopsies were fixed in 4% neutral-buffered formalin, dehydrated and paraffin embedded. Sections of 4 µm were cut and stained using haematoxylin and eosin for light microscopic evaluation. The non-transplanted contralateral kidneys were prepared for histological examination following the same protocol. Morphological characteristics of the contralateral kidneys after

24 hours of preservation were graded as (a) tubular degeneration; (b) severe tubular degeneration with cast formation (necrotic remnants of tubular cells which have passed into the tubular lumen); (c) tubular obstruction by cast. Grading was performed per individual kidney in five randomly selected fields (magnification x400).

Data analysis

Animals that survived up to 14 days were considered to be survivors. All values reported are medians with interquartile ranges (25th and 75th percentiles). Statistical analysis was carried out with the non-parametrical Kruskal Wallis One-way ANOVA test for differences between two groups, and the Friedman Two-way ANOVA for differences within one group. A p value of less than 0.05 was considered statistically significant.

RESULTS

Hypothermic perfusion

Figure 1 demonstrates the changes in systolic perfusion pressure and flow (medians and interquartile ranges) during the 22 hours of hypothermic perfusion. The initial systolic pressure was set at 60 mm Hg. The median perfusate flow (mL/min per 100 g of tissue) was 75 (67-85). During the first hour of hypothermic perfusion the systolic pressure dropped to a level of 44-51, median 50 mm Hg. Subsequently the systolic pressure was adjusted to 60 mm Hg resulting in a median perfusate flow of 93 (89-105). This flow was continued until implantation. After 22 hours of machine perfusion median systolic pressure had dropped to 46 mm Hg (39-49). Median IRR decreased during machine perfusion from 0.7 to 0.3 ($p < 0.05$).

The LDH concentration of the perfusate increased, in all cases, during the course of perfusion. However, large differences in LDH release in the perfusate were observed (1176 to 4440 U/L per 100 g of tissue) at the 22 hours of perfusion. The amount of LDH in the perfusion fluid correlated with the systolic pressure drop during the 22 hours of machine perfusion in a way that a small pressure drop was associated with a high LDH release in the perfusate ($r = 0.89$, $p < 0.05$) (Figure 2).

In all experiments the pH of the perfusion fluid (measured at 37 centigrade) decreased, starting from 7.3 to about 7.1 after 22 hours of machine preservation. Correction of the pH was not necessary.

Figure 1. Perfusate systolic pressure and flow (median values and interquartile ranges; 25th to 75th percentiles) during 22 hours of hypothermic perfusion (n=6).

Figure 2. Correlation between the systolic perfusate pressure drop and the amount of LDH release per 100 g of tissue in the perfusate after 22 hours of hypothermic perfusion (n=6), ($r=0.89$, $p<0.05$).

Survival rate and renal function after transplantation

One animal in the cold storage group (group I) survived although the serum creatinine remained high (above 500 $\mu\text{mol/L}$) at 14 days after transplantation. The other five dogs all died on day five post-operative. In the machine perfusion group (group II) a rapid return of the renal function was observed, with median serum creatinine values decreasing from day 4-5 onward and returning to normal values at day 14. One animal in group II, which had aspirated stomach contents during surgery, died on day 5 with signs of pneumonia. As from day 2 onward, the median serum creatinine in group I was higher when compared to the median values in group II (Figure 3).

Figure 3. Daily serum creatinine measurements, before and subsequently during 14 days after transplantation (median values and interquartile ranges; 25th to 75th percentiles). * means significant ($p < 0.05$) difference between the two groups, † refers to death of an animal.

Histology of the transplanted kidneys

Dogs with life sustaining function were sacrificed two weeks after implantation. At autopsy these kidneys showed normal morphology in all cases. Light microscopic examination revealed multiple calcium deposits and lypofuscine pigmentation in the tubules, especially in the cortical area. On bisecting the non-viable

kidneys, a large haemorrhagic zone was observed between the cortex and the medulla. In the histological sections of these kidneys, blood congestion in the corticomedullary area was associated with massive tubular necrosis in the medulla as well as in the cortex. No signs of glomerular damage were seen. The diagnosis of pneumonia of one dog in group II was confirmed histologically.

Contralateral kidneys

Median weight of the contralateral kidneys before preservation did not differ between group III and group IV (46 vs 50 g, NS). In contrast to the kidneys in group III a significant increase in weight of the kidneys in group IV was observed (median 16%, interquartile ranges 12%-18%, $p < 0.05$).

Median IRR after 24 hours of preservation was 0.8 in group III versus 0.4 in group IV ($p < 0.05$).

Light microscopic analysis revealed no signs of vascular or glomerular damage in group III and IV. However, red blood cell trapping throughout the renal cortex, severe tubular cell damage (predominantly of proximal tubules), and cast formation leading to tubular obstruction was observed in group III (Figure 4). Kidneys in group IV showed less tubular degeneration (Figure 5). The morphological characteristics of both groups, scored as mentioned above, are shown in Table 2.

Figure 4. Light micrograph of a cold storage preserved contralateral kidney (group III) showing severe tubular damage and cast formation leading to obstruction. Red blood cell trapping can be observed. Paraffin embedment, hematoxylin and eosin (magnification x400).

Figure 5. Light micrograph of a machine preserved contralateral kidney (group IV). Loss of brush border integrity and slough of apical tubular cytoplasm. No obstruction of the tubules is observed. Paraffin embedment, hematoxylin and eosin (magnification x400).

Table 2. Morphological characteristics of contralateral kidneys. Score: (a) tubular degeneration; (b) severe tubular degeneration with cast formation; (c) tubular obstruction by cast. (RBC = red blood cell)

Kidney No.	Cold storage		Machine perfusion	
	Score	RBC trapping	Score	RBC trapping
1	c	+	a	-
2	c	+	a	-
3	b	-	a	-
4	b	+	a	-
5	b	-	b	-
6	c	-	b	-

DISCUSSION

At present it is widely believed that kidneys from Heart-Beating (HB) donors can be preserved equally well with simple CS or with MP (16,17). In Europe, therefore, the relatively expensive and more time-consuming method of MP is abandoned. With the use of kidneys from NHB donors however, a renewed interest towards this method of preservation has come forward. Kidneys from NHB donors have all been subjected to a period of warm ischemia. As a result, delayed function of the graft after transplantation is frequent (14,18,19). In a prior publication we stated that, in spite of higher percentages of delayed function, there is no statistical difference between the long term function of HB donor kidneys versus a matched group of NHB donor kidneys (20). However, the need for dialysis, the loss of clinical markers heralding rejection, the longer hospitalisation, and, consequently the negative effects on the patient's emotional response after the transplant procedure are major drawbacks in the use of kidneys from asystolic donors. These important disadvantages require one to seek out for the most efficient manner to maximize immediate function.

Several groups have reported the advantages of machine preservation for kidneys with prolonged warm or cold ischemia (21,22,23). However, one of the major obstacles of MP was that, in order to prevent excessive cellular edema during perfusion, addition of human blood products in the preservation solution for colloid support was necessary. The colloid originally used was serum albumin, either contained in plasma (9), protein plasma fraction (10), silica gel fraction (11) or added in the form of serum albumin to saline or Ringer's Lactate (12). Unfortunately, due to its low molecular size, the albumin is not restricted to the vascular space, and thus during the course of time intravascular oncotic pressure is diminished (24). In addition, perfusion induced denaturation of albumin can occur and produce endothelial damage (25). In plasma, immune complexes have shown to induce vasculitis which predisposes to rejection (26). In order to overcome these, problems the laboratory of Belzer developed a new solution based on HES (27). HES is a completely synthetic colloid. It has an average molecular weight of 250,000 and can, therefore, not diffuse out of the vascular space. Further advantages of HES in comparison to human albumin include the ease of preparation, lower costs, and no risk of immunogenicity or transfer of infectious disease.

In this study we compared CS preservation in HTK solution to MP with the Belzer perfusion solution. An autotransplantation model was used to preclude the problem of rejection. The kidneys were subjected to 30 minutes warm ischemia, then flushed, and subsequently preserved for 24 hours either with CS

or MP. In our opinion 30 minutes is the maximum safe warm ischemic interval in human kidney transplantation. All kidneys were flushed with HTK preservation solution. HTK is standardly used in our hospital for ischemically damaged kidneys. It is thought to be an excellent flush-out solution due to its low viscosity (28). Mean cold ischemia time within the Euro-Transplant region is 24 to 25 hours (29). With this critical model, a clear difference in survival rate and post-transplant serum creatinine values between the CS group (group I) and the MP group (group II) was observed. Histological examination of the dogs who died revealed massive tubular necrosis causing renal failure. The results emphasise the beneficial effect of MP on post-transplant renal function, in the case of ischemically damaged kidneys.

One of the effects of ischemia on a kidney is an increase in vascular resistance (30). The pathogenesis of phenomenon is complex. Nervous stimulation (31), local release of vasoconstrictive agents (32), the loss of ability of the renal vascular endothelium to release Endothelium-derived-relaxing factor (33), and swelling of endothelial and perivascular cells causing vascular blockage (34) have all been purported to play a role in the process. In our study, we found a higher IRR in kidneys preserved with CS (group III) as compared to kidneys preserved by means of MP (group IV). In contrast to the CS preserved kidneys (group III) an increase in weight of the kidneys in the MP group (group IV) was observed. This increase in weight, reflecting the amount of edema of the renal tissue, seemed to have no determined effect on the IRR since the IRR decreased during MP.

