Six day kidney preservation : combined hypothermic and normothermic perfusion

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SIX DAY KIDNEY PRESERVATION

combined hypothermic and
normothermic perfusion

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PROEFSCHRIFT

Ter verkrijging van de graad van doctor in de geneeskunde
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door

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PART I

INTRODUCTION

During the last fifteen years much research has been done in preservation of kidneys for transplantation. However no major improvements have been made since Belzer developed the hypothermic continuous perfusion in 1967 and Collins introduced the "cold storage" method in 1969. Nowadays kidneys can be preserved satisfactory 24 to 48 hours by one of these basic techniques. This preservation time allows for tissue typing, transport of the organ, preparation of the recipient and the surgical procedure.

There are several advantages if long-term kidney preservation will be available. The shortage of donor organs can be reduced if less kidneys are discarded because the preservation time has exceeded safe limits. In addition more time will be provided for preparing the recipient and for elective surgery. The introduction of new techniques in organ preservation may also enable improved viability testing of kidneys before transplantation. Moreover, further research in organ preservation may eventually lead to the ultimate goal of "organ banking", that has not been achieved as yet.

Much has been written about organ preservation. To give here a comprehensive review of the literature would be beyond the scope of this thesis. In chapter I an overview is presented of the development and current status of various methods for extracorporeal kidney preservation which are employed in the clinical and experimental situation. Further, the subject of this thesis will be introduced, i.e. the combination of hypothermic- and normothermic perfusion as a method for intermediate-term kidney preservation.

section 1: Normothermic perfusion

Throughout human history people have been interested in preservation of organs outside the body. In ancient Egypt (2700-1000 B.C.) most organs of human cadavers were carefully preserved and stored outside the body in vases (canopes). However, this kind of preservation took place as part of a mummification procedure for magical-religious reasons and had nothing to do with scientific interest in living organs. In later dynasties, as reported by Herodotus (1), separate embalment of organs had been abolished.

It was not before the nineteenth century that the first attempts at experimental preservation of living organs were made from physiological point of view. In 1849 Löbell (2) initiated the isolated perfusion of kidneys with defibrinated blood to investigate the process of urine formation. In 1881 Martin et al. (2) studied the activity of a heart-lung preparation. They succeeded in excluding the heart-lung circulation from the rest of the body by diverting the total cardiac output to the right auricle. In 1903 Brodie (3) designed an apparatus for isolated organ perfusion in which the oxygenation of the blood was effected by mixing it with air in its passage through the pump. However, in most of such experiments, especially in kidney perfusions, it was difficult to obtain a sufficient flow rate through the organ. In 1912 Knowlton and Starling (4) simplified and improved the heart-lung perfusion method of Martin. They succeeded in measuring the output of the left heart ventricle under nearly normal conditions. Hereby the amount of blood needed for one experiment was greatly reduced. This was an important contribution to the better quality of the perfusion.

In 1914 Bainbridge and Evans (5) employed the heart-lung preparation of Knowlton and Starling for the perfusion of kidneys both in situ and isolated. In this system, as opposed to the apparatus of Brodie, the kidneys seemed in a nor-

mal functional condition and had a good blood flow. So these conditions were more physiological although in this form of perfusion the investigation of the renal function was complicated by variations induced by metabolism of the heart-lung preparation itself.

In 1920 Belt et al. (6) described the artificial perfusion of different organs and body tissues. They emphasized that different factors in the perfusion procedure, such as composition of the perfusate, method of oxygenation, ischaemia and pressure were important in relation to injury to the perfused organ. The difference in results of the experiments between Brodie and Brainbridge becomes more obvious by the work of Hemingway (7), who developed a lung-kidney preparation mechanically perfused by a pump. He demonstrated that defibrinated blood, which had been stored for a while, caused vasoconstriction in the perfused kidney. The vasoconstriction properties of the blood disappeared within five to ten minutes when a lung was included in the perfusion circuit. When a steady renal blood flow had been established, the lung could be removed without recurrence of vasoconstriction.

In 1957 Brull et al. (8) showed that also heparinized blood had vasoconstrictive properties when employed in artificial kidney perfusions. Since this vasoconstriction seemed to be the most limiting factor in the completely isolated kidney perfusion, Nizet, Cuypers and other coworkers of the group of Brull investigated the circumstances in which the vasoconstriction occurred. They detected vasoactive material in plasma of extravasated blood which had been stored for only a few minutes. The circulation through an isolated kidney could be blocked almost entirely by perfusion with blood that had been stored for 40 minutes (9). It was concluded that the vasoconstriction was caused by humoral factors which should be released mainly by erythrocytes (see also chapter IX). Moreover the traumatically handling of the blood perfusate induced a more severe vasoconstriction in

the perfused kidneys. In additional experiments Cuypers and Nizet (10) investigated the ability of different organs to neutralize the vasoconstrictive material. Their results confirmed the observation of Hemingway (7) that the vasoconstrictive substances were eliminated or inactivated by the lungs. However, this neutralizing effect was also seen in the liver and in the kidney itself. Taking into account these data, Cuypers and Nizet designed a significantly improved apparatus for isolated kidney perfusion (11). With this device, approximately normal kidney function could be achieved provided three important conditions were fulfilled (12):

- a) The reduction of mechanical trauma of the blood was achieved by using a film oxygenator and a Dale Schuster type pump with two light valves (13),
 - b) The perfusion started immediately after filling of the circuit with fresh blood,
 - c) The amount of blood volume in the perfusion machine was kept low and did not exceed 15 ml per gram tissue weight.
- By frequent recirculation of the blood through the kidney, the released vasoconstricting material could be eliminated continuously. A more detailed description of this perfusion machine is given in Chapter V.

Other or modified techniques for normothermic blood perfusion of isolated kidneys were described by several authors (14,15,16). Rosenfeld et al. (17,18) achieved satisfying function in rabbit kidneys by using whole heparinized blood in an entirely plastic perfusion system including a film oxygenator. Berkowitz et al. (19) obtained good results in perfused canine kidneys: injury to the heparinized blood was reduced by employing a silicone rubber circuit and a membrane oxygenator. Waugh and Kubo (20) described improved function in isolated dog kidneys. In their perfusion apparatus with a film oxygenator, normal blood flow rates were measured within the first hour of the perfusion although, as distinct from one of the principles mentioned by Cuypers and

Nizet, they used defibrinated blood that had been stored up to 1.5 hour.

Although some of the main problems in normothermic perfusion of isolated kidneys have been solved certain drawbacks remain. After 2 to 3 hours isolated perfusion, a progressive loss of vascular tone, a decrease of glomerular filtration and a dissipation of urine acidification are observed (21). Moreover alterations in the intrarenal blood flow distribution in favour of the medulla are probably responsible for an increasing loss of concentration ability of the kidney (22).

To avoid the difficulties inherent to blood perfusions several investigators (23,24,25) performed normothermic isolated kidney perfusions with an artificial cell-free perfusate. These perfusions are best suited for kidneys of small animals and yield satisfying results in metabolic and pharmacologic studies. Very high perfusate flow rates commonly observed in these perfusions are due to the low viscosity of the cell-free perfusate. However, other physiological functions of cell-free perfused kidneys seem to deteriorate more rapidly than in blood perfused organs. Within two hours Bahlmann et al. (23) found a decreasing glomerular filtration rate (GFR) and a low sodium and water reabsorption in isolated rat kidneys perfused with a bovine-albumin solution. Ross et al. (24) showed improved ability of sodium reabsorption in rat kidneys by the addition of glucose as substrate to a dialyzed albumin solution. Fuller et al. (25) obtained GFR values within the normal range by using Pluronic F108 as colloid in two hours perfused rabbit kidneys. In all these cell-free perfusions, a high oxygen pressure was maintained in the perfusate.

Another approach to achieve adequate oxygen supply in normothermic bloodless perfused organs is the use of fluorocarbon. Micro-emulsions of fluorocarbon particles (two to three micron), which can be considered as artificial erythrocytes, have a high oxygen-carrying capacity. Kowalewski

et al. (26,27) described fluorocarbon perfusion of isolated gastroduodenopancreatic preparations in dogs which maintained secretory function up to five hours. However, Endrich et al. (28,29) showed that fluorocarbon emulsions had a detrimental effect on microcirculation and endothelial cells, unless used in small concentrations. Dunn et al. (30) perfused dog kidneys with fluorocarbon emulsions up to 18 hours. In these perfusions the loss of organ viability was related to swelling and considerable weight gain (>50%) in combination with increasing vascular resistance.

Oxygen transport in extracorporeal organ perfusion can also be obtained with stroma free haemoglobin (SFH) solutions. In these perfusions the free haemoglobin does not only play a role in oxygen delivery but also creates colloid osmotic pressure. Schrier et al. (31) reported sufficient oxygen release in isolated dog kidneys perfused with a 6 g% SFH solution. However, during six hours perfusion they found a continuing decrease in perfusate flow rate. Histological examination of the kidneys demonstrated haemoglobin deposits in the glomeruli. Suaudeau et al. (32) described isolated lamb heart perfusions with an 8 g% SFH solution for eight hours. In their experiments the addition of plasma-proteins was essential for a good functional and morphologic preservation of the heart.

It can be concluded that so far the functional integrity of an isolated organ cannot be preserved longer than seven hours by one of the described methods of normothermic perfusions (33). Lack of substrates, hormones and oxygen supply, the cumulation of waste products and damage caused by the mechanical way of perfusion are all factors which may be responsible for this failure. Therefore organ preservation by only normothermic perfusion seems to be unsuitable for application in transplantation procedures.

Some investigators (34,35,36) have used normothermic perfusions to assess the viability of kidneys after different methods of preservation. A new application of normo-

thermic perfusion in kidney preservation will be introduced at the end of this chapter.

section 2: Cold storage

In the early sixties cadaver kidneys were used for the first time in human transplantation (37). A reliable method was necessary for preservation of the organ while the prospective recipients were prepared for surgery.

It was known by experiments of Lefèbre and Nizet, published in 1952 (38), that after initial cooling by brief perfusion with a "physiological" solution, kidneys could be stored up to 24 hours at low temperatures. After autotransplantation the kidneys recovered functional activity. In 1959 Levy (39) demonstrated a marked but reversible reduction of oxidative metabolism in hypothermic perfused dog kidneys. So the use of hypothermia seemed to be an attractive and simple approach for practical organ preservation.