Failure of the circulation to return to a tissue, which has suffered from a period of ischemia, has been termed the "no reflow phenomenon" (35). It is generally accepted that this phenomenon is a major factor in the causation of reperfusion injury (36,37). Obstruction of the capillaries with red blood cells has been proposed to play a role in the development of the "no reflow phenomenon" (38). High vascular resistance of the renal circulation causes insufficient washout of red blood cells after harvesting. In addition, red blood cells become more rigid by ischemia. Ischemia causes the deterioration of Adenosine Tri-Phosphate (ATP). Depletion of ATP (which is known to chelate calcium) induces this cell membrane rigidity through calcium binding to membrane proteins (39). Subsequently, these rigid red cells might obstruct capillaries in which they become trapped. Magnesium in the preservation fluid can competitively inhibit calcium binding and thus prevent red blood cell trapping. Both HTK and Belzer solution contain magnesium. However, MP has the advantage over CS preservation that it allows delivery of agents to where they are needed. In contrast to the light microscopic observations of CS preserved kidneys (group III), we found

no signs of red blood cell trapping in the histological sections of MP kidneys (group IV).

Light microscopic analysis revealed tubular obstruction in three of the six CS preserved kidneys (group III). Tubular obstruction is caused by damage of the proximal tubular cells, whose brush borders are particularly sensitive to ischemia (40). The importance of an early relief of the tubular obstruction in acute renal failure has been demonstrated by Tanner and Steinhauzen (41). High intratubular pressures proximal to the obstruction will counteract the hydraulic pressure in the glomerular capillaries and thus hamper the glomerular filtration. In their study, relief of tubular obstruction led to an early restoration of nephron function. No tubular obstruction was observed in the histological sections of the MP kidneys (group IV). Apparently, all cast was flushed out by filtrated perfusion fluid during MP.

The amount of enzyme release from damaged cells has been thought to be a marker for ischemic injury and thus a possible tool for viability testing during MP (42). Nevertheless, in our study, large differences in LDH release in the perfusate were observed, although the ischemical damage was similar in all kidneys. An interesting observation is the correlation between the amount of LDH released in the perfusate and the extent of the systolic pressure drop during MP. This relation suggests that an accumulation of toxic metabolites in the preservation fluid during MP might play a role in the maintenance of the ischemia induced vascular resistance. It supports the idea that intermediate refreshing of the perfusion fluid could even improve the results of MP (43,44).

In conclusion, using a dog autotransplantation model, we have been able to prove the beneficial effect of machine perfusion using Belzer solution in case of ischemical damaged kidneys. Machine perfusion can counteract the high vascular resistance, induced by ischemia, and therefore ameliorates the expulsion of red blood cells from the renal microcirculation. Prevention of erythrocyte trapping might encourage a more uniform reflow after implantation and thus limit reperfusion damage. Furthermore, machine perfusion prevents tubular obstruction by cast, which might precipitate restoration of nephron function after reperfusion.

Taken together, we believe that machine preservation can decrease the high percentage of delayed function after transplantation of ischemically damaged kidneys.

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BENEFICIAL EFFECT OF MACHINE PERFUSION ON THE PRESERVATION OF RENAL MICROCIRCULATORY INTEGRITY IN ISCHEMICALLY DAMAGED KIDNEYS

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ABSTRACT

The aim of this study was 1) to evaluate the suitability of scintigraphy for viability testing of kidneys prior to transplantation and 2) to compare the ability of cold storage (CS) in Histidine Tryptophan Ketoglutarate (HTK) to machine perfusion (MP) using Belzer perfusate to preserve the renal microcirculatory integrity in ischemically damaged and control kidneys. 16 dog kidneys were divided over four experimental groups of four kidneys each. In group I, kidneys were subjected to 0 min of warm ischemia (WI) and subsequently preserved for 2 hrs with CS and for 22 hrs with MP at 4°C. Group II; 0 min of WI followed by 24 hrs of CS. Group III; 30 min of WI, 2 hrs of CS and 22 hrs of MP. Group IV; 30 min of WI and 24 hrs of CS. Intrarenal perfusion was assessed by two successive radionuclide flow studies in an isolated perfusion model. Perfusion pressure was fixed at 60 mm Hg. The radioactive tracers were injected in the renal artery. First, the total renal flow and the intrarenal flow distribution were determined quantitatively by use of Xenon-133. Second, perfusion defects were visualised with technetium labelled Hexamethyl Propylene Amine Oxime (^{99m}Tc-HMPAO). *Results:* WI to the kidney was associated with a marked decrease in total renal flow, an intrarenal flow redistribution with a relative increase in flow to the sub-cortex, and with perfusion defects of the renal cortex. In contrast to the CS kidneys, MP kidneys showed an enhanced ability to resist to these effects of WI on the renal microcirculation. *Conclusion:* Our results indicate that, radionuclide flow studies using Xenon-133 and ^{99m}Tc-HMPAO

might be valuable complementary tools for viability testing in renal preservation. Furthermore, in case of ischemically damaged kidneys, MP with Belzer's solution is superior to CS in HTK as far as preservation of the renal microcirculation is concerned.

INTRODUCTION

A homogeneous reperfusion of the graft is a prerequisite for successful transplantation. Several studies have shown good correlation between the intactness of the renal microcirculation and subsequent post-transplant function (1,2,3).

The present study was designed to compare the ability of cold storage (CS) preservation in Histidine Tryptophan Ketoglutarate (HTK) solution (4) and machine perfusion (MP) using Belzer's perfusate (5), to preserve renal microcirculatory integrity in ischemically damaged and control kidneys.

Intrarenal perfusate flow was assessed by two successive radionuclide flow studies in an isolated perfusion model. Total renal flow and flow distribution was determined by use of radioactive Xenon-133 (6). The Xenon washout technique provides a method to measure quantitatively the amount of flow throughout different compartments of the kidney. To visualise perfusion defects technetium labelled Hexamethyl Propylene Amine Oxime (^{99m}Tc -HMPAO) was applied (7). HMPAO is a lipophilic complex that has a high first-passage extraction rate proportional to the regional flow. This complex exhibits a sufficiently long retention in the renal parenchyma in order to permit static gamma camera images of the organ.

The results of this study indicate that, in case of ischemically damaged kidneys, MP with Belzer's solution is superior to CS in HTK as far as preservation of the renal microcirculation is concerned. Furthermore, it is postulated that radionuclide flow studies using Xenon-133 and technetium labelled HMPAO are valuable complementary tools for viability testing in renal preservation.

MATERIALS AND METHODS

All experiments were performed after approval by the responsible ethics committee, and principles of laboratory animal care according to the NIH standards were followed.

Nephrectomy of the right kidney was performed in 16 heparinized (100 IU/kg), female Beagle dogs, age 2-4 years, and weighing 10-12 kg. After removal, the kidney was flushed either immediately or following a period of 30 minutes storage in the warm body cavity, with 200 ml of cold (4°C) HTK solution. Flushing was performed by gravity, from a height of 100 cm, using a tapered catheter placed in the renal artery. Subsequently, the kidney was weighed and packed in a sterile plastic bag containing 500 mL of HTK solution.

Hypothermic preservation

Eight kidneys were stored for 24 hours on melting ice. The other eight kidneys were stored in the same way for two hours, then connected to a preservation machine (PF 200, Gambro, Lund, Sweden), and perfused for 22 hours with 500 ml of Belzer solution at 4°C. The initial systolic perfusion pressure was set at 60 mm Hg and this pressure was reinstalled only once after one hour. During MP, 100% filtered oxygen (200 mL/min) was blown over the surface of the perfusate.

Experimental groups

Four experimental groups of kidneys subjected to various periods of warm and cold ischemia were studied.

- Group I: 0 minutes of warm ischemia followed by 2 hours CS and 22 hours of MP (n=4).
- Group II: minutes of warm ischemia followed by 24 hours of CS preservation (n=4).
- Group III: 30 minutes of warm ischemia followed by 2 hours of CS and 22 hours of MP (n=4).
- Group IV: 30 minutes of warm ischemia followed by 24 hours of CS preservation (n=4).

Scintigraphy

After CS or MP respectively, all kidneys were placed in an organ chamber, connected to a perfusion circuit, and placed under a gamma camera (Figure 1). The perfusion circuit consists of a small roller pump (Gambro BP 10) into which a perfusion tube is inserted. The pump provides a pressure controlled pulsatile flow to the renal artery. The perfusate used was HTK solution. The systolic perfusion pressure was fixed at 60 mm Hg. Venous and ureteral outflow were collected in a container. Absolute perfusate flow (APF) (mL/min per 100 g of tissue) was determined by constant weighing of the effluent. The camera used (Technicare 420) was equipped with a low energy collimator and linked

to a computer system (Technicare MCS 560). The distance between the camera and the kidney was approximately 2 cm.

Figure 1. Schematic picture of the isolated kidney perfusion model. Venous and ureteral effluent are collected in a container.

Two successive measurements, each of a 10 minutes duration, were performed. First, a bolus of 0.5 mCi of Xenon-133 dissolved in 1 mL isotonic saline was injected into the renal artery in approximately 2 seconds. The washout rate of the radioactive Xenon from the renal parenchyma was monitored during 10 minutes by acquiring 120 frames of each 5 seconds. The matrix size was 64 by 64. A 20% window was centred on the 80 Kev photopeak of the Xenon-133. Intrarenal perfusate flow was determined by the compartmental analysis technique as described by Thorburn (8). The isotope washout curve was described as the sum of two exponential functions reflecting the disappearance of the radioactivity from two compartments {cortex (compartment I) and sub-cortex (compartment II)} in the kidney (Figure 2).

The formula for the calculation of the total renal flow (TRF) in ml/min per 100 g of tissue was by:

$$\text{TRF} = \frac{A1 + A2}{A1/Y1 + A2/Y2} * p * 60 * 100 \text{ (ml/min per 100 g)}, \quad [1]$$

where A1 and A2 represent the amplitude of the radioactive activity measured in compartment I and II; Y1 and Y2 the slope of the washout function line of compartment I and II, and p the partition coefficient of Xenon. The partition coefficient is the ratio of the Xenon distribution between tissue and perfusate.