In 1963 Calne and Pegg (40) described experiments in which the influence of simple surface cooling on dog kidneys was studied. After perfusion with 20 ml of heparinized blood the kidneys were stored at 4°C in a Hanks' solution for periods up to 24 hours. Kidneys preserved for 12 and some up to 17 hours had life-sustaining function after implantation followed by immediate contralateral nephrectomy. The preservation time could be extended successfully to 24 hours if the kidneys were perfused with cold arterial blood prior to surface cooling. The cold blood itself seemed to protect the kidney for ischaemia, because in the perfusion experiments of Calne the cooling rate was the same as in those with surface cooling alone. This was due to the additional technical manipulations required in the perfusion with cold blood (41).

To investigate further the effect of rapid cooling low molecular weight dextran solutions were used. However, after

24 hours preservation no life-sustaining kidney function was observed. Histological examination of these kidneys showed hyaline deposits in the glomeruli.

Similar histological findings were reported by Kiser et al. (42) using dextran solutions for rapid cooling of dog kidneys followed by surface cooling. In their series they obtained life-sustaining kidney function after seven to eight hours preservation.

Another method of initial flushing with subsequent cooling was described by Brunius et al. (43). The preservation method of kidneys obtained from cadaver donors involved three stages:

- a) injection of a vasodilator and an anticoagulant in the renal artery,
- b) flushing of the organ with a 5% low molecular weight dextran solution at 5°C,
- c) cold storage after perfusion with a solution containing equal amounts of 10% fructose and 1.4% sodium bicarbonate.

Eight out of 46 kidneys, preserved by this technique up to seven hours, never functioned. In dog experiments the authors obtained satisfactory preservation up to 20 hours using this procedure. In Europe this technique has been used in clinical preservation for some years.

In 1966 Keeler et al. (44) described some important experiments. They analysed the changes in water, sodium, potassium, magnesium and phosphate content of rat kidneys after perfusion with a physiological solution of sodium chloride at 0°C. After three hours perfusion a considerable increase in tissue-water and sodium was measured. Tissue potassium content decreased rapidly whereas loss of tissue magnesium was only observed during the third hour. No loss of phosphate occurred in any perfused kidney. The mechanisms (a.o. slowing down of Na/K pump at low temperatures) responsible for these ion and water shifts will be discussed in section 4 of this chapter.

To find out if the damage caused by the diffusion of water and electrolytes across the cell membrane could be prevented, Keeler et al. used perfusates containing high concentrations of potassium and magnesium. After three hour perfusion of dog kidneys at 0°C followed by implantation good renal function was observed. The idea to use intracellular-like solutions was elaborated further by Collins et al. (45). Dog kidneys were flushed with 100 to 150 ml of various cold solutions with an intracellular composition and stored for 24 to 30 hours at 4°C. After implantation and immediate contralateral nephrectomy, the animals survived with excellent renal function. The composition of the various preservation fluids is listed in Table I,1. The solution without glucose (C_1) caused swelling and low perfusion-rate of the kidneys. Preservation with this solution was not superior to simple surface cooling. The C_2 , C_3 and C_4 solutions protected the kidney adequate for hypothermia. Good results were achieved in clinical preservation of cadaver kidneys up to 12 hours using the C_3 solution (46). However, the additives procaine (C_3 and C_4) and phenoxybenzamine (C_4) were found to be harmful for short periods of hypothermic storage (47,48), whereas the addition of heparin seemed to be of little value. In other experiments Collins et al. (49) studied the influence of changes in cation composition on the efficacy of the preservation fluid. In presence of $MgSO_4$ the exchange of K^+ by Na^+ in the preservation fluid was equally effective in the preservation up to 24 hours. This indicated that the beneficial effect of the Collins solution was not due to its intracellular like composition. However, a high potassium content did have a beneficial effect in preservations with solutions free of magnesium. Apparently magnesium prevented loss of intracellular potassium.

Lateron Collins et al. (50) investigated the role of the anion content and the osmolarity of the flush solutions. They demonstrated that the presence of osmotically active, non-diffusible anions (phosphate) and non-electrolytes (glu-

Table I,1: COMPOSITION OF FLUSH SOLUTIONS FOR "COLD STORAGE" KIDNEY PRESERVATION

Ions meq	C ₁	C ₂	C ₃	C ₄	Euro-Collins	Sacks I	Sacks II	Hyperosmolar citrate sol.
Na ⁺	10	10	10	10	10	10	10	80
K ⁺	115	115	115	115	115	115	126	80
Mg ²⁺	60	60	60	60	-	-	16	70
Cl ⁻	15	15	15	15	15	15	16	-
HCO ₃ ⁻	10	10	10	10	10	10	20	-
SO ₄ ²⁻	60	60	60	60	-	-	-	70
Phosphate*	100	100	100	100	100	100	120	-
Citrate	-	-	-	-	-	-	-	160
Additives g/l								
Glucose	-	25	25	25	35	-	-	-
Mannitol	-	-	-	-	-	50	37.5	34
Heparine U/l	5000	5000	5000	5000	-	-	-	-
Procaine HCL	-	-	0.10	0.10	-	-	-	-
Phenoxybenzamine	-	-	-	0.025	-	-	-	-
Osmolality								
mOsm/kg H ₂ O	300	320	320	320	355	410	430	400

*) HPO₄²⁻: 85 meq/l and H₂PO₄⁻: 15 meq/l

cose) was of major importance in the prevention of cell swelling under hypothermic conditions (see section 4). The "Collins-solutions" are now widely used in clinical preservation. Watkins et al. (51) showed that similar solutions without magnesium-sulphate yielded equally good results in 24 hour preservation of dog kidneys. This simplification of the preservation fluid prevents the precipitation of magnesium-phosphate which might cause vascular occlusion (52,53).

Since 1976 most centres of the Eurotransplant organisation obtain satisfactory results in clinical preservation using a modified Collins solution. This "Eurocollins"-solution (Table I,1) (54) contains no magnesium and has an increased osmolarity by the use of a higher glucose concentration.

In 1973 Sacks et al. (55) introduced a new renal perfusate which was successfully used in preservation of canine kidneys up to 72 hours. Remarkably good results were obtained in 48 hour preservation of dog kidneys which had suffered 30 minutes warm ischaemia in situ before perfusion with the flush solution. The solutions had also an intracellular ion composition and were made strongly hyperosmolar with mannitol: one solution (Sacks I) was free of magnesium and the other (Sacks II) contained magnesium in the form of $MgCl_2$ (Table I). These solutions proved to be also suitable for preservation of kidneys at normothermia allowing two to three hours "work-bench surgery". Particularly in the protection for warm ischaemia the Sacks' solutions were superior to the Collins solutions, which failed in 24 hour preservation of kidneys exposed to 30 minutes of warm ischaemia (56). However, other authors were not able to reproduce these favourable results. Some investigators (57,58) could not preserve canine kidneys with Sacks' solutions for periods longer than 24 hours.

In 1976 Ross et al. (59) described successful 72 hour preservation with a hyperosmolar citrate solution which differed from the intracellular solutions employed by Collins

and Sacks. The good results obtained with this solution (Table I,1) clearly demonstrate that a high osmolarity and the presence of slowly permeable ions are of more importance than an "intracellular" composition (see section 4). However, other authors (60,61) have not been able to reproduce these results.

In 1978 Toledo-Pereyra et al. (62) introduced a colloid hyperosmolar solution with high concentrations of proteins, potassium and glucose. This solution was found to be superior to Sacks II solution in 24 hour preservation of dog kidneys with a preceding warm ischaemia time of 30 to 60 minutes.

It can be concluded that most of the cold storage solutions, which have been discussed, can be safely used for preservation up to 36 hours. Among several investigators different and reverse results are obtained when these flushing solutions are used for preservation periods of 48 to 72 hours. A considerable warm ischaemia time antecedent to hypothermic storage greatly reduces the efficacy of the flush solutions. The mechanisms which might be responsible for this fact will be discussed in the next section.

section 3: Hypothermic continuous perfusion

In the early stages of kidney preservation most attention was paid to hypothermic continuous perfusion of the organ. Indeed this technique has some advantages above cold storage.

In 1959 Levy (39) demonstrated that in perfused kidneys hypothermia induced a reduction in oxygen consumption which was proportional to the centigrade temperature. Hence, cell metabolism and so the need for substrate and oxygen supply are not completely abolished under hypothermic conditions. Burg et al. (63) indicated that even at 0°C an active transport of cations can be detected in renal tubular cells. This

persistence of active ion transport at low temperatures was confirmed later by Lambotte (64). Therefore it seems to be most likely that only a continuous perfusion can account for the cell requirements under hypothermia. The method of hypothermic continuous perfusion was first described by Humphries et al. in 1963 (65,66). They perfused dog kidneys with 50% diluted blood at 10°C for 24 hours. After autotransplantation five out of seven kidneys had life-sustaining function. The perfusion circuit included a glass wool filter and a membrane lung. In a few experiments successful three day preservation was achieved (67).

In 1967 Belzer et al. (68) were the first who described consistent successful results in 24 and 72 hour hypothermic continuous perfusion of canine kidneys. Essentially their perfusion circuit resembled that of Humphries', but as perfusate they used cryoprecipitated plasma obtained from citrated blood to which magnesium sulphate, dextrose, insulin, penicillin and hydrocortisone were added (Table I,2). In this cryoprecipitated plasma flocculations of lipoproteins, generated by a procedure of freezing and rapid thawing, were removed by microfiltration. Preservation of canine kidneys for 72 hours using the Belzer technique was also described by Woods in 1971 (69). However, his experiments were only successful if 250 mg methylprednisolone was added to one litre of perfusate. Abouna et al. (70) were not able to obtain good results unless the amount of perfusate (600 ml) was exchanged by fresh cryoprecipitated plasma at 12 to 16 hour intervals during the three day preservation period. He suggested that high levels of ammonia concentration up to 1.5 mmol/L measured in the perfusate were responsible for failure when the perfusate was not exchanged. Indeed in vitro studies of Preuss and Murdaugh (71) have indicated that ammonia is a toxic substance to renal tubular cells. Abouna perfused the canine kidneys at 12 to 14°C whereas Belzer perfused at 8°C. The higher temperature might explain the rapid accumulation of waste products measured by Abouna.