The percentage of the TRF which is received by a specific compartment was evaluated by:

$$\text{Percentile flow (PF) compartment I} = \frac{A1}{A1 + A2} \text{ (\%)} \quad [2]$$

The actual flow (AF) through a compartment was determined using:

$$\text{AF compartment I} = Y1 * 60 * 100 \text{ (ml/min per 100 g)}. \quad [3]$$

The tissue-perfusate partition coefficient (p) for Xenon-133 was determined empirically by comparing the APF, calculated by weighing the effluent during perfusion, with the measurements for the TRF found by the Xenon-133 washout curve. Correlation between the two, was assessed by calculating the Pearson correlation coefficient. A *p* value of less than 0.05 was considered significant.

Second, 100 MBq (in 4 ml of saline 0.9%) of ^{99m}Tc -HMPAO was injected as abolus into the perfusate. Energy was set at 140 KeV with a 20% window and data acquisition was performed in 120 dynamic frames, each of 5 seconds duration, with a 64x64 matrix. After 10 minutes static images of each 30 seconds were taken from six different directions (anterior, posterior, medial, lateral, cranial and caudal). In four cases additional to the planar images single photon computer tomography (SPECT) was performed. In these four cases, radioactivity from the kidney was computed by a 360 degree rotating gamma-camera (Diacam, Siemens) taking 64 images of 2 minutes in a 128x128 matrix and transformed in tomographic slices. Visual interpretation of all ^{99m}Tc -HMPAO scintigrams was performed by two independent observers. A

predefined scale from 0 to 2 was used, with 0 representing no perfusion defects visible, and 2 denoting large defects in regional perfusion.

Figure 2. The disappearance of radioactivity from the renal parenchyma following injection of a bolus of Xenon-133 into the renal artery is characterized by a multiexponential curve. Radioactivity in counts (cts) per second (sec) is plotted against time. Two compartments within the kidney, each with their own flow rate, contribute to the curve.

Histology

After the scintigraphic exploration, the kidneys were bisected for gross macroscopic examination. Wedge-shaped biopsies were fixed in 4% neutral-buffered formalin, dehydrated and paraffin embedded. Sections of 4 μ m were cut and stained using haematoxylin and eosin for light microscopic evaluation.

RESULTS

Xenon-133 washout studies

The partition coefficient for Xenon-133 in our study was 1.70. The Pearson correlation coefficient between the APF and TRF was: $r = 0.926$; $p < 0.05$. Results of the Xenon-133 washout curve analysis are shown in figures 3 and 4. Regardless the method of preservation, warm ischemic damage (group III and IV) resulted in a decrease of the TRF (caused by a reduction of the cortical AF), and a relative shift of intrarenal distribution from compartment I to compartment II. Sub-cortical AF was not affected. Comparing the two methods of preservation; in non-ischemically damaged kidneys as well as in kidneys subjected to 30 minutes of warm ischemia, cold storage resulted in a lower TRF, lower cortical PF, and higher sub-cortical PF compared to machine preservation. However, changes were more apparent in the ischemically damaged kidneys.

Figure 3. Total renal flow (mL/min per 100 g of tissue) and intrarenal flow distribution (percentile flow through compartment I and compartment II) (%). Median values with their interquartile ranges (25th to 75th percentiles) are given.

Figure 4. Absolute flow (mL/min per 100 g of tissue) through compartments I and II. Median values with their interquartile ranges (25th to 75th percentiles) are given.

Figure 5. Typical time-activity curve after injection of a bolus of ^{99m}Tc -HMPAO in the renal artery. Note the steady state equilibrium which allows static gamma-camera acquisition.

^{99m}Tc-HMPAO imaging

Figure 5 shows a typical time-activity curve of ^{99m}Tc-HMPAO (of 10 minutes duration) after injection of the tracer in the renal artery. Within 10 minutes a steady state equilibrium is achieved which allows static gamma camera acquisition. Kidneys in both groups I and II showed uniform uptake of the tracer throughout the entire cortex (perfusion score 0). In group III only one kidney had a minor perfusion defect (perfusion score 1), whereas the other three had no perfusion defects visible. However, large perfusion defects (perfusion score 2) varying from 20% to 80% of the total renal cortex were observed in all four kidneys of group IV. Figures 6 and 7 represent the tomographic images obtained by SPECT analysis in groups III and IV, respectively.

Figure 6. SPECT imaging of a kidney which was subjected to 30 minutes of warm ischemia and subsequently preserved for 2 hours with cold storage and 22 hours with machine perfusion (Group III).

TRANSVERSE

posterior
lateral medial
anterior

SAGITTAL

cranial
posterior anterior
caudal

FRONTAL

cranial
lateral medial
caudal

Figure 7. SPECT imaging of a kidney which was subjected to 30 minutes of warm ischemia and subsequently preserved by cold storage for 24 hours (Group IV).

TRANSVERSE

SAGITTAL

FRONTAL

Histology

On section, no focal abnormalities were observed. Light microscopic analysis revealed an overall insufficient red blood cell expulsion in kidneys of group IV. No clear relation between the perfusion defects on the HMPAO scan and intravascular obstruction caused by erythrocyte trapping could be found.

DISCUSSION

The effect of 24 hours' hypothermic preservation on renal hemodynamics was studied in ischemically damaged and control kidneys using scintigraphy. Scintigraphic evaluation of renal perfusion is a routine procedure in the post-transplant period. In addition, attempts have been made in order to utilize this method to assess the viability of kidneys before they are transplanted. Anaise et al (9) reported of a direct correlation between the integrity of the cortical microcirculation during preservation, and a life-sustaining function of the kidney after transplantation.

Xenon-133 is a diffusable flow-limited tracer which allows a quantitative assessment of the total renal perfusion and the relative intrarenal perfusion distribution (10). After injection of a bolus of radioactive Xenon the gas rapidly distributes in the renal tissue. Being highly fat soluble and poorly soluble in water, a reversal of the concentration gradient develops, which results in a net diffusion of xenon from the kidney into the perfusate. Subsequently the gas is washed out of the organ. The disappearance of Xenon from the kidney can be characterized by a multiexponential curve (11). Various compartments within the kidney, each with its own flow rate contribute to this curve. In a normal dog kidney four such compartments have been determined of which compartment I is the most important since it represents the renal cortex (12). A lot of discussion surrounds the interpretation of the meaning of the other three compartments (13). Therefore, we preferred to describe the gas washout curve as a two compartment system (cortex and sub-cortex). Our study shows a good correlation between the TRF, calculated by this two compartmental analysis method, and APF obtained by weight measurements of the effluent during perfusion.

Vascular integrity and major defects in the regional perfusion can be evaluated by visual interpretation of radionuclide flow studies. The potential of a relatively new radiopharmaceutical, technetium-99m HMPAO was demonstrated in cerebral perfusion studies (14). This highly lipophilic complex passively penetrates biological membranes, as shown by a high brain uptake after intravenous injection. After passing the cell membrane an intracellular reaction takes place, converting the agent into a more hydrophilic species. It is suggested that due to this intracellular metabolism, the compound remains in the cell and is not cleared from the tissue as easily as, for instance, the widely used perfusion marker in kidney transplantation diethylene triamine penta-acetic acid (^{99m}Tc -DTPA) (15). It is therefore possible to make static scintigraphic images in which extraction of HMPAO is proportional to the regional perfusion of the graft.

Two methods of preservation were compared in this study; cold storage preservation using HTK and machine perfusion with Belzer's solution. The results of our study show, that the beneficial effect of machine preservation becomes apparent in kidneys which have encountered considerable warm ischemic damage. In a normal dog, the cortical flow averages 85% of the total renal flow (16). Ischemic injury to the kidney is associated with a marked decrease in flow to the cortex (17). Our results confirm this observation. Furthermore, an intrarenal flow distribution takes place in concomitance with relative increase in flow to the subcortical area. In contrast to the cold storage kidneys, machine preserved kidneys showed an enhanced ability to resist these effects of warm ischemia on the renal microcirculation.

In addition, large perfusion defects were observed in ischemically damaged kidneys preserved for 24 hours by simple cold storage. These perfusion deficits might be due to the insufficient clearing of red blood cells from the kidney which consequently have become trapped in the capillaries causing obstruction of flow. However, light microscopic evaluation revealed that the red blood cell trapping in these kidneys was not restricted to the non-perfused areas on the scan. Therefore, the above-described phenomenon can not be explained by erythrocyte trapping alone. Other pathogenic factors such as endothelial and perivascular cell swelling, causing vascular narrowing, and arteriolar spasm might play a role in the process.

In conclusion our results indicate that, in case of ischemically damaged kidneys, MP using Belzer solution is superior to CS in HTK as far as preservation of the renal microcirculation is concerned. Furthermore, nucleotide flow studies using ^{99m}Tc -HMPAO and Xenon-133 might be valuable complementary tools for renal viability assessment in renal preservation.

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PROTECTION OF CANINE RENAL GRAFTS BY RENIN-ANGIOTENSIN INHIBITION THROUGH NUCLEOSIDE TRANSPORT BLOCKADE

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ABSTRACT

The aims of this study were 1) to investigate the effect of R 75231, a nucleoside transport inhibitor, on renin-angiotensin release after renal ischemia-reperfusion and 2) to establish a possible protective effect of this drug on renal function. We used a canine model for autotransplantation of kidneys that had been subjected to 30 min of warm ischemia and subsequently 24 hrs of cold storage in HTK preservation solution, with immediate contralateral nephrectomy. R 75231 was injected intravenously into six dogs in two equal portions of 0.05 mg/kg both 30 min and 10 min before reanastomosis was established. Another group of six dogs were used as controls. *Survival*: at two weeks post-transplantation, five out of six dogs in the R 75231 group and one out of six in the control group were still alive. *Renal function*: Starting on day four, serum creatinine was lower in the R 75231 group than in the control group ($p < 0.05$). *Energy metabolism*: In contrast to the control group, an inversion of the median pre-ischemia adenosine/inosine ratio was observed in the R 75231 group after reperfusion (0.4 preischemia vs 4.0 after 60 min of reperfusion). *Renin-angiotensin system*: Reperfusion of the graft resulted in an immediate increase in renin, angiotensin I and angiotensin II venous blood levels in the control group. In the R 75231 group, renin, angiotensin I and angiotensin II levels were significantly lower. *Conclusion*: Administration of R 75231 before reperfusion has a protective effect on post-transplant function of kidneys that have been subjected to prolonged warm ischemia. This effect may, at least in part, be ascribed to inhibition of the breakdown and disposal of endogenous adenosine which, in turn, inhibits the

excessive stimulation of the renin-angiotensin system in the early phase of reperfusion.

INTRODUCTION

Since the availability of nonischemic donor organs is limited, the use of ischemically damaged kidneys for transplantation is increasing (1). Unfortunately post-transplant outcome of these so-called Non-Heart-Beating donor kidneys is characterized by a high percentage of delayed recovery of normal function due to the ischemic damage to the organ (2). The theory that a significant portion of the organ injury after ischemia develops during the reperfusion phase offers possibilities for therapeutic intervention.

R 75231 is a nucleoside transport inhibitor. The effect of such an agent is to delay the breakdown and disposal of endogenous adenosine, large amounts of which are produced during ischemia (3,4,5,6,7). Cessation of the oxygen supply to a tissue causes breakdown of nucleotides (ATP,ADP,AMP) in the cells. As a result adenosine is released in the interstitium. The accumulation of adenosine in the interstitial space is transient. Functional nucleoside transporters at the interstitial side of the endothelial cell carry the adenosine across the endothelial membrane into the endothelial cell, where it is metabolized to inosine and hypoxanthine. Moreover, adenosine is rapidly released from the interstitium into the vessel lumen upon reperfusion. This process is also mediated by nucleoside transporters. By inhibiting the nucleoside transporters it is possible to prolong the physiological actions of adenosine. One of the physiological actions of adenosine is a direct inhibition of the renin secreting cells (8,9). Excessive stimulation of the renin-angiotensin system is known to have an adverse effect on renal viability (10).

The aims of this study were 1) to investigate the effect of R 75231 on renin-angiotensin release after renal ischemia-reperfusion and 2) to establish a possible protective impact of this drug on renal function in a dog autotransplant model of kidneys subjected to prolonged warm ischemia.

MATERIALS AND METHODS

R 75231.

R 75231, {(±)-2-(aminocarbonyl)-N-(4-amino-2,6-dichlorophenyl)-4-[5,5-bis(4-fluorophenyl)pentyl]-1-piperazineacetamide}, which was kindly provided by H.

van Belle, Janssen Pharmaceutics, (Beerse, Belgium), is a racemic mixture of which the l-enantiomer is the active substance. R 75231 is a specific inhibitor of nucleoside transports with no other known relevant pharmacological action. Based on previous experiments with R 75231 a dosage of 0.1 mg/kg was chosen to treat the dogs (11).

Animals and anesthesia

Experiments were performed on 12 female beagle dogs, aged 2-4 years, and weighing 10-12 kg. The animals were fasted for 12 hrs prior to surgery with free access to water. Anesthesia was induced with thiopentotal (20 mg/kg body weight) and maintained with a nitrous oxide-oxygen gas mixture delivered continuously by a positive pressure respirator via an endotracheal tube. An antibiotic prophylaxis of 500 mg ampicillin was given before each operation. During surgery the dogs were hydrated by intravenous infusion of 500 mL Ringers lactate solution and body temperature was kept constant (37°C) using a thermostatic watermattress. All experiments were performed after approval by the responsible ethics committee, and principles of laboratory animal care according to the NIH standards were followed.

Surgical protocol

The kidneys were exposed via a midline abdominal incision. The left kidney was carefully dissected from the surrounding tissue. Heparin (1000 IU) was given 10 min prior to induction of warm ischemia in order to prevent thrombosis. Ischemia was induced by crossclamping the left renal vessel pedicle. During the ischemic interval the abdomen was kept closed to maintain the kidney at body temperature. After 30 min the kidney was removed and flushed with 200 mL 4°C Histidine Tryptophan Ketoglutarate (HTK) solution (12). Flushing was performed by gravity from a bottle at a height of 100 cm. Subsequently the kidney was wrapped in a sterile plastic bag containing 500 mL HTK solution, and stored for 24 hrs on melting ice. The following day, the operation started with the introduction of a catheter into the left carotid artery to monitor the arterial blood pressure and of a catheter into the left jugular vein for blood sampling. Morphine (0.5 mg/kg) was administered intravenously in order to prevent intestinal invagination. After removal of the right kidney, the preserved left kidney was reimplanted autologously onto the iliac vessels in the right lower abdomen. In six dogs, R 75231 was injected intravenously in two equal portions of 0.05 mg/kg in 10 mL saline 30 min and 10 min before unclamping was established. The other six dogs did not receive R 75231 and were used as a control group. During vascular anastomosis rewarming was prevented by maintaining the kidney at 4°C with sterile saline. Sorbitol 20%, 150 mL, was administered intravenously after completion of the anastomoses in order to

induce osmotic diuresis. The ureter was implanted in the bladder using a technique described by Politano and Leadbetter (13). A 10 French catheter was positioned in the bladder and connected to a urine bag for urine production measurement. After recovery from surgery the dogs were allowed free access to food and water.

Cortical biopsies for measurement of energy metabolites

Wedge shaped cortical biopsies were taken from the left kidney at two time points: before induction of warm ischemia and 60 min after reperfusion of the graft. All tissue samples were immediately frozen in liquid nitrogen and stored at -80°C . The concentration of energy metabolites in the cortical biopsies was determined using an HPLC method described by Maessen et al (14). In short, before analysis, the tissue samples were lyophilized at -30°C . Adherent blood was removed. The samples were weighed and extracted in 25-50 $\mu\text{L}/\text{mg}$ tissue HClO_4 (2 mol/L) containing 5 mmol/L dithiothreitol. The tissue HClO_4 mixture was centrifuged at 1200 G for 5 min at 4°C and the supernatant was frozen in liquid nitrogen. Following the addition of 40-80 $\mu\text{L}/\text{mg}$ tissue KHCO_3 (2 mol/L) to the supernatant, this mixture was allowed to thaw during centrifugation at 1200 g at 4°C for 1 hour, during which neutralization took place. A Varian Vista 5000 (Varian, Walnut Creek, California, USA) equipped with a narrow bore, stainless steel column filled with spherical liChrosorb RP-18 particles of 5 μm (Merck, Darmstadt, Germany) was used for a gradient HPLC analysis. Injection volume of standard or sample extract was 10 μL . After each single run which took 30 min the column was re-equilibrated for 15 min. All chemicals were obtained from Merck. Peaks were quantitated at 254 nm with a Varian 604 data-system using a conversion factor of peak area per concentration of a known external standard. Standards were of the highest purity available. ADP, AMP, and IMP were purchased from Boehringer (Mannheim, Germany), hypoxanthine (HX) and adenosine (ADO) from Merck and ATP, inosine (INO) and xanthine (X) from Sigma (St. Louis, Mo, USA).

Renin, angiotensin I and angiotensin II measurements

Venous blood samples were taken before implantation, and subsequently 1,2,4,6,8 and 24 hrs after reperfusion of the graft. The concentration of angiotensin I and angiotensin II was determined by radioimmunoassay. During two incubation periods, radiolabeled angiotensin I or angiotensin II competed with unlabelled angiotensin I or angiotensin II in test samples, standards, and controls (0.1 or 0.3 mL, respectively) for a limited number of specific antibody binding sites (angiotensin I or angiotensin II). At the end of the incubation periods antibody bound angiotensin I or angiotensin II was separated from unbound angiotensin I or angiotensin II using anti-sheep (donkey) coated cellulose in

suspension as the solid phase. Following a brief incubation and centrifugation, the unbound angiotensin I or angiotensin II is measured in a gamma counter. Using a standard curve sample concentrations were expressed in pg/mL. The lower limit of detection was 1.1 fmol/tube for angiotensin I and 0.7 fmol/tube for angiotensin II. Intra-assay coefficient of variation was 7.3% and inter-assay variation was 14.1%. Plasma renin activity (ng/L per min) was measured by incubation of plasma at 37°C and 0°C for two hrs in the presence of renin substrate and was expressed as the formation of angiotensin I, measured by radioimmunoassay.

Post-transplant function and survival

Renal function was determined by measuring the daily serum creatinine concentration (in $\mu\text{mol/L}$) using a Dimension creatinine kit (DuPont de Nemours, Wilmington, USA) and the daily 24-h urine production (in mL). Animals with life-sustaining function up to 14 days were considered to be survivors.

Data analysis

All values reported are medians with interquartile ranges (25th and 75th percentiles). Energy metabolite measurements are given in $\mu\text{mol/g}$ dry weight of tissue. Total adenine nucleotide (TAN) levels were defined as the sum of ATP, ADP, and AMP. The total levels of degradation products (TDP) were determined by summing IMP, ADO, INO, HX, and X. For statistical analysis of differences between the two experimental groups, the non-parametric Kruskal Wallis test was used. The level of significance was set at 5%.