Table I,2: COMPOSITION OF PERFUSATES FOR HYPOTHERMIC CONTINUOUS PERFUSION OF KIDNEYS

	Plasma (Belzer)	PPF (Johnson)	Albumin (Claes)	Albumin perfusate ^a (Belzer)
Na ⁺	145	130	107	Sodiumgluconate 80 mmol
K ⁺	7	15	5	25 mmol
Mg ²⁺	5	6	6	8.3 mmol
Ca ²⁺	3.5	4.4	-	- mmol
Cl ⁻	95	105	100	- mmol
HCO ₃ ⁻	5	27	12	H ₂ PO ₄ ⁻ 25 mmol
SO ₄ ²⁻	4	-	6	8.3 mmol
Protein	50 g/l	-	-	Glutathione 3.0 mmol
PPF	-	37 g/l	-	Adenosine 4.9 mmol
Albumin	-	-	43	37.5 g/l
Dextrose	250 mg/l	-	-	10 ml/l
Glucose	3	1.5	34	1.5 g/l
Insulin	80	100	-	40 U/l
Hydrocortison	100	250	100	Dexamethason 8 mg/l
Penicillin	200.000	-	10 ⁶	200.000 U/l
Ampicillin	-	1.25 g/l	-	Phenosulphatelein 12 mg/l
Osmolality	300	275	300	290 mOsm/ kgH ₂ O

a) Perfusate recently developed by Belzer et al. (see p.36 and 39)

These observations were confirmed by Grundmann et al. (72). They achieved successful hypothermic continuous perfusion up to 96 hours by exchanging the perfusate volume of 1200 ml every 24 hour. Good results were also obtained when the perfusate volume was doubled and not exchanged during the preservation period. However, a controlled study of Williams et al. (73) showed equally good results in 24 hour canine kidney preservation with a perfusate which had been already used for 72 hours.

In 1972 Johnson et al. (74) introduced a new perfusate containing plasma protein fraction (PPF). PPF is prepared by extracting heat-labile fibrinogen and gamma-globulin from plasma; subsequently the remaining heat-stable protein solution is incubated at 60°C for ten hours to inactivate eventual serum hepatitis agent. Their perfusion apparatus included a pulsatile pump and a disc-oxygenator. The perfusion was carried out at 6-8°C with a total perfusate volume of 500 ml. Using this PPF solution (Table I,2) good renal function was observed after 72 and 96 hours preservation (75).

In 1972 Claes and Blohmé (76) described successful 72 and 96 hour hypothermic continuous perfusion using a human albumin (KabiVitrum) containing perfusate (Table I,2). Dog kidneys were perfused with 0.8 litre of this solution at 6-10°C in a Gambro[®] machine including a roller pump and initially a membrane oxygenator. However, in preservation experiments up to 96 hours equally good results were obtained when the membrane oxygenator was omitted and the oxygenation was established by blowing the gas mixture above the surface of the perfusate in the organ chamber. This simplification was also applied by Johnson (77).

In 1974 Abouna et al. (78) described 24 hour hypothermic perfusion in dogs. In four experimental groups they compared cryoprecipitated plasma and albumin using pulsatile and non-pulsatile perfusion. Although the overall survival was similar in all groups, better perfusate flow characteristics were obtained with albumin perfusate using either pulsatile

or non-pulsatile perfusion. Kidneys perfused with plasma showed higher perfusate flow and better function immediately after implantation when pulsatile perfusion had been used. In 1974 Toledo-Pereyra et al. (79) introduced a perfusate containing silica gel-treated plasma, which was found to be superior to cryoprecipitated plasma. By the silica gel treatment all cholesterol, lipoproteins and fibrinogen are removed.

The first clinical applications of hypothermic continuous perfusion was reported by Belzer et al. (80). Since that time this technique has been widely used in preservation of human cadaver kidneys up to 3 days using either cryoprecipitated plasma (81,82,83), plasma protein fraction (84,85) or albumin (76,86,87). Recently, Belzer et al. (88) obtained very good results with a new albumin perfusate containing a.o. sodium-gluconate, adenosine and phosphate (Table I,2).

In hypothermic kidney preservation there has always been a kind of "cold war" between followers of the cold storage- and the continuous perfusion technique. In 1969 Collins introduced his preservation solutions as a counterpart of the "bulky, complex and expensive Belzer apparatus". Sending dog kidneys all over the world (45), he gave more information about the size of the Belzer machine at that time, than about the quality of his own preservation solutions. As mentioned before, simplifications in the method of pumping and oxygenation did not influence the results of hypothermic continuous perfusion. By these contributions the development of much smaller perfusion systems was possible (76,89,90). Today portable perfusion machines containing disposable organ-chamber cassettes are commercially available. These machines can be handled easily by one person and are suitable for transport by air. Of course, from a practical point of view, perfusion preservation still remains more laborious than cold storage. However, there are several more important differences between these two techniques which may be relevant for the choice of the best suited method for clinical

preservation.

There has been some controversy about the question whether the method of preservation influences the long term graft survival in clinical kidney transplantation. In a multicentre study Opelz and Terasaki (91) indicated that the overall survival rate of cold storage kidneys was significantly higher than in the perfused group. In this study the two groups are not strictly comparable since the cold storage group had a mean preservation time of nine hours as opposed to 23 hours in the perfused group. In the perfused group an unusual low immediate function rate (45%) was reported. This may be due to technical errors in the use of preservation machines. Centres with high experience in perfusion techniques obtained immediate function rates up to 80% (92) and in their series long term graft survival was similar or even improved after perfusion preservation (93,94,95). In another more recent study Opelz and Terasaki (96) reported superior one month and one year graft survival of human cadaver kidneys, preserved by cold storage up to 48 hours, with or without a preceding warm ischaemia time up to 60 minutes. Hypothermic continuous perfusion was only better in kidneys preserved for longer than 48 hours. From this study they concluded that cold storage was superior to continuous perfusion. However, it must be admitted that the percentages of one month and one year graft survival refer only to the rate of rejection. The immediate kidney function after transplantation would be a better parameter to evaluate the preservation technique. Rather one might conclude from the study of Opelz and Terasaki that kidneys preserved by perfusion techniques are likely to be rejected. Several authors (97,98) have reported endothelial damage and denuding of the basement membrane following perfusion preservation. This perfusion nephropathy may "unmask" antigenic sites and thus accelerate allograft rejection. Perfusion nephropathy itself is morphologically identical to hyperacute rejection (98). This perfusion-related injury can vary from minute breaks in

the glomerular endothelium to complete denudation of the basement membrane. After transplantation the exposed collagen activates the clotting sequence resulting in platelet, fibrin and white cells deposition in glomerular capillary loops, whereas in the hyperacute rejection the triggering mechanism is cytotoxic recipient antibody.

In an experimental study Cerra et al. (99) showed that endothelial damage induced by perfusion preservation could be minimized by using low perfusion pressures and perfusates with high colloid osmotic pressure. Perfusion nephropathy was not observed by van der Wijk et al. (100) in dog kidneys preserved for 96 and 144 hours. In these experiments a low perfusion pressure (20 mm Hg) and an albumin perfusate were used.

A general approved difference between cold storage and hypothermic continuous perfusion is the ability of perfusion techniques to preserve kidneys which have suffered a considerable time of warm ischaemia. This was clearly shown by Johnson et al. (101). After 60 minutes of warm ischaemia dog kidneys were perfused for 24 hours. All these kidneys returned to normal function within two weeks following autotransplantation. On the other hand kidneys preserved by cold storage for 24 hours did not function if the preceding warm ischaemia time exceeded 30 to 40 minutes.

These observations have been confirmed by Halasz (102) who reported successful 48 hour perfusion preservation in dog kidneys exposed to 20 minutes of warm ischaemia. No survival was obtained after preservation of ischaemic damaged kidneys with Collins or Sacks solutions. Denham et al. (103) also described improved results with continuous perfusion after warm ischaemia. Moreover in these experiments combinations of perfusion and cold storage were superior to cold storage alone, but inferior to continuous perfusion. In kidneys subjected to prolonged periods of warm ischaemia, Johnson et al. (56) observed an increased vascular resistance during hypothermic continuous perfusion, which was propor-

tional to the warm ischaemia interval. This was evidenced by low flow rates of the wash-out solution and higher perfusion pressures. During the first 12 hours of the hypothermic continuous perfusion, the pressure decreased to values measured in kidneys preserved without antecedent warm ischaemia. The initial wash-out was less effective since the perfusate was stained with blood. Johnson et al. concluded that the continuous perfusion had further opened up the renal circulation. Kidneys injured by warm ischaemia and preserved by cold storage for 24 hours were not completely refilled with blood after reimplantation. This so-called "no-reflow" phenomenon after renal ischaemia was first described by Sheehan and Davis (104) in 1959. The possible mechanism of this "no-reflow" phenomenon can be explained by the work of several investigators.

Flores et al. (105) observed swelling of endothelial cells after 60 minutes of warm ischaemia in rat kidneys. They suggested that during anoxia depletion of ATP inactivated the sodium pump in the cell membranes, causing perivascular cell swelling and finally vessel blockade.

Summers and Jamison (106) demonstrated perfusion defects in rat kidneys subjected to 60 minutes of warm ischaemia by perfusion of the renal artery with carbon particles (600A). When the renal vessels were emptied of blood by saline flushing prior to the warm ischaemia, the carbon distribution was similar to that in non-ischaemic kidneys. On the other hand, if saline flushed kidneys were reperfused with blood, the "no-reflow" phenomenon occurred. This suggested that the capillary lumina were too narrow to permit passage of red cells but large enough for the much smaller carbon particles. These authors also showed that the endothelial cell swelling could not account entirely for the "no-reflow" phenomenon. Large perfusion defects, present in blood-filled kidneys subjected to only 15 minutes warm ischaemia, were prevented if heparin was given before clamping of the renal vessels. It was suggested that in absence of heparin, fi-

brin-dependent rouleaux formation of erythrocytes and fibrin clots might contribute to capillary occlusion. However, anticoagulation with heparin did not prevent the "no-reflow" phenomenon after 60 minutes of warm ischaemia. This might be due to the decrease in pH caused by anaerobic metabolism in the anoxic renal tissue. Hardaway et al. (107) have shown that heparin is less effective at low pH levels. Another mechanism which might also cause the "no-reflow" phenomenon was described by Weed et al. (108). Warm ischaemia not only induces ATP depletion in endothelial and tubular cells, but also in blood cells stagnant in renal vessels. These investigators demonstrated a decreased deformability in ATP depleted red-cells. They suggested that in absence of ATP, which normally chelates calcium, the cell membranes become more rigid by calcium binding to membrane protein. These rigid cells could be easily trapped in capillaries of ischaemic kidneys. The calcium binding could be inhibited competitively by Mg^{2+} ; this would explain the beneficial effect of Mg^{2+} in wash-out solutions and perfusates. Bergstrom et al. (109) described rapid decrease of ATP levels in dog kidneys during 20 minutes warm ischaemia. After 24 hours of hypothermic continuous perfusion the ATP levels of those kidneys were increased, whereas a further decrease was observed if the kidneys were preserved by cold storage with Collins solution. The data described by these authors give a plausible explanation for the decrease in the high vascular resistance in ischaemic damaged kidneys during hypothermic continuous perfusion.