RESULTS

Post-transplant renal function and survival

A clear difference in the survival rate between the two experimental groups was observed. One animal in the control group survived although the serum creatinine remained high ($> 500 \mu\text{mol/L}$) throughout the 14 day study period. The other five control dogs all died of renal insufficiency on day 5 postoperatively. In the R 75231 group five dogs survived. One dog died on day 4 postoperatively after renal artery thrombosis. Daily serum creatinine values up to 14 days post-transplantation and daily 24-h urine production up to 7 days post-transplantation in both groups are shown in Fig 1.

Figure 1. Daily serum creatinine values ($\mu\text{mol/L}$) during the first 14 days post-transplantation (A) and daily 24-h urine production (mL) during the first 7 days post-transplantation (B) in both groups. Data are given as medians with interquartile ranges (25th and 75th percentiles). In the R 75231 group a rapid return of renal function was observed. Serum creatinine values decreased from day 4 onward and returned to almost normal values at day 14 post-transplantation. Starting on day 1, the daily 24-h urine production was significantly higher in the R 75231 group than in the control group. (* means significant ($p<0.05$) difference between the two groups, † refers to death of an animal)

Energy metabolites

Data are shown in Table 1. Except for a statistically significant higher level of IMP in the control group, tissue samples taken before induction of warm ischemia showed no differences in energy metabolite content between the two groups. After 60 min of reperfusion, TAN levels were 43% (control group) and 52% (R 75231 group) of preischemic values. This difference between the two groups was however not statistically significant. In the R 75231 group the level of ADP was higher than that in the control group ($p < 0.05$). Both groups showed a marked decrease in TDP compared to the preischemia levels. Although the levels of ADO and INO were not statistically different between the two groups, an inversion of the preischemia adenosine/inosine ratio was observed in the R 75231 group after 60 min of reperfusion (0.4 before induction of warm ischemia vs 4.0 60 min after reperfusion). This phenomenon was absent in the control group (0.5 preischemia vs 0.7 60 min after reperfusion).

Table 1. Energy metabolites ($\mu\text{mol/g}$ tissue dry weight) in renal cortical tissue before warm ischemia (0 min WI) and one hour after reperfusion (60 min REP) in the control group and the R 75231 group. Median values with interquartile ranges (25th and 75th percentiles) are given.

	0 MIN WI		60 MIN REP	
	CONTROL	R 75231	CONTROL	R 75231
TAN	9.53 (7.63-11.96)	8.97 (8.11-9.69)	4.06 (3.40-4.65)	4.67 (4.16-5.52)
ATP	3.34 (2.40- 5.58)	3.94 (2.84-3.92)	1.45 (1.13-1.78)	1.59 (1.35-1.93)
ADP	3.97 (2.88- 4.43)	3.52 (3.26-3.90)	1.63 (1.37-1.73)	2.02 (1.69-2.26)*
AMP	2.18 (2.00- 2.45)	1.67 (1.49-2.37)	0.98 (0.79-1.23)	1.02 (0.86-1.20)
TDP	3.33 (2.76- 4.37)	2.30 (1.76-3.02)	0.54 (0.36-1.22)	0.86 (0.43-1.45)
IMP	1.26 (1.15- 1.53)	0.91 (0.65-1.23)*	0.00 (0.00-0.27)	0.30 (0.09-0.43)
ADO	0.26 (0.00- 0.82)	0.16 (0.00-0.64)	0.06 (0.00-0.23)	0.20 (0.05-0.50)
INO	0.53 (0.42- 0.79)	0.43 (0.21-0.95)	0.09 (0.00-0.29)	0.05 (0.00-0.12)
HX	0.74 (0.00- 0.83)	0.29 (0.00-0.76)	0.00 (0.00-0.26)	0.00 (0.00-0.36)
X	0.70 (0.00- 0.79)	0.00 (0.00-0.75)	0.00 (0.00-0.63)	0.00 (0.00-0.52)

Abbreviations (TAN, total adenine nucleotides; TDP, total level of degradation products; ADO, adenosine; INO, inosine; HX, hypoxanthine; X, xanthine).

*, significantly ($p < 0.05$) different from corresponding values in the control group.

Systemic side effects

Administration of R 75231 resulted in a slight decrease in mean arterial blood pressure and a concomitant increase in heart rate. However, changes in both parameters were not significantly different from corresponding measurements in the control group (Fig 2).

Figure 2. Percentile changes in mean arterial pressure and heart rate measured 5 min after administration of R 75231 and subsequently 30 minutes and 60 min after reperfusion of the graft. Data are given as medians with interquartile ranges (25th and 75th percentiles). No significant changes in either parameter were observed in the R 75231 group (A) compared to the corresponding measurements in the control group (B).

Renin-angiotensin system

Data are shown in Fig 3. In the control group, reperfusion of the graft resulted in a marked increase in renin, angiotensin I, and angiotensin II levels. Maximum values were measured in the first 2 hrs after reperfusion. In the R 75231 group, renin, angiotensin I, and angiotensin II levels were statistically significantly lower than the levels measured in the control group. In both experimental groups, all three parameters returned to normal values within 24 hrs after reperfusion.

Figure 3. Venous blood levels of renin (A) (ng/L/min), angiotensin I (B) (pg/mL), and angiotensin II (C) (pg/mL) measured before induction of warm ischemia and subsequently 1,2,4,6,8 and 24 hrs after reperfusion of the graft. Data are given as medians with interquartile ranges (25th and 75th percentiles). In the R 75231 group renin, angiotensin I, and angiotensin II levels were significantly lower than the corresponding levels measured in the control group. (* means significant ($p < 0.05$) difference between the two groups)

DISCUSSION

The renin-angiotensin system is considered to be an important mediator in the development of postischemic renal failure (10). Following renal ischemia, renin release is stimulated as a compensatory response in order to preserve the glomerular filtration rate, urea excretion and the ability to concentrate urine. On the other hand, excessive stimulation of the system might have an adverse effect on renal viability. Gravas et al showed that intravenous administration of high doses of angiotensin II induced acute renal failure with the histological manifestations of ischemic tubular necrosis in rabbits (15). Moreover, elevated plasma concentrations of renin and angiotensin were observed by several investigators in patients with acute renal failure (16,17,18). The present study shows an excessive stimulation of the renin-angiotensin system immediately following transplantation of kidneys that have been subjected to prolonged warm ischemia. Maximum venous blood levels of renin, angiotensin I and angiotensin II were observed in the first 2 hrs after reperfusion of the graft. Although the high levels of renin and angiotensin returned to normal values after 24 hrs of reperfusion, all except one dog in the untreated control group developed severe renal failure, resulting in death of the animals within 5 days post-transplantation.

Pretreatment with R 75231, administered 30 min and 10 min before reperfusion, successfully inhibited the excessive activation of the renin-angiotensin system in our model. Moreover superior post-transplant renal function and survival was seen in the R 75231 group. The direct pharmacological effect of R 75231 is the inhibition of the active transport (transporter mediated) of endogenous adenosine across the endothelial membranes (7). Following tissue ischemia, nucleoside transport inhibition results in the accumulation of adenosine in the interstitial space. This adenosine can only be released slowly into the circulation, bypassing the endothelial cell layer, by paracellular passage. The net result is a continuous prolonged action of adenosine in the early phase of reperfusion. Although the inversion of the preischemia adenosine/inosine ratio, measured in cortical tissue samples taken after reperfusion of the graft in the R 75231 group, indicates inhibition of the adenosine transport in our model, we did not observe an accumulation of adenosine in these samples. This discrepancy probably results from the fact that the renal biopsies were taken 60 min after the start of reperfusion. By that time the major part of the adenosine pool might already have leaked out from the tissue and into the renal circulation. Pretransplant administration of R 75231 had no effect on the recovery of TAN during reperfusion, which makes it unlikely that adenosine was used to rebuild adenine nucleotides through the so-called salvage pathway (19). Therefore, all protective effects of nucleoside transport inhibition in our model must be attributed to the physiological actions of adenosine itself, inhibition of renin release being one of them.

The use of exogenous adenosine as a therapeutic tool to prevent single organ ischemia-reperfusion damage is restricted, due to the extremely short half-life of adenosine and the risk of severe hemodynamic and cardiac side effects. No statistically significant systemic side effects of R 75231 administration were observed in our study. This suggests that the action of the endogenous adenosine was restricted to the local ischemic area.

Angiotensin II antagonists and angiotensin I converting enzyme inhibitors have been shown to lessen the severity of acute renal failure in both experimental and clinical models including kidney transplantation (20,21,22). Therefore it is tempting to ascribe the superior post-transplant renal function and survival in the R 75231 group exclusively to the inhibition of the excessive renin release in our model. Other physiological actions of adenosine might, however, have played an important additional role. Theoretically, adenosine is well suited to tackle many deleterious influences causing ischemia-reperfusion damage: it acts as a platelet aggregation inhibitor (23), modulates the presynaptic release and post-synaptic actions of catecholamines (24,25), and has a direct effect on renal vessel wall tension (26). Moreover, adenosine is known to be a potent antagonist of leukocyte activation (27,28). Studies concerning the impact of these actions of adenosine on the development of ischemia-reperfusion injury as seen in grafted kidneys are currently being carried out in our department.

In conclusion, the administration of R 75231 before reperfusion has a protective effect on the post-transplant function of kidneys that have been subjected to prolonged warm ischemia. This effect may, at least in part, be ascribed to the inhibition of the breakdown and disposal of endogenous adenosine which, in turn, inhibits excessive stimulation of the renin-angiotensin system in the early phase of reperfusion.