Clinically the experimental evidence has been confirmed that perfusion is superior to cold storage in kidneys subjected to warm ischaemia. Slooff et al. (110) reported a higher percentage of immediate function in kidneys obtained from non-heart beating donors preserved by continuous perfusion compared to those preserved by cold storage.

It can be concluded that hypothermic continuous perfusion is a reliable method for preservation of kidneys up to

72 hours. Moreover perfusion preservation should be the method of choice for kidneys which have suffered considerable time of warm ischaemia.

VIABILITY TESTS

It would be a great advantage to have the disposal of reliable tests predicting the viability of cadaver kidneys before transplantation. In this respect the history and pretreatment of the donor may be important.

Belzer et al. (111) described the detrimental effect of renal vasospasm in the donor due to shock in the agonal period. After transplantation of these kidneys a high incidence of acute tubular necrosis was observed. Even in cadaver kidneys with minimal warm ischaemia this vasospasm caused insufficient wash-out and high perfusion pressures during preservation. Excessive manipulation and traction on kidneys during nephrectomy could also induce vasoconstriction. Belzer emphasized the need for administration of phenoxylbenzamine to the donor before cardiac arrest to prevent this vasoconstriction. Filoso et al. (112) have confirmed the importance of cadaver donor pretreatment with vasodilating drugs.

In kidneys harvested from heart-beating donors under optimal conditions, a high flow rate of initial wash-out is generally correlated with good viability after preservation up to 24 hours with Collins solution. Kidneys obtained from non-heart beating donors are mostly selected for hypothermic perfusion. It will be obvious that this preservation technique offers the opportunity to evaluate certain mechanical and biochemical parameters during the preservation period. Clinical (81) and experimental (103) data indicate that low perfusion pressures and high perfusate flow rates during the initial phase of the preservation period tend to correlate well with viability after transplantation. However, low pressures and high flow rates measured during later phases

of perfusion are not reliable in predicting renal function. This was shown by Proctor (113). In irreversibly damaged dog kidneys he observed initial high vascular resistance. After 12 to 24 hours these pressures decreased to values within the range of undamaged control kidneys. This equal resistance may be explained by a changed pattern of corticomedullary flow as observed by Miller et al. (114) in kidneys subjected to warm ischaemia.

In their initial studies Johnson and Baxby (56,115) showed that a decrease in pH and rise in lactate levels in the perfusate during the first hour of perfusion correlated with the viability after 24 hours preservation. Throughout the remaining preservation period these values did not change very much. Later on they argued that the lactate level was only suitable to assess the extent of injury by warm ischaemia. Further deterioration of the kidney during perfusion was found to be related to the release of N-acetyl glucosaminidase (NAG), a tubule-specific lysosomal enzyme (75). However, the tubular release of NAG could not reflect the presence of glomerular lesions observed in kidneys preserved for 5 to 8 days (77).

Grundmann et al. (116) described good correlation between lactic dehydrogenase (LDH) concentration in perfusate and viability of dog kidneys subjected to 30 minutes of warm ischaemia prior to hypothermic perfusion up to 72 hours. In their series the LDH concentration correlated not only with the length of warm ischaemia but also with the duration of perfusion. Other authors (Johnson and Belzer) have objections to LDH as predictor of viability. In some kidneys with high perfusate levels of LDH, they observed good renal function after transplantation. An irregular release pattern of LDH, which is a cytoplasmic enzyme, could explain the large overlap in perfusate levels of LDH between viable- and non-viable kidneys. In a retrospective study Modgill et al. (117) evaluated some viability tests in cadaver kidneys preserved by hypothermic continuous perfusion up to about 24

hours. No significant difference in pH, LDH or free fatty acid levels could predict kidney function after transplantation.

Feinfeld et al. (118) showed that high perfusate levels of ligandin, an enzyme having glutathione S-transferase activity, correlated with damage of the proximal tubule after transplantation. Recently this finding has been confirmed by Cho et al. (119).

However, in conclusion it can be said that the prediction of viability is not likely to be correlated to the change of one single factor. The injury on preserved kidneys, being complex and multifocal, should be evaluated by all disposable data: the history of the donor, the method of preservation, perfusion characteristics and biochemical parameters. In combination with the duration of the preservation period, these factors will allow a more reliable assessment of viability after transplantation.

section 4: Mechanisms of preservation fluids

In preservation techniques as described in section 2 and 3, the vascular and interstitial space are filled with preservation fluid. The interaction between the extracellular and intracellular space may be described by the following approach.

Some billions of years ago life on earth began in the primordial ocean. It has been proposed that a relatively high concentration of potassium was present in this ocean. Evolutionary speculations suggest that the cytoplasm of early organisms closely resembled the composition of the primordial sea (120). During long-range ecological changes potassium precipitated out in the form of silicates on the bottom of the ocean. Finally the sea had a high sodium and low potassium composition. By the formation of cell membranes the living organisms protected themselves against their

	INTRACELLULAR		EXTRACELLULAR	
(a)	100 Prot ⁻		150 Cl ⁻	before equilibrium
	50 Cl ⁻		145 Na ⁺	
	40 Na ⁺		5 K ⁺	
	110 K ⁺			
(b)	100 Prot ⁻		120 Cl ⁻	Gibbs-Donnan equilibrium
	80 Cl ⁻		74 Na ⁺	
	111 Na ⁺	← H ₂ O	46 K ⁺	
	69 K ⁺			
(c)	100 Prot ⁻		150 Cl ⁻	active transport osmotic equilibrium
	50 Cl ⁻		145 Na ⁺	
	40 Na ⁺	Na ⁺ →	5 K ⁺	
	110 K ⁺	← K ⁺		

Figure I,1: Hypothermia, as applied in preservation techniques, will slow down the Na/K pump activity in cell membranes. Since these membranes are impermeable for (negatively charged) proteins, the diffusible ions will distribute themselves in a predictive way. This figure shows a simplified scheme of this situation, known as the Gibbs-Donnan equilibrium.

- Distribution of ions at the onset of hypothermia before equilibration is established.
- Gibbs-Donnan equilibrium across the cell membrane under hypothermic circumstances (see text). The excess of ions inside the cell membrane induces osmotic imbalance and subsequent cell swelling.
- For comparison the normothermic situation is illustrated here. Equal osmotic pressure on both sides of the cell membrane is maintained by active transport of sodium ions out of the cell.

changed environment. In addition, these membranes had the advantage of maintaining a desirable configuration of proteins and organic molecules. However, the development of membranes involved the need for active transport mechanisms. By active extrusion of sodium osmotically cell swelling could be prevented. This interaction between intra- and extracellular solutions will be explained here in more detail.

Cell membranes are impermeable for intracellular organic acids and proteins. At normal pH (7.4) these proteins are negatively charged (anions). Hence, if there are no active transport mechanisms within the cell membranes, the diffusible ions will distribute themselves in a predictable way. A simplified scheme of this situation, known as the "Gibbs-Donnan" equilibrium (121) is shown in figure I,1.

Before the establishment of the equilibrium, as depicted in figure I,1^a, the chemical concentration gradient for Cl^- will induce a net movement of diffusible anions (Cl^-) into the cell. By this Cl^- influx the cell will become negative. This potential difference in turn results in a redistribution of cations (Na^+, K^+). After the establishment of an equilibrium (figure I,1^b) the potential difference for diffusible ions is described by the Nernst equation (122):

$$E_d = \frac{R}{z} \frac{T}{F} \ln \frac{(C_1^+)}{(C_e^+)} = \frac{R}{z} \frac{T}{F} \ln \frac{(A_e^-)}{(A_1^-)} *$$

* E_d = electrical potential difference

R = universal gas constant

T = absolute temperature

z = valency of ion

F = Faraday's constant

C_1 = cation intracellular

C_e = cation extracellular

A_1 = anion intracellular

A_e = anion extracellular

Simplification of this expression gives the following relationship between the diffusible (univalent) ions:

$$\frac{(C_1^+)}{(C_e^+)} = \frac{(A_e^-)}{(A_1^-)}$$

If the amount of mmols Cl^- which have moved into the cell is called x , the ion concentration values in the equilibrium situation can be calculated by solving the equation:

$$\frac{(Na_1^+) + (K_1^+) + x}{(Na_e^+) + (K_e^+) - x} = \frac{(Cl_e^-) - x}{(Cl_1^-) + x}$$

After substitution of the ion concentration values of figure I,1^a (the numbers are chosen for explanatory purposes):

$$\frac{150 + x}{150 - x} = \frac{150 - x}{50 + x} \quad x = 30$$

So in absence of active transport (Gibbs-Donnan equilibrium), the concentration of Na^+ , K^+ and Cl^- in and outside the cell can be calculated.

The Na^+ and K^+ concentration ratios are reciprocal to the Cl^- concentration ratio.

$$\frac{(Na_1^+)}{(Na_e^+)} = \frac{(K_1^+)}{(K_e^+)} = \frac{(Cl_e^-)}{(Cl_1^-)} \text{ resp. } \frac{111}{74} = \frac{69}{46} = \frac{120}{80} \text{ (figure I,1^b)}$$

In this equilibrium situation a concentration difference exists for non-diffusible ions (by definition (121)). Moreover, the total amount of diffusible ions inside the cell is also greater (260 inside versus 240 outside). By this osmotic gradient water will move into the cell. In the vegetable kingdom the cell can protect itself against these osmotic forces by a rigid cell-wall of cellulose. However, the animal cell membrane would lose its motility and deformability under this high osmotic pressure. Hence, as illustrated in

figure I,1^c, there is a need for active transport mechanisms to prevent cellular swelling. Equal osmotic pressure on both sides of the cell membrane is maintained by active extrusion of sodium ions out of the cell. In mammalian species this energy dependent Na/K pump requires oxygen, substrates and normothermic conditions for optimal function.

Hypothermia, as applied in preservation techniques, will induce a slowing down of the Na/K pump which results in osmotic cell swelling. Initially the effectiveness of Collins solutions for cold storage of kidneys was attributed to the high potassium content of this "intracellular" fluid. However, surrounding the cell with a K⁺ rich solution (like the primordial ocean) would cause as much osmotic cell swelling due to the Gibbs-Donnan equilibrium (figure I,1^b). Indeed, as demonstrated later by Collins himself (50), the beneficial effect of this solution must be ascribed to the presence of poorly diffusible molecules (glucose) and slowly permeant anions (phosphates).

Previously this was also shown very clearly by Acquatella et al. (123). In dog kidneys perfused with a Collins solution without glucose (C₁), enormous cellular swelling leading to tubular damage was observed. After 48 hours cold storage with a hypertonic solution containing K₂SO₄, NaHCO₃ and glucose, histological sections showed excellent preservation of renal tissue (SO₄²⁻ is a less permeant anion). Downes et al. (124) showed that a hyperosmolar solution with high sodium and low potassium and sucrose was as effective as Collins in 24 hour preservation of dog kidneys. They concluded that during cold storage it was cellular swelling and not intracellular potassium loss which primarily caused the poor renal function after implantation. States et al. (125) demonstrated that the Na/K pump restored the intracellular potassium content in kidney slices which had been rewarmed and oxygenated after preservation in an extracellular like solution for 48 hours. Later Green and Pegg (126) also emphasized the importance of osmotically active and impermeant

neutral solutes and anions in wash-out solutions.

These neutral molecules, which counteract osmotic cell swelling, cannot prevent a net movement of diffusible electrolytes into the cell (see figure I,1^b). In addition glucose, as applied in Collins solutions, will slowly penetrate the cell during prolonged periods of cold storage (124). Therefore these preservation fluids are made hyperosmolar. However, by ion shifts across the cell membrane and osmotic imbalance, these hyperosmolar preservation solutions may even lead to cell shrinkage. Southard et al. (127) developed an iso-osmolar solution that would effectively prevent both cellular swelling and shrinkage during hypothermia. They observed no increase in slices of dog kidneys incubated in an iso-osmotic solution in which the major part of the anions was composed of the large impermeant anion lactobionate (4- β -D galactosido-D-gluconic acid). Later on good results were obtained with an iso-osmolar perfusate containing sodiumgluconate, which was effective in preventing hypothermic-induced cell swelling (128,129).

After hypothermic perfusion and revascularisation the osmotic equilibrium across the cell membrane will again be fully dependent on active transport mechanisms. This implies that the mitochondria will have to remain intact to account for the energy requirements of the cell, i.e. adenosine-triphosphate (ATP). Many investigators have emphasized the need for adequate preservation of energy generating functions.

Southard et al. (130) studied mitochondrial functions in kidney slices obtained from kidneys preserved up to 48 hours by cold storage. The ability to generate ATP decreased in proportion to the preservation time. During respiration, in absence of ADP and with succinate as substrate, the oxygen consumption observed in slices from 48 hour stored kidneys was higher than in control slices. This suggests an uncoupling of oxidation and mitochondrial phosphorylation (ATP synthesis). Kidneys exposed to warm ischaemia before hypothermic preservation showed a more rapid decline in mito-

chondrial function. In another study Southard et al. (131) investigated the effects of hypothermia on the function of isolated mitochondria prepared from fresh dog kidneys. Under hypothermic conditions (5-10°C) the respiratory rate was suppressed; however, the oxidation remained fully coupled to the phosphorylation. In the presence of the uncoupler dinitrophenol (DNP) the respiration rate did not decrease after lowering the temperature, especially when succinate was used as substrate. From these data it was concluded that at low temperatures not the respiratory chain is the rate limiting factor, but rather the translocation of adenosine-di-phosphate (ADP) across the mitochondrial membrane (Fig. I,2). Impaired rephosphorylation of ADP to ATP will induce conversion of ADP into adenosine-mono-phosphate (AMP) and ATP. So at the cost of ADP the ATP level is maintained (Fig. I,2).

Indeed Buhl and Jörgensen (132) observed relatively normal ATP-, decreased ADP and increased AMP levels in rabbit kidneys subjected to several hours of hypothermic ischaemia. During 24 hours hypothermic preservation the amount of AMP decreased while at the same time a progressive accumulation of hypoxanthine and inosine was measured in the renal tissues. This was explained by catabolism of AMP: after deamination of AMP into IMP (inosine acid) the nucleotide is hydrolyzed by 5'-nucleotidase to inosine, which in turn is converted into hypoxanthine by nucleoside-phosphorylase. The cell membrane is permeable to both inosine and hypoxanthine, so the catabolism of AMP finally leads to a decrease of the sum of adenine nucleotides (SAN). In addition the diffusion of hypoxanthine and inosine out of the cell means a loss of intracellular precursors for the postischaemic regeneration of nucleotides. Buhl and Jörgensen also demonstrated that the catabolism rate of mono-nucleotides during normothermia was about 50 times higher than during hypothermic conditions. So after implantation the ATP synthesis may be impaired due to a low level of adenine nucleotides in the cell,

PRESERVATION INJURY ON ENERGY METABOLISM

Figure I,2: Scheme of preservation injury on energy metabolism.

The ATP synthesis may be impaired at several sites:

1. Ischaemia, O_2 lack.
2. Suppressed translocation of ADP across mitochondrial membrane.
3. Uncoupling of oxidative phosphorylation.
4. Catabolism of adenosine-mono-phosphate.

(See text)

even though the mitochondria are intact.

Several investigators (133,134) have suggested that the SAN value correlates well with the viability of the kidney. Indeed Fischer et al. (135) found higher SAN levels in dog kidneys stored for 48 hours by "ROP"-preservation than in kidneys preserved by cold storage. In conformation with others (136) using this technique, they had previously observed improved renal function compared to simple cold storage (137). The technique consists of cold storage with Collins solution (4°C) combined with retrograde oxygen perfusion (ROP) through the renal vein: the oxygen diffuses out through the capsular veins which are opened by multiple pricks with a thin needle.

However, in kidneys predamaged by warm ischaemia the SAN content did not correlate with viability. In these kidneys the better quality of ROP-preservation did not result in recovery of the SAN loss, which had rapidly occurred during the warm ischaemia. Apparently the level of intracellular nucleotides does not reflect the degree of mitochondrial integrity. It is suggested that after prolonged periods of preservation a slow decrease of adenine nucleotides within the mitochondria may lead to irreversible damage and uncoupling of oxidative phosphorylation (130).

Bore et al. (138) indicated that the ability of mitochondria to produce ATP immediately upon reperfusion was a prerequisite for viability. In rat kidneys subjected to warm ischaemia the ATP level measured after ten minutes reperfusion correlated well with subsequent function. Accounting the need for rapid resynthesis of ATP Belzer et al. (88) added adenosine and phosphate to the perfusate during hypothermic perfusion. In human cadaver kidneys they obtained 80% immediate function after preservation of 22 to 58 hours and a mean warm ischaemia time of 24 minutes (range 0-55 min). Some investigators have attempted to reduce the catabolism and loss of adenine nucleotides by exogenic supplement of inosine and adenosine. Fernando et al. (139) demon-

strated that rat kidneys were protected from 60 minutes warm ischaemia by prior intra-arterial infusion of inosine. Pretreatment with inosine resulted in a more rapid resynthesis of ATP upon reperfusion. This beneficial effect of inosine was also found by Buhl et al. (140) in rabbit kidneys, although the improved renal function in the inosine treated group was not attended with higher postischaemic ATP levels. On the other hand in cold-stored kidneys the pretreatment with inosine had unfortunate results because it produced further damage to the kidney. It was suggested that crystallization of inosine at hypothermia might be responsible for this failure.

By addition of allopurinol or adenosine to the preservation fluid, Buhl et al. (141) observed improved function in rabbit kidneys after 48 hours of cold storage. Allopurinol was found to reduce the loss of intracellular nucleotides by an inhibitory effect on 5'-nucleotidase.

From the results of the above mentioned studies it can be concluded that certain steps in the energy metabolism are affected during and after hypothermic ischaemia. Since the reactions in the intracellular metabolic pathways are all linked to each other, one cannot regard one of these steps to be critical to the viability of the kidney. The data of several investigators rather suggest a time sequence for metabolic changes to occur (142).

section 5: Preservation at sub-zero temperatures

Although "cryopreservation" is not the subject of the present study, some remarks about freezing of organs should not be omitted in this thesis. The complete arrest of metabolism by storage at very low temperatures (e.g. -196°C) would enable an unlimited preservation time. However, there are some important drawbacks involved in this seemingly attractive approach to organ banking. The main problems will

be mentioned here.

a. Freezing of cells

During freezing of salt solutions the concentration of solutes increases. As ice formation goes on, this rise in salt concentration progressively lowers the freezing point of the remaining solution until the so-called "eutectic temperature" is reached. Below this temperature water solidifies together with the solute either as a hydrated salt or as crystalline salt and crystalline water (143). For example, an isotonic sodium chloride solution (0.15 molar) reaches a concentration of 5.2 molar (concentration factor: 35) at -21.6°C until the last liquid freezes (143). This implies that between the beginning of water-crystallization and eutectic freezing, the concentration of a solute depends only on the temperature. In 1953 Lovelock (144) showed that this increase in salt concentration is the principal cause of injury which occurs after freezing of cell suspensions. High concentrations of electrolytes would damage the lipoproteins of cell membranes, which increase the permeability to cations (145). As a result the cells become hypertonic which leads to osmotic lysis upon thawing and resuspending in an isotonic medium.

During subzero-cooling of cell suspensions the formation of ice crystals starts in the extracellular space at -2 to -5°C . Intracellular crystallization does not occur at these temperatures. The cell interior would not contain effective nucleators to form ice crystals above -10°C (146). Since the vapour pressure of supercooled water is higher than that of ice at the same temperature, the simultaneous existence of extracellular ice and intracellular supercooled fluid induces a thermodynamic imbalance across the cell membrane. This difference results in a water movement out of the cell, which leads to cell shrinkage and increase in concentration of intracellular solutes. Hereby the freezing point is depressed and the intracellular formation of ice is prevented. A high cooling rate allows insufficient time for water to

leave the cell, and results in intracellular ice formation, which is usually lethal to the cell. It has been suggested that subcellular organelles are disrupted by ice crystals (147). However, the amount and size of intracellular ice crystals can be reduced by extremely rapid cooling. The survival of such cells depends highly on the rewarming rate. During slow rewarming there is sufficient time for recrystallization, which has a damaging effect as yet. So the optimal cooling rate of cells should be slow enough to prevent intracellular ice formation and fast enough to avoid a severe increase in concentration of electrolytes. Since the cell membranes of various cell types have a different permeability for water, each cell type has its optimal cooling rate. However, in most cells it is not possible to prevent the injury by increase of concentration as well as the damage by ice formation (148).