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INHIBITION OF CD18-DEPENDENT LEUKOCYTE ADHERENCE BY Mab 6.5 E DOES NOT PREVENT ISCHEMIA-REPERFUSION INJURY AS SEEN IN GRAFTED KIDNEYS

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ABSTRACT

The present study was designed to determine whether β -2 integrin-mediated leukocyte adherence to the endothelium is involved in renal ischemia-reperfusion damage and to evaluate the therapeutic intervention potency of monoclonal antibody (MAb) 6.5 E, directed against leukocyte CD18 adhesion molecule. To answer these questions, we used a clinical relevant canine model for auto-transplantation of kidneys that had been subjected to 30 minutes of normothermic ischemia, followed by 24 hours of cold storage preservation. Intravital fluorescence microscopy of capsular microvessels showed that substantial leukocyte adherence occurred after renal ischemia and reperfusion. Leukocyte adherence was observed both in arterioles and in venules but predominantly in the latter. Reperfusion of the graft resulted in a statistically significant reduction of the venular red blood cell velocity (RBCV). Moreover, the venular diameter increased. No significant changes in arteriolar RBCV and arteriolar diameter were observed. Administration of MAb 6.5 E, one hour before reperfusion, inhibited leukocyte adherence to the renal microvascular endothelium, resulting in an improved venular flow two hours after reperfusion. However, we observed no beneficial effect of MAb 6.5 E pretreatment on post-transplant graft function and survival. We conclude that leukocyte adherence does not play a critical role in the development of renal injury following reperfusion of kidneys that have been subjected to prolonged warm and cold ischemia.

INTRODUCTION

Leukocytes are considered to be among important mediators in the development of organ injury after ischemia and reperfusion (1,2,3). Activated leukocytes become less deformable which may cause capillary obstruction and prevent full restoration of blood flow at reperfusion. This results in prolonged tissue ischemia (4,5). In larger microvessels a prerequisite for leukocyte-mediated injury is adhesion of the cells to the endothelium (6,7). Leukocyte adhesion is thought to be a signal required for the leukocytes to release proteolytic enzymes and oxygen metabolites into the extracellular environment (8,9). This leads in post-capillary venules to increased permeability due to interendothelial gap formation and to migration of the leukocytes into the tissue (10).

Firm and prolonged adhesion (sticking) of leukocytes to the endothelium is mediated by an interaction between the intercellular adhesion molecule-1 (ICAM-1) on endothelium and β -2 integrins on leukocytes (11,12). Therapeutic intervention using antibodies to CD18, the common β -2 leukocyte membrane molecule, has been shown to reduce ischemia-reperfusion injury in the heart (13), lung (14), liver (15), and small bowel (16) significantly.

As for the kidney, there is a debate over whether or not ischemia-reperfusion injury is leukocyte-dependent. Infusion of human neutrophils into postischemically isolated perfused rat kidneys was shown to augment reperfusion injury when compared to kidneys perfused with albumin alone (17). Further evidence of a leukocyte involvement in renal ischemia-reperfusion injury was provided by studies with neutrophil-depleted rats that developed less azotemia 24 hours after ischemia than non-neutropenic controls (18). In contrast, Thornton et al. failed to show any functional or morphological protective effect of anti-CD18 monoclonal antibody (MAb) 60.3 after renal artery occlusion in the rabbit (19). A potential problem in interpreting the latter results was that they could not confirm whether the antibody in fact prevented leukocyte adherence within the kidney.

The major objectives of this study were 1) to determine whether leukocyte adherence is involved in renal ischemia-reperfusion and 2) to evaluate MAb 6.5 E, directed against leukocyte CD18 membrane protein, as a therapeutic intervention. To answer these questions, we employed a clinical relevant canine model for transplanting kidneys that had been subjected to warm ischemia and then placed in cold storage for 24 hours. Besides a survival protocol, we also incorporated intravital fluorescence microscopy in the study to assess leukocyte adherence in microvessels at the surface of the kidney.

MATERIALS AND METHODS

Animals and anesthesia

Experiments were performed on 16 female Beagle dogs, aged 2-4 years, and weighing 10-12 kg. The animals were fasted during 12 hours prior to surgery but had free access to water. Anesthesia was induced with thiopental (20 mg per kg body weight) and maintained with a nitrous oxide-oxygen gas mixture delivered continuously by a positive pressure respirator via an endotracheal tube. An antibiotic prophylaxis of 500 mg ampicillin was given before each operation. During surgery the dogs were hydrated by intravenous infusion of 500 mL Ringer's lactate solution, and body temperature was kept constant (37°C) using a thermostatic watermattress. All experiments were done only after approval by the responsible ethics committee, and the 'Principles of laboratory animal care', according to the NIH standards, were followed.

Surgical protocol

The kidneys were exposed by a midline abdominal incision. The left kidney was carefully dissected from the surrounding tissue. Heparin (1000 IU) was given 10 minutes before induction of warm ischemia to prevent thrombosis. Ischemia was induced by cross-clamping the left renal vessel pedicle. During the ischemic interval the abdomen was kept closed to maintain the kidney at body temperature. After 30 minutes the kidney was removed and flushed with 200 mL 4°C Histidine Tryptophan Ketoglutarate (HTK) solution (20). Flushing was performed by gravity from a bottle at 100 cm height. Subsequently the kidney was wrapped in a sterile plastic bag containing 500 mL HTK solution, and stored for 24 hours on melting ice. The next day, the operation started with an intravenous administration of morphine (0.5 mg per kg) to prevent intestinal invagination. After a right nephrectomy the preserved kidney was reimplanted autologously onto the iliac vessels in the right lower abdomen. During vascular anastomosis rewarming was prevented by maintaining the kidney at a temperature of 4°C with sterile saline. We administered 150 mL 20% sorbitol intravenously after completion of the anastomoses to induce osmotic diuresis after implantation. The ureter was implanted into the bladder with a technique described by Politano and Leadbetter(21). A catheter for blood sampling was placed in the right jugular vein.

Monoclonal antibody

Dr. M. Robinson of Celltech (Berkshire, UK) kindly provided the MAb 6.5 E, which is a murine IgG1 antibody that is specific for human CD18 and strongly inhibits CD18-mediated adhesion of human leukocytes to a plastic substratum. The antibody cross reacts with canine CD18 and also inhibits adhesion of canine

leukocytes to a plastic substratum (data not shown). Based on findings from earlier experiments with MAb 6.5 E in the rabbit (22) and in the guinea-pig (23) and based on our own in vitro experiments, the dogs were treated with a single intravenous injection of 1.5 mg/kg, administered one hour before reperfusion of the graft.

Intravital fluorescence microscopy study

We studied intravital microscopy in 4 dogs (two Mab 6.5 treated animals and two untreated controls).

Equipment

The experimental setup consisted of a modified Leitz Orthoplan microscope in combination with a television circuit, including a highly sensitive television camera (Bosch TYC 9A, 1 inch SIT-tube RCA 4804), a monitor screen (Philips LDH 2122; 12 inch) and a video cassette recorder (JVC VHS HR-D910E). A Leitz 4x objective lens (Numerical Aperture 0.14) or Leitz 10x objective lens (Numerical Aperture 0.30) were used. The surface of the kidney was incidentally illuminated through the objective lens with light from a 100W mercury arc. For bright field microscopy we used in the incident illuminator (Leitz Ploemopak) a POL (polarizer/analyzer)-cube and for fluorescence microscopy the I₂ filter set (excitation filter: BP 450-490; dichroic mirror: RKP 510; barrier filter: LP 515).

Observations

Observations were made in the renal capsula. During the whole experiment the renal capsula was kept moist with warm (37°C) saline. A solution of acridine orange in saline (5 mg/mL; Sigma, Deisenhofen, Germany) was injected intravenously (5 mL) to label the leukocytes fluorescently (24). This was repeated for each recording session, i.e. prior to nephrectomy, and subsequently at 15 minutes, 60 minutes, and 120 minutes after reperfusion. At each time of data collection we studied 5 arterioles and 5 venules, each approximately 150 µm long.

Measured parameters

We analyzed video-recordings offline to assess leukocyte-endothelial adherence, microvascular diameters (in µm) and red blood cell velocity (RBCV) (in µm/-sec) as a measurement of microvascular flow. The incidence of leukocyte adherence was expressed as the percentage of the vessels in which one or more leukocytes remained stationary for at least 20 seconds (25). RBCV was determined by means of the flying spot technique (26). In brief, this technique is based on the principle of synchronizing the velocity of dots moving over the

monitor screen with the velocity of blood cells and plasma gaps in the vessel. Vessel diameter was computed by measuring the distance between two dots placed outside the vessel on either side and touching the inner wall.

Survival study

Six dogs treated with MAb 6.5 E and six untreated control dogs were followed for 14 days post-transplantation. Daily serum creatinine values were determined with a Dimension creatinine kit (DuPont de Nemours, Wilmington, USA). On day 15 post-transplantation all animals were sacrificed. Histology data were obtained after premature death or after sacrifice. At autopsy we bisected kidney, liver, pancreas, heart and lungs for gross macroscopic examination. Wedge-shaped biopsies were fixed in 4% neutral buffered formalin, dehydrated and paraffin-embedded. Sections (4 μ m thick) were cut and stained with haematoxylin/eosin for light microscopic evaluation.

Statistics

Intravital fluorescence microscopy study

At each time of data collection 5 arterioles and 5 venules were studied. Data are presented per individual animal as medians together with their interquartile ranges (25th and 75th percentiles). Per animal, differences in time related samples were tested with the Friedman Two-way ANOVA test. The level of significance was set at 5%.

Survival study

Data are presented per experimental group. To characterize group values, medians are given together with their interquartile ranges (25th and 75th percentiles). The survival rate was defined as the percentage of the dogs alive 14 days after transplantation. The nonparametric Kruskal-Wallis one-way ANOVA test was used to test for differences between treated and untreated groups in daily serum creatinine values. The level of significance was set at 5%.