In presence of cryoprotectants, such as glycerol and dimethyl sulphoxide (DMSO), the increase in electrolyte concentration can be reduced. For example, a solution containing 2M glycerol solidifies at -45.5°C . The concentration of glycerol at this temperature amounts 8.45 M. So during slow cooling the concentration increase of the electrolytes is the same as that of glycerol, i.e. only 4.23 times. Hence in presence of cryoprotectants optimal freezing of cells can be obtained at lower cooling rates since both the concentration rises and intracellular ice formation can be avoided.

At the eutectic temperature freezing is modified probably by the strong hydrogen binding capacity of glycerol. The high viscosity of the solution prevents crystal formation, and the liquid tends to solidify in an amorphous state (143).

Low molecular cryoprotectants such as glycerol may have a damaging effect themselves. These substances penetrate the cell at a much lower rate than water, so they may damage the cell by osmotic effects when they are added or removed in high concentrations. This implies that in presence of cryo-

protectants the rewarming of frozen cells should be performed slowly.

By the utilisation of glycerol or DMSO, successful cryopreservation of a number of cells can be achieved. Long term storage of red blood cells (149), lymphocytes (150), bone marrow (151), spermatozoa (152) and mammalian embryos (153) has been performed without unacceptable loss of viability.

b. Freezing of organs

Freezing and thawing of whole organs involves special problems. First, organs consist of various cell types which may have different optimal cooling rates. Secondly, the cooling rate of bulk organs is limited due to their thermodynamic properties. The low ratio of surface area to volume prevents rapid cooling. So high concentrations of cryoprotecting substances are necessary to obtain a compromise cooling rate for the survival of all different cells.

The addition and removal of cryoprotecting agents require organ perfusion techniques. Jacobsen (154) showed that rabbit kidneys perfused with glycerol in concentrations up to 4M recovered normal renal function after autotransplantation. Osmotic damage to the kidneys could be prevented by gradual introduction and wash-out of glycerol using hypertonic perfusates. However, kidneys equilibrated with 3M glycerol and frozen to -80°C for 30 minutes showed no sign of function after removal of glycerol and implantation. Pegg et al. (155) also reported unsuccessful attempts to preserve rabbit kidneys and hearts by the use of high concentrations of cryoprotectants with slow freezing and thawing. Halasz et al. (156) observed life sustaining function in 25 per cent of dog kidneys protected with glycerol and frozen to -50°C for 15 minutes. Guttman et al. (157) reported survival of dog kidneys protected with DMSO, frozen by intra arterial helium to -80°C for 15 minutes and thawed by microwave irradiation. However, these good results could not be repeated by Pegg et al. (158).

The mechanisms of freezing injury in whole organs have been partially elucidated. Jacobsen (154) observed cessation of blood flow in autotransplanted rabbit kidneys protected with 3M glycerol and frozen to -80°C . Histological examination of the kidneys 30 to 60 minutes after revascularisation revealed glomerular damage and bleeding into the tubulus, suggesting a primary vascular injury. This observation was supported by the high vascular resistance measured during the deglycerolisation perfusion after freezing. The vascular damage was explained as follows: after the perfusion with glycerol before freezing the renal capillaries are widely dilated since the colloid in the perfusate draws water from the interstitium into the vessels as soon as the perfusion pressure is removed. So the capillaries are likely to be disrupted by the mechanical effect of intravascular ice during freezing. The occurrence of extracellular ice is generally considered as rather innocuous in freezing of diluted cell suspensions. However, in freezing of organs the formation of extracellular ice may disrupt the morphological structure, since the expansion is prevented by the architecture of the tissues and the high density of interconnected cells.

In conclusion the characteristics in which whole organs distinguish from cell suspensions, namely a fixed geometry, a high density of various cells and a vascular system, are mainly responsible for the disappointing results so far obtained in freezing of organs (154).

Another approach to preservation of tissues at low sub-zero temperature was reported by Fahy and Hirsch (159). In the presence of very high concentrations of cryoprotectants solidification without ice formation occurs. This so-called vitrification refers to solidification of a liquid into a non-structured, amorphous state (glass transition) upon cooling to sufficiently low temperatures. In theory this offers an attractive approach to organ preservation. During vitrification all mechanisms which cause "freezing injury", such

as electrolyte concentration elevation and mechanical damage by intra- and extracellular ice formation, are avoided. However, the concentrations of cryoprotecting agents required for vitrification are toxic to living organs. Fahy and Hirsch reported that high pressures of 1000 and 2000 atmospheres reduced the cryoprotectant concentration required for vitrification. This discovery of baroprotection provides hope for eventual success in cryopreservation of organs by vitrification.

section 6: Combination of different preservation methods

The preservation methods, so far discussed in this chapter, all have their limitations, advantages and drawbacks. From the two methods currently used in kidney preservation, the cold storage technique has a maximal preservation time of about 48 hours, whereas the hypothermic continuous perfusion can be successfully employed up to four days. The exact mechanisms responsible for the limitations in preservation time are not completely understood. There is evidence that changes in the metabolism of parenchymal cells are restrictive to the duration of cold storage (130). According to some authors prolongation of hypothermic continuous perfusion seems to be prevented mainly by progressive endothelial damage induced by the mechanical perfusion itself (160). So from theoretical point of view the combination of the two methods could have a synergistic effect, making long-term kidney preservation possible. However, as reported by Belzer and Southard (160), attempts at intermittent hypothermic perfusion and cold storage have been disappointing. Kidneys preserved with this procedure were inferior to kidneys either cold stored or perfused separately for the same time.

In clinical kidney preservation a combination of the two methods is often employed for technical and practical reasons. Mostly hypothermic continuous perfusion of kidneys

which have suffered a considerable warm ischaemia time (see section 3) starts after several hours of cold storage, because the centre performing the donor-nephrectomy does not have the disposal of a perfusion machine.

To evaluate the effectiveness of this preservation procedure Denham et al. (103) compared the combination of continuous perfusion and cold storage with both techniques separately in an experimental model. Canine kidneys subjected to 15 minutes of warm ischaemia were preserved for 24 hours by either hypothermic continuous perfusion, six hours perfusion followed by 18 hours cold storage, six hours cold storage with subsequent 18 hours perfusion, or cold storage alone. Two hours after autotransplantation renal function was assessed by creatinine clearance determinations. In these experiments hypothermic continuous perfusion was found to be superior to the other techniques. The combination perfusion - cold storage was slightly better than cold storage followed by perfusion, which was in turn much better than cold storage alone.

Milner and Lakely (161) confirmed that the combination of hypothermic continuous perfusion and Collins technique yielded inferior results compared to hypothermic perfusion alone. Similar results were obtained by Scott et al. (162) and Woods et al. (163). In 24 hour preservation of dog kidneys they combined hypothermic perfusion with cold storage in the same perfusate (cryoprecipitated plasma). The combination of 18 hours hypothermic perfusion followed by six hours cold storage (Scott et al.) had a remarkable bad result: this combination revealed no life-sustaining kidney function. Summarizing the data of combined techniques during 24 hour kidney preservation, it can be concluded that the best results were obtained when kidneys were perfused as long as possible until the time of revascularization.

section 7: The present thesis

A new approach to kidney preservation was introduced by Kootstra et al. in 1980 (164). They showed that the results of four days hypothermic continuous perfusion of canine kidneys could be significantly improved by a normothermic perfusion of four hours halfway the preservation period. During the normothermic perfusion, the preserved kidney was connected with the femoral vessels of the again-anaesthetised donor dog. The possibilities of this so-called "ex-vivo" perfusion were investigated further by Van der Wijk et al. (165).

The combination of four hours normothermic ex-vivo perfusion and six days hypothermic continuous perfusion revealed life-sustaining kidney function in five out of six experiments. On the contrary, one out of six dogs survived autotransplantation in the control group with only hypothermic perfusion. In other experiments the six day hypothermic perfusion was combined with one, two and three hours intermediate perfusion. In this six day preservation model the best results were obtained with three hours ex-vivo perfusion.

The experiments presented in this thesis are based on the experiences and results obtained by Van der Wijk and Kootstra. The study is a continuation of their previous work. The results of six day experimental kidney preservation will be presented. The combination of hypothermic continuous perfusion with various types of three hour normothermic intermediate perfusion will be compared with hypothermic perfusion alone.

In chapter II the experimental model is described. Furthermore details are given about the laboratory animals and the surgical procedure. In the next chapters four series of preservation experiments are described. The kidney function was assessed by measurement of blood flow, urine production, glomerular filtration and tubular secretion rate. The serum creatinine concentration values and histological data after

implantation are presented.

The results of each experimental group are discussed separately.

In chapter VI the development of a new heart-lung machine, especially adapted for the perfusion of preserved kidneys, is described. A number of pilot studies with the newly built heart-lung machine is reported in chapter VII. These experiments were useful for the technical improvement of the isolated blood perfusion. In chapter VIII a final series of preservation experiments is described. In this group successful six day kidney preservation was achieved by combined hypothermic and normothermic perfusion. In chapter IX special attention will be paid to the blood flow during the intermediate blood perfusion, which played an important role in the ultimate results of the experiments.

Finally the possible mechanisms involved in the beneficial effect of the intermediate normothermic perfusion on hypothermic perfusion will be discussed in chapter X.

PART II

CHAPTER II

MATERIALS AND METHODS

Laboratory animals: For the kidney preservation experiments mongrel dogs of both sexes were used with a body weight of 20 to 30 kg. The animals had not been used previously for other experiments and were apparently healthy. During the experiments the animals were kept in separate cages at room temperature. They were fed on a normal laboratorial diet and were allowed to drink water at liberty.

Experimental model: In all preservation experiments the kidneys were preserved for six days (Fig. II,1). After unilateral nephrectomy the right kidney was immediately flushed through the artery with Eurocollins solution at 4°C. Subsequently hypothermic continuous perfusion was performed in a preservation machine (Gambro[®], Lund, Sweden). After six days hypothermic continuous perfusion the preserved kidney was implanted in the donor dog. The auto-transplantation model was selected to avoid immunological damage of the preserved kidney. One hour after implantation the contralateral kidney was removed and the animal was now fully dependent on the preserved kidney.

Anaesthesia: The animals did not receive food and water during the night before operation. As premedication 0.5 mg atropin i.m. was given half an hour prior to the operation. Anaesthesia was induced with thiopentotal (Nesdonal[®]) 20 mg per kg body weight i.v. Immediately thereafter the animal was intubated and anaesthesia was continued by ventilation with a nitrous oxide/oxygen mixture supplemented with halothane (Fluothane[®]). During the operation the dogs were hydrated by intravenous infusion of 500 ml glucose solution (5 g/100 ml) and 500 ml NaCl solution (0.9 g/100 ml). Through-

EXPERIMENTAL MODEL

Figure II,1: Experimental model.

After six days preservation autotransplantation was performed followed by contralateral nephrectomy.

out each operation the animals lay on a thermostatic water mattress (37°C).

Surgical procedure: Nephrectomy: The anaesthetised dog was placed on his left side on the operating table. The right subcostal region was carefully shaven and the skin was disinfected with an alcohol solution containing two per cent iodine. Subsequently the dog was covered with sterile towels. A subcostal incision was made. The external oblique muscle was transected. The internal oblique muscle and the transversus abdominis muscle were split parallel to their fibres and the abdomen was opened. So the right kidney was exposed intraperitoneally. The subcostal incision was preferred above a midline abdominal incision. By a subcostal incision the kidney can be exposed without handling of intestinal loops, which might prevent postoperative intussusception to a large extent.

First the fibrous fibres between Gerota's fascia and the diaphragma were dissected. Hereby the upper pole of the kidney was mobilised. The perirenal fat was removed. During this procedure small vessels between the perirenal fat and the kidney were ligated as much as possible. Subsequently the renal artery and vein were dissected free on the dorsal side of the kidney. The ureter was divided about 10 cm distally to the kidney and freed upwards to the hilus. A small silicon tubing (0.096 in O.D., 0.068 in I.D., Down Corning Corp. Midland, Michigan) was inserted into the ureter. Five minutes before clamping of the renal vessels, 100 ml mannitol solution (20 g/100 ml) was given intravenously. First the renal artery was clamped and dissected and subsequently the renal vein. Within two minutes the kidney was weighed and a plastic cone-shaped cannula was inserted in the renal artery. The kidney was flushed with 500 ml Eurocollins solution (Table III,1) at 4°C and transferred to the Gambro® preservation machine. The renal vessels in the donor dog were ligated with silk and abdomen was closed in two layers

Figure II,2: Implantation of the preserved kidney was performed at the left side of the neck. The renal vein was anastomosed end to side to the external jugular vein, the renal artery end to end to the carotid artery. The kidney was placed in a subcutaneous pocket and a uretero-cutaneostomy was made.

with chromic catgut. The skin was closed with interrupted mersilene sutures.

Implantation: After six days the donor dog was anaesthetised as mentioned before. The animal was placed on its right side on the operating table. The left flank and left collar region were carefully shaven and disinfected. The remaining parts of the dog were covered with sterile towels. First a subcostal incision was made and the left kidney was dissected free according the same technique as applied for the right kidney. The kidney was left in situ, only attached by its vessels, and a cannula was inserted in the ureter to collect the urine. Subsequently implantation of the preserved kidney was performed. A longitudinal incision was made on the lateral side of the neck. The external jugular vein was dissected free the length of about 5 cm. By separation of the musculus sternocephalicus and the long muscles of the neck the common carotid artery was exposed. The renal vein was anastomosed end to side to the external jugular vein and the renal artery end to end to the carotid artery. The time for performing the anastomoses (6-0 silk, Ethicon) was about 25 minutes. The kidney was placed in a subcutaneous pocket and a uretero-cutaneostomy was made (Fig. II,2). The silastic cannula was left in the ureter and fixed with one suture to the skin near the uretero-cutaneostomy. All animals received antibiotic prophylaxis (amoxicilline, 1 g/day) during the first five days after implantation. Besides technical facility, the implantation of the kidney in the animal's neck has several advantages:

First, the risk of intusseption is avoided which is a frequent complication after abdominal operations in dogs.

Secondly, the diuresis can be observed continuously and clearance studies can be performed after implantation. However, the technique is not suitable for long-term postoperative follow-up as in immunological experiments since the kidney is likely to be damaged by ascending infections after

a few weeks.

Statistics:

A non-parametric method was used for statistical analysis of the results. The Wilcoxon test was chosen for comparing the results of the different groups.

In this chapter the results of six day kidney preservation are presented. In eight dogs the kidneys were preserved by hypothermic continuous perfusion. The series (group I) served as control group for the other preservation experiments described in the next chapters.

Materials and methods:

According to the experimental model described earlier, the right kidney was removed under general anaesthesia. The renal artery was cannulated and the kidney was flushed with Eurocollins solution (4°C). The composition of this Collins solution is given in Table III,1. Subsequently the kidney was transferred to a preservation machine (Gambro[®], Lund, Sweden). The kidney was placed in the organ chamber of a disposable sterile preservation cassette, which was connected with the Gambro machine (Fig. III,1 and III,2). As perfusate 800 ml albumin solution (Kabi Vitrum, Amsterdam, The Netherlands) was used. The composition of the perfusate is given in Table III,1. The temperature of the perfusate was kept between 5 and 7°C . The systolic perfusate pressure was indicated by a pressure manometer and the perfusate flow could be estimated by calibration of the pump speed.

Pure filtered oxygen (200 ml per minute) was blown into the organ chamber above the surface of the perfusate. During the first minutes of the hypothermic perfusion the perfusion pressure was adjusted to 60 mm Hg. After one hour perfusion the perfusion pressure was readjusted to 20 mm Hg.

Daily pH measurements of the perfusate were made at 37°C (ABL Blood gas analysator, Radiometer, Copenhagen, Denmark).

After the six day preservation period the kidney was autotransplanted. One hour after recirculation of the pre-

Figure III,1: Gambro[®] preservation machine used for hypothermic continuous perfusion of kidneys. In this apparatus the kidneys were perfused for six days with an albumin perfusate (see Fig. III, 2).

Figure III,2: Scheme of Gambr[®] preservation machine applied for hypothermic continuous perfusion of kidneys. Two disposable perfusion circuits can be placed in this apparatus (see Fig. III,2).

Table III,1: COMPOSITION PER LITER OF THE EUROCOLLINS SOLUTION USED FOR FLUSHING THE KIDNEYS AND OF THE PERFUSATE USED IN THE GAMBRO MACHINE

EUROCOLLINS			PERFUSATE		
Na^+	10	mmol	Na^+	107	mmol
K^+	115	mmol	K^+	5	mmol
Cl^-	15	mmol	Mg^{2+}	6	mmol
HCO_3^-	10	mmol	Cl^-	100	mmol
H_2PO_4^-	15	mmol	HCO_3^-	12	mmol
HPO_4^{2-}	42.5	mmol	SO_4^{2-}	6	mmol
glucose	132	mmol	glucose	34.24	mmol
			albumine	5%	g/V
osmolality	355	mOsm/KgH ₂ O	osmolality	300	mOsm/kgH ₂ O

served kidney, the contralateral kidney was removed and 100 ml mannitol solution (20 g/100 ml) was given intravenously. Kidney function after implantation was assessed by daily serum creatinine measurements for two weeks. Clearance studies were performed at one hour after implantation. The glomerular filtration rate (GFR) and the effective renal plasma flow (ERPF), the latter as tubular parameter, were determined by clearance calculations of respectively ^{125}I iothalamate and ^{131}I -hippurate (166). One hour after implantation a single injection of 0.010 mCi ^{125}I iothalamate and 0.005 ^{131}mCi ^{131}I -hippurate (Amersham, Buckinghamshire, England) was given intravenously and the clearances of these labelled compounds was measured during half an hour after ten minutes equilibration. All samples were counted in a dual channel gamma counter (Hewlett Packard). The clearances of ^{125}I iothalamate and ^{131}I -hippurate were calculated using the standard formula $\frac{[U] V}{[P]} = C \text{ ml/min}$ (167). By dividing the GFR by the ERPF the filtration fraction (FF) was calculated.

Histology: At autopsy the implanted kidneys were bisected for gross macroscopic examination. Wedge-shaped biopsies were fixed in formaline. Light microscopy of these biopsies was performed on 4 μm sections stained with hematoxylin and eosin. Tubular damage was correlated to the assessed amount of casts; these casts are supposed to be necrotic remnants of tubular cells which have been passed into the tubular lumen.

Results:

Hypothermic perfusion: At the start of each perfusion the systolic perfusion pressure was adjusted to 60 mm Hg. Among the eight experiments the perfusate flow varied from 70 to 80 ml/min/100 g kidney weight at that perfusion pressure. During the first hour of hypothermic perfusion the pressure

Figure III,3: Perfusate pressure and flow during 6 day hypothermic perfusion of canine kidneys. At the start of the perfusion the pressure was set at 60 mmHg. After 60 minutes the perfusion pressure was readjusted at 20 mmHg.

fell to about 20 mm Hg or even lower. The perfusate flow amounted 80 to 100 ml/min/100 g kidney weight at this perfusion pressure (Fig. III,3).

So in all experiments the renal vascular resistance (quotient of mean perfusion pressure and mean perfusate flow) decreased to one third - one fourth of the initial value. In all experiments the pH of the perfusate increased from 7.0 to about 7.2 (measured at 37°C).

Survival after implantation: Upon revascularisation all kidneys regained normal colour. However, the diuresis remained low or anuria occurred. The mean urine production measured at one hour after implantation was 2 ml/h/100 g (range 0.5 - 3.5 ml). Only one out of eight dogs survived after auto-implantation of the preserved kidney and contralateral nephrectomy. The others died uraemic within five days after implantation. The only survivor had a maximum serum creatinine concentration of 1080 $\mu\text{mol/l}$ on day five after implantation (Fig. III,4).

Kidney function after implantation: All kidneys regained normal colour upon revascularisation. However, as mentioned before, the urine production remained low (range 0.5 - 3.5 ml, measured one hour after implantation).

Clearances of ^{125}I iothalamate (GFR) and ^{131}I -hippurate (ERPF - tubular secretion) revealed low values at one hour after implantation (Table III,2). In seven dogs without life-sustaining kidney function, the ERPF was equal or slightly higher than the GFR, resulting in a high filtration fraction ($\text{FF} = \text{GFR}/\text{ERPF}$). In the only survivor a low FF (0.17) was measured (Fig. III,5).