RESULTS

Intravital microscopy study

Leukocyte adherence

Leukocyte adherence to the microvascular endothelium was not observed in the pre-nephrectomy recordings (Table 1). Shortly after reperfusion, leukocyte adherence was seen in almost all venules. After one hour, leukocyte adherence was observed in all venules and arterioles. Figure 1 gives characteristic photo-

graphs taken before and immediately after injection of acridine orange, showing massive leukocyte adherence in arterioles. The MAb 6.5 E completely prevented leukocyte adherence in arterioles, and reduced it in venules during the observation time (Table 1).

Figure 1. Characteristic photographs from a recording before (A) and immediately after (B) injection of acridine orange showing massive leukocyte adherence in arterioles 120 minutes after reperfusion in an untreated control graft. The fluorescence dye has not yet arrived on the venous side.

Table 1. Percentage of vessels in which leukocyte adherence was observed before nephrectomy and subsequently 15, 60, and 120 minutes after reperfusion. Reperfusion of the graft induces leukocyte adherence in venules as well as in arterioles. This phenomenon is inhibited by pre-treatment with monoclonal antibody 6.5 E (MAb). Ten vessels in each group.

Observation time (min)	Control		MAb	
	Venules	Arterioles	Venules	Arterioles
Pre-nephrectomy	0%	0%	0%	0%
15 min reperfusion	90%	0%	0%	0%
60 min reperfusion	100%	100%	20%	0%
120 min reperfusion	100%	100%	40%	0%

Figure 2. Median venular red blood cell velocity (RBCV) in $\mu\text{m}/\text{sec}$ (with interquartile ranges; 25th and 75th percentiles) per individual animal measured before nephrectomy and subsequently 15, 60 and 120 minutes after reperfusion in two control dogs. Reperfusion of the graft results in a significant decrease in venular flow. (* means $p < 0.05$)

Microvascular flow

Reperfusion of the graft resulted in a statistically significant reduction of venular RBCV as compared to the pretransplant value in the control animals (Figure 2), but not in the MAb treated animals (Figure 3). In both the MAb treated animals and the control animals, no significant changes over time were observed in arteriolar RBCV.

Figure 3. Median venular red blood cell velocity (RBCV) in $\mu\text{m}/\text{sec}$ (with interquartile ranges; 25th and 75th percentiles) per individual animal measured before nephrectomy and subsequently 15, 60 and 120 minutes after reperfusion in two MAb 6.5 E treated dogs. The venular RBCV did not change significantly during reperfusion.

Vessel diameter

In the control animals, venular diameter remained constant until 60 minutes after reperfusion (Figure 4) and subsequently increased. This phenomenon was not observed in the MAb treated animals (Figure 5). In both the MAb treated animals and the control animals, the arteriolar diameter did not change significantly during reperfusion.

Figure 4. Median venular diameter in μm (with interquartile ranges; 25th and 75th percentiles) per individual animal measured before nephrectomy and subsequently 15, 60 and 120 minutes after reperfusion in two control dogs. In both dogs an increase of venular diameter can be observed after 60 minutes of reperfusion. (* means $p < 0.05$)

Figure 5. Median venular diameter in μm (with interquartile ranges; 25th and 75th percentiles) per individual animal, measured before nephrectomy and subsequently 15, 60 and 120 minutes after reperfusion in two MAb 6.5 E treated dogs. The venular diameter did not change significantly during reperfusion.

Survival rate and renal function after transplantation

In the MAb 6.5 E treated group, one dog survived the whole study period of two weeks (Figure 6). Two dogs died approximately eight hours after transplantation. Autopsy revealed severe lung edema accompanied by leukocyte sequestration in the pulmonary circulation. The other three MAb treated dogs died on day five postoperatively. Histologic sections of the graft showed massive tubular necrosis in all three cases, especially in the corticomedullary area. Of the six untreated controls, one dog survived. The other five dogs died on day five due to renal insufficiency. Histology showed severe tubular damage, similar to that described for the MAb treated dogs that died on day five postoperatively. No significant differences were found in daily median serum creatinine values between the two groups during the first five days postoperatively (Figure 6).

Figure 6. Daily median serum creatinine values ($\mu\text{mol/L}$) with interquartile ranges (25th and 75th percentiles) measured before nephrectomy and subsequently for the first 14 days after implantation of the graft. The figure shows that there is no significant difference in renal function between the group treated with MAb 6.5 E (MAb) and the control group. († refers to death of an animal)

DISCUSSION

Our intravital microscopic study shows that leukocyte adherence in fact occurs after renal ischemia and reperfusion. Although similar observations have been made in several other organs (3,27), we have found no reports in literature showing this phenomenon in the kidney. All observations were made in the renal capsula. The thick capsula of a canine kidney prevents a direct visualisation of subcapsular cortical vessels as in other experimental animals like the mouse (28) or the rat (29). Decapsulation would influence the measurements. Since the capsular vessels originate from perforating arteries coming from the kidney itself, capsular microcirculation was considered representative for the perfusion of the parenchyma of the kidney.

Monoclonal antibody 6.5 E inhibited leukocyte adherence to the renal microvascular endothelium in our model. However, no beneficial effect of MAb 6.5 E pre-treatment on post-transplant renal function and survival was observed. Moreover, autopsy of the dogs that died five days post-transplantation showed similar morphological damage of the graft in the MAb group and in the control group. These findings confirm Thornton et al.'s (19) suggestion that leukocyte adherence does not play a critical role in the development of renal ischemia-reperfusion injury. The use of monoclonal antibodies that inhibit leukocyte adhesion to prevent ischemia-reperfusion damage in grafted kidneys is, therefore, questionable.

Two dogs treated with MAb 6.5 E died eight hours after reperfusion. The exact reason for this remains obscure. Such early death was not observed in the untreated control group suggesting MAb 6.5 E as a likely cause. Histology of both animals showed severe pulmonary edema and leukocyte accumulation in the lungs. Pulmonary sequestration of leukocytes after reperfusion of a large mass of previously ischemic tissue has been reported in literature. Anner et al. observed severe pulmonary hypertension and leukosequestration after 4 hours of lower torso ischemia in dogs (30). Similar observations have been made following ischemia-reperfusion of the liver (31) and small intestine (32). In addition, there is substantial evidence that in the lung, unlike in other organs, leucocyte adherence is mediated not only by CD18 dependent, but by CD18 independent mechanisms as well. Bishop et al. showed that MAb 60.3, directed against CD18, improved reperfusion and limited alveolar infiltrate following unilateral pulmonary artery occlusion in the rabbit (14). In contrast, Vedder et al. found that pulmonary injury was not prevented by MAb 60.3 in rabbits resuscitated after haemorrhagic shock, although MAb 60.3 did protect them from hepatic and gastrointestinal injury (33). Doerschuk et al. compared the effect of

MAb 60.3 on neutrophil accumulation in the systemic and pulmonary circulation of rabbits by using different inflammatory stimuli (34). Mab 60.3 inhibited neutrophil accumulation in the systemic microvasculature in all cases. In the pulmonary circulatory bed, however, the effect of MAb 60.3 depended on the inciting stimulus responsible for the inflammatory reaction. It is therefore possible that the premature deaths already mentioned in our study result from a massive release of activated leukocytes into the circulation, caused by the inhibition of CD18-dependent leukocyte adherence in the graft by MAb 6.5 E. Subsequently, these released leukocytes lodged in the lungs by a CD18-independent mechanism. Pulmonary sequestration of these cells may have caused lung tissue edema, respiratory insufficiency and cerebral anoxia.

Leukocyte adherence was more apparent in venules than in arterioles. In the control dogs, leukocyte adherence to the arteriolar endothelium was not observed in recordings made 15 minutes after reperfusion, whereas incidence in venules at that time was 90%. Similarly, in the MAb treated dogs, leukocyte adherence was not observed until 120 minutes after reperfusion in arterioles, although on the venular side adherence was present to some extent in the 60-min (20%) and the 120-min (40%) recordings. This difference in leukocyte adherence between arterioles and venules is probably related to a difference in hydrodynamic dispersal forces (e.g. wall shear rates) in both type vessels. The high shear rates in arterioles tend to sweep leukocytes away from the vascular wall, making adherence more difficult (35). Another important factor involved might be the induction of adhesion molecules on the surface of the endothelium. Differences in leukocyte adherence between arterioles and venules as seen in exposed mesentery are in this tissue attributed to such differences (36).

Reperfusion of the graft in the two control animals resulted in an inferior venular flow as compared to the pre-nephrectomy situation. In both animals, even full cessation of venular flow was seen in more than 50% of all vessels under observation two hours after reperfusion. Moreover, the venular diameter had increased significantly during the same time interval. The changes in venular flow and venular diameter can not be explained by the presented. The fact that pre-treatment with MAb 6.5 E prevented these hemodynamic changes suggests, however, that the pathophysiological mechanism responsible involves leukocyte adherence.

The present data show that leukocyte adherence is induced after renal ischemia and reperfusion. Pre-treatment with MAb 6.5 E inhibits this process. Inhibition of leukocyte adherence was, however, not sufficient to secure organ viability in our model. Therefore, we conclude that leukocyte adherence is not a critical

factor in the development of renal injury following reperfusion of kidneys which have been subjected to prolonged warm and cold ischemia.

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SUMMARY AND CONCLUSIONS

To alleviate the shortage of kidneys for transplantation, criteria for organ donation are gradually liberalized. Donors which are considered less 'ideal' are now being accepted for organ donation. The subject of this thesis is the role of the Non-Heart-Beating donor as a new source of kidneys for transplantation. Kidneys from donors who have sustained irreversible circulatory arrest are not yet utilized frequently, primarily because of the concern that long warm ischemia times in these donors might have caused irreversible damage to the organs.