Histology: The dog with life-sustaining kidney function was sacrificed three weeks after implantation. At autopsy the kidney showed normal morphology. However, light-microscopic examination of biopsies showed signs of pyelonephritis, due

Figure III,4: Serum creatinine concentration after six days hypothermic perfusion (mean values $\pm$ S.D.). Only one out of eight dogs survived after implantation.

Table III,2: GLOMERULAR FILTRATION RATE, EFFECTIVE RENAL PLASMA FLOW AND FILTRATION FRACTION MEASURED IN SIX DAYS PRESERVED KIDNEYS ONE HOUR AFTER IMPLANTATION

GROUP I (controls)

<u>dog</u>	<u>GFR^a</u>	<u>ERPF^a</u>	<u>FF</u>
1	0.11	0.11	1
2	0.11	0.01	1
3 ^b	0.04	0.24	0.17
4	0.17	0.21	0.81
5	0.09	0.17	0.53
6	0.09	0.11	0.82
7	0.03	0.04	0.75
8	0.12	0.15	0.80

a GFR and ERPF values were calculated in ml/min/100 g kidney weight.

b Only survivor.

Figure III,5: One hour after implantation of 6 days hypothermic-preserved kidneys, high filtration fractions were measured in all non-viable kidneys.

to ascending infection, which is inherent to the implantation in the neck (168).

In the other non-viable kidneys severe tubular damage attended with many casts was observed. Dispersed tubular regeneration was seen. The glomeruli and vessels had a normal aspect (Fig. III,6).

Discussion:

Successful experimental kidney preservation of three to four days by hypothermic continuous perfusion has been achieved by several investigators (72,73) (Chapter I,4). However, consistent good results have not been reported after longer preservation periods (75,69). Similar results were obtained with the "standard" hypothermic preservation technique as described in this chapter.

In earlier experiments by van der Wijk et al., successful four day canine kidney preservation was achieved with hypothermic perfusion in a Gambro[®]-machine and an albumin perfusate (165). Attempts to preserve kidneys for longer than four days failed. The results in this group (I) clearly show that a six day hypothermic perfusion is not likely to maintain the viability of the kidney. Only one out of eight dogs survived with a high serum creatinine concentration after implantation.

At the start of the hypothermic perfusion in all experiments the perfusion pressure dropped from 60 mm Hg to about 20 mm Hg within half an hour, while the perfusate flow remained constant. This initial high vascular resistance is probably due to the high potassium concentration of the Eurocollins flush solution. Scott et al. (169) demonstrated that potassium infusion in concentrations exceeding 0.69 mmol/min caused vasoconstriction by a direct action on the vessel walls. By flushing with Eurocollins solutions in

Figure III,6: After 6 days hypothermic preservation the non-viable kidneys showed severe tubular damage with many casts. The glomeruli and vessels had a normal aspect (Hematoxylin/Eosin, ca. 250 x).

tery in concentrations of approximately 6 to 10 mmol/min!

During the hypothermic continuous perfusion the pH of the perfusate increased from 7.0 to about 7.2 (measured at 37°C). This phenomenon has also been described by Johnson et al. (75). The changes in pH were due to a decrease in carbon dioxide concentration, since no carbon dioxide was added to the oxygen which was blown into the organ chamber.

In all experiments of this group hyperosmolar mannitol was given after surgical manipulation just before nephrectomy and after revascularisation at the end of the preservation period. Mannitol was supposed to improve the renal blood flow by reducing swelling of tissues and endothelial cells, and thereby restoring circulation to ischaemic areas (170, 171).

In all experiments the preserved kidneys were subjected to clearance studies at one hour after implantation. The absolute values of GFR and ERPF did not correlate with the serum creatinine concentration after implantation but their quotient, the filtration fraction (FF), had a predictive value regarding the viability of the kidney. In the surviving dog a low FF was measured, indicating the presence of active functioning tubules. In the seven non-viable kidneys absence of tubular function resulted in a high filtration fraction.

Histological examination of the non-viable kidneys at autopsy showed severe tubular damage. However, the blood vessels and glomeruli were intact. Signs of endothelial damage leading to intravascular coagulation and fibrin thrombi in the glomerular capillaries were not observed. This so-called "perfusion nephropathy" following hypothermic continuous perfusion has been described by several authors (97, 98) (chapter I,3). It has been suggested that perfusion nephropathy can be prevented by low perfusion pressure in hypothermic perfusion (100) as applied in all preservation experiments described here.

EXPERIMENTAL MODEL

Figure IV,1: Modified experimental model. In a series of 7 experiments (group II) kidneys were preserved by hypothermic perfusion as in group I (controls). Halfway the preservation period, kidneys were normothermic perfused for 3 hours. The kidneys were connected ex-vivo with the same donor dogs (Fig. IV,2).

The results, described in chapter III, clearly show that successful six day kidney preservation cannot be achieved by hypothermic perfusion only.

A new approach to kidney preservation was introduced by Kootstra and van der Wijk (164,165). They showed that the results of four and six day hypothermic continuous perfusion could be significantly improved by a few hours normothermic perfusion. During this perfusion the preserved kidney was connected "ex-vivo" with the femoral vessels of the again-anaesthetised donor dog. In the six day preservation model the best results were obtained with three hours ex-vivo perfusion. The experiments described in this chapter are based on the results of van der Wijk and Kootstra. In a series of seven experiments six day hypothermic perfusion was combined with three hours ex vivo perfusion. The beneficial effect of the normothermic ex vivo perfusion was assessed by determination of GFR and ERPF during the ex-vivo perfusion.

Materials and methods:

In a series of seven experiments kidneys were preserved for six days by the same method as described in chapter III. However, halfway the preservation period the experimental model was changed (Fig. IV,1). On the third day the hypothermic perfusion was interrupted and the preserved kidney was connected "ex-vivo" with the same donor dog for three hours. For this ex-vivo perfusion a special circuit was constructed (Fig. IV,2,3). Under general anaesthesia a femoral artery and vein were cannulated. Before the cannulation heparine, 300 IU/kg body weight was given intravenously followed by 100 IU/kg per hour during the ex-vivo perfusion. The cannulae in the femoral vessels were connected with silastic tu-

Figure IV,2: Scheme of "ex-vivo" perfusion in group II. Halfway the six day preservation period, the kidneys were connected with silastic tubing to the femoral vessels of the donor dog. After three hours blood perfusion, the kidneys were placed back on the Gambro[®] machine (see Fig. IV,2).

Figure IV,3: Ex-vivo perfusion in group II. To maintain humidity and temperature, the kidney was placed in a disposable organ chamber filled with 500 ml saline and heated with water of 38°C through the original cooling system of the organ chamber (see Fig. IV,2).

bing to the cannulae in the artery and vein of the preserved kidney. To maintain humidity and temperature the kidney was placed in a disposable organ chamber (Gambro[®]), filled with 500 ml saline solution and heated with water of 38°C through the cooling system of the organ chamber. The cannulated ureter was led out through a separate hole in the organ chamber. The arterial and venous blood pressure were measured and recorded on a Sanborn 7700 series recorder (Hewlett Packard). The venous pressure was kept between 0 and 5 mm Hg by varying the height of the organ chamber. The blood flow through the kidney was measured in the arterial line and recorded continuously (Directional Doppler Model 806, Parks Electronics Laboratory). After the ex-vivo perfusion the kidney was flushed with 500 ml Eurocollins solution 4°C and the kidney was placed back on the Gambro[®] machine for another three days. The same perfusion pressures were used during the second period of hypothermic perfusion. After the ex vivo perfusion protamine chloride was given intravenously to the donor dog to counteract the effect of heparine until the clotting time (measured with Hemochron 1200) reached a normal value.

After six days auto-implantation of the preserved kidneys was performed, followed by contralateral nephrectomy. As in the control group mannitol was given at one hour after implantation. Kidney function was assessed by daily serum creatinine measurements for two weeks. One hour after implantation GFR and ERPF were determined as in the control group (Chapter III).

During the normothermic perfusion on the third day of the preservation period, GFR and ERPF were also determined by clearance calculations of respectively ¹²⁵Iothalamate and ¹³¹I-hippurate. During this ex vivo perfusion a loading dose of 0.012 mCi ¹²⁵Iothalamate and 0.005 mCi ¹³¹I-hippurate was given intravenously. Thereafter a saline solution with 0.040 mCi ¹²⁵Iothalamate and 0.050 mCi ¹³¹I-hippurate was infused in an anticubital vein of the donor dog. The speed of the

infusion pump was 9 ml/hour. Urine and blood samples were taken at hourly intervals.

Results:

Hypothermic perfusion: During the hypothermic perfusion the same values for perfusion pressure, perfusate flow and pH were measured as in the control group (I) (Fig. III,1). After the normothermic ex-vivo perfusion, the same perfusion parameters were measured as during the first part of the hypothermic perfusion.

Survival after implantation: Upon revascularisation all kidneys regained normal colour and produced immediately urine. At one hour after implantation the urine production was significantly higher ($p < 0.01$; Wilcoxon test) than in the control group and had a mean value of 53 ml/h/100 g (range 10-100 ml). In this group all seven dogs survived in a healthy condition. After implantation the mean serum creatinine concentration reached a maximum value of 580 $\mu\text{mol/l}$ (range 425-824 $\mu\text{mol/l}$) on day two and returned to normal values within two weeks (Fig. IV,4).

Kidney function after implantation: The GFR and ERPF values measured at one hour after implantation were higher than in the control group (Table IV,1). In the ex-vivo perfused group much variation was found among these values and the individual values did not correlate with the level of serum creatinine concentration after implantation. As shown in figure IV,5 in all kidneys low filtration fractions ($\text{FF} = \text{GFR} / \text{ERPF}$) were measured at one hour after implantation (for comparison the FF values of the control group are also given).

Figure IV,4: Serumcreatinine concentration in group II (mean values $\pm$ S.D.). In the ex-vivo perfused group all kidneys had life-sustaining function after 6 days preservation. The results of the control group (I) with hypothermic perfusion only are also presented.

Table IV,1: GLOMERULAR FILTRATION RATE, EFFECTIVE RENAL PLASMA FLOW AND FILTRATION FRACTION MEASURED IN SIX DAYS PRESERVED KIDNEYS ONE HOUR AFTER IMPLANTATION

GROUP II (ex-vivo perfused)			
dog	GFR ^a	ERPF ^a	FF