In part I (chapter 1) the Maastricht Non-Heart-Beating donor protocol is presented. Using an in situ preservation technique, it is possible to limit the first warm ischemia time and to salvage the kidneys from this type of donor for transplantation. The concept is to introduce a double balloon triple lumen catheter through the femoral artery. The catheter is designed to block the aorta at its bifurcation and at the level of the diaphragm by means of the two balloons. Selective perfusion of the part of the aorta where the renal arteries take their origin is hereby possible. After introduction of the catheter, rapid cooling and adequate flushing of the kidneys is performed with a cold solution. The method is simple and can be applied in every hospital. Furthermore, a retrospective analysis of the post-transplant outcome of 57 Non-Heart-Beating donor kidneys, harvested in the Maastricht procurement region between 1980 and 1992 and transplanted in the Eurotransplant area, is reported. A matched group of 114 Heart-Beating donor kidneys is compiled for comparison. Despite the higher incidence of delayed function following transplantation of kidneys from Non-Heart-Beating donors, long-term graft and patient survival are equivalent to the outcome of Heart-Beating donor grafts. It is concluded in this chapter that when the high incidence of delayed function of the graft can be diminished, the Non-Heart-Beating donor can be a valuable asset to reduce the current shortage of kidneys for transplantation.

Part II deals with the effect of warm ischemia on renal viability and possibilities to protect the organ from ischemia-reperfusion damage as seen after transplantation of ischemically damaged kidneys. Using a critical auto-transplantation model in the dog, different preservation methods and pharmacologic agents to limit the reperfusion damage, are evaluated. In chapter 2 a comparison is made between two preservation solutions, UW and HTK, to determine which one is the most effective in cold storage preservation of kidneys which have been subjected to prolonged warm ischemia. It is shown that UW is superior to HTK,

demonstrating better survival, better recovery of kidney function, better protection against ultrastructural damage, and better preservation of energy metabolism indicated by (a faster) regeneration of adenine nucleotide levels after reperfusion of the graft. In chapter 3 two methods of preservation, cold storage and machine perfusion are compared. In contrast to cold storage, machine perfusion counteracts the high intrarenal resistance, induced by the warm ischemic damage, which ameliorates the expulsion of red blood cells from the renal microcirculation. Moreover, machine perfusion prevents tubular obstruction by cast formation. Improved recovery of post-transplant kidney function and survival is observed after machine preservation. Chapter 4 deals with the effects of cold storage and machine preservation on renal microcirculatory integrity in ischemically damaged kidneys and control kidneys. Intrarenal perfusion is assessed by two successive radionuclide flow studies in an isolated perfusion model using Xenon-133 and ^{99m}Tc -HMPAO respectively. It is shown that warm ischemic damage is associated with a decrease in total renal flow, an intrarenal flow redistribution with a relative increase in flow to the sub-cortex, and with perfusion defects of the renal cortex. In contrast to cold storage kidneys, machine perfused kidneys show an enhanced ability to resist the effects of warm ischemia on the renal microcirculation. Chapter 5 describes the effect of R 75231, a nucleoside inhibitor, on renin-angiotensin release after renal ischemia and reperfusion. R 75231, administered shortly before reperfusion of the graft, delays the breakdown and disposal of endogenous produced adenosin which, in turn, inhibits the excessive stimulation of the renin-angiotensin system in the early phase of reperfusion. Improved recovery of post-transplant kidney function and survival is observed after R 75231 pre-treatment. In chapter 6 the role of leukocyte adherence in the development of renal ischemia-reperfusion injury is determined using intravital fluorescence microscopy. Moreover, the therapeutic intervention potency of monoclonal antibody MAb 6.5 E, directed against leukocyte CD18 membrane protein, is evaluated. Pre-treatment with MAb 6.5 E (administred one hour before implantation) succesfully inhibits leukocyte adherence after reperfusion of the graft. However, no beneficial effect of MAb 6.5 E on post-transplant renal function or survival is seen. This suggests that leukocyte adherence does not play a critical role in the development of ischemia-reperfusion damage as seen after transplantation of kidneys which have been subjected to prolonged warm and cold ischemia.

SAMENVATTING EN CONCLUSIES

Om het huidig tekort aan donornieren voor transplantatie te verminderen, worden de criteria voor orgaandonatie steeds ruimer gesteld. Ook minder 'ideale' donoren komen tegenwoordig in aanmerking. Het onderwerp van dit proefschrift is de betekenis van de hartdode donor als nieuwe bron van nieren voor transplantatie. Nieren van donoren met een circulatiestilstand worden door een aantal centra voorlopig ongeschikt geacht voor transplantatie. Met name is men bang dat de nieren onherstelbare schade hebben opgelopen, door de lange warme ischemietijd bij deze donoren. De ervaring verkregen in de achterliggende jaren maakt duidelijk dat deze angst onterecht is.

In deel I (hoofdstuk 1) van dit proefschrift wordt het Maastrichtse 'Non-Heart-Beating' donorprotocol beschreven. Door middel van een speciale koeltechniek is het mogelijk de duur van de warme ischemietijd te beperken en zo de nieren alsnog te beschikbaar te houden voor transplantatie. Bij deze techniek wordt een dubbele balloncatheter via de liesarterie in de aorta van de overleden patiënt opgeschoven. De catheter is zo gemaakt dat de onderste ballon de aorta afsluit bij de bifurcatie en de bovenste ballon de aorta blokkeert ter hoogte van het diafragma. Door het opblazen van de beide ballonnen wordt een selectief perfusiegebied verkregen waarbinnen de nierarteriën gelegen zijn. Hierdoor is het mogelijk de nieren adequaat door te spoelen en te koelen met een koude vloeistof. De procedure is eenvoudig en kan in elk ziekenhuis worden toegepast. Tevens wordt in dit hoofdstuk een retrospectieve vergelijking gegeven van de resultaten van 57 Non-Heart-Beating donornieren en een controlegroep van 114 hersendode ('Heart-Beating') donornieren. Alle NHB donornieren werden in de periode 1980-1992 in de Maastrichtse regio verworven. Transplantatie vond plaats in een centrum binnen de Eurotransplant regio. Alhoewel het percentage vertraagd functionerende nieren hoger was in de groep van Non-Heart-Beating donornieren, waren er met betrekking tot transplantaat- en patiëntoverleving op de lange termijn geen verschillen tussen beide groepen. Concluderend kan gesteld worden dat de hartdode donor een waardevolle aanvulling vormt op de conventionele, hersendode donor, vooropgezet dat het hoge percentage vertraagd functionerende nieren bij de hartdode groep verminderd kan worden.

Deel II van dit proefschrift beschrijft experimenteel onderzoek naar het effect van warme ischemie op de nier en de nierfunctie en mogelijkheden om de nier te beschermen tegen de ischemie-reperfusieschade zoals die gezien wordt na het transplanteren van ischemisch beschadigde nieren. Gebruik wordt gemaakt van een kritisch auto-transplantatiemodel in de hond. In hoofdstuk 2 worden twee

preservatievloeistoffen (UW en HTK) met elkaar vergeleken. UW is superieur aan HTK voor 'cold storage' preservatie van ischemisch beschadigde nieren, getuige de betere overleving, het betere herstel van de nierfunctie, de betere bescherming tegen cellulaire schade en de betere preservatie van het cellulaire energiemetabolisme, welke blijkt uit een (snellere) regeneratie van de adenine nucleotide concentraties na reperfusie. In hoofdstuk 3 worden twee preservatiemethodes ('cold storage' en machinale perfusie) met elkaar vergeleken. In tegenstelling tot 'cold storage' preservatie, verlaagt machinale perfusie de hoge intrarenale weerstand die als gevolg van de ischemische schade optreedt. Hierdoor is een betere nierdoorspoeling mogelijk en kunnen alle nog aanwezige bloedcellen uit de renale microcirculatie verwijderd worden. Bovendien voorkomt machinale perfusie obstructie van de niertubuli door afgestoven tubuluscelresten. Machinale preservatie resulteert zodoende in een betere nierfunctie en overleving na transplantatie. In hoofdstuk 4 worden de effecten beschreven van 'cold storage' preservatie en machinale perfusie op de renale microcirculatie in ischemisch beschadigde- en controlenieren. Veranderingen in intrarenale perfusie worden geëvalueerd door middel van perfusiescintigrafie met Xenon-133 en ^{99m}Tc -HMPAO in een geïsoleerd nierperfusiemodel. Ischemische schade resulteert in een afname van de totale nierdoorstroming en een relatieve toename van de doorstroming van de sub-cortex. In de cortex van de nier worden meerdere gebieden zonder perfusie gezien. In machinaal gepreserveerde nieren zijn deze veranderingen duidelijk minder geprononceerd dan in 'cold storage' bewaarde nieren. In hoofdstuk 5 wordt de invloed van de nucleoside transportremmer R 75231 op excretie van renine en angiotensine na renale ischemie en reperfusie beschreven. R 75231, toegediend kort voor reperfusie van het transplantaat, vertraagt de afbraak van het endogeen geproduceerde adenosine en de uitwas van dit adenosine uit het interstitium. Adenosine remt de schadelijke, excessieve stimulatie van het renine-angiotensine systeem in de vroege reperfusiefase. R 75231 toediening resulteert zodoende in een betere nierfunctie en overleving na transplantatie. In hoofdstuk 6 wordt het belang van de adherentie van leucocyten aan het endotheel in het ontstaan van nierschade na ischemie en reperfusie onderzocht door middel van intravitaal fluorescentie microscopie. Bovendien wordt het therapeutisch effect van het monoclonale antilichaam MAb 6.5 E, gericht tegen het leucocyten membraan eiwit CD18, geëvalueerd. MAb 6.5 E, toegediend 1 uur voor reperfusie, remt de leucocyten adherentie maar heeft geen invloed op de nierfunctie of overleving na transplantatie. Dit lijkt erop te duiden dat de adherentie van leucocyten aan het endotheel geen kritische factor is voor het ontstaan van ischemie-reperfusieschade na preservatie en transplantatie van ischemisch beschadigde nieren.

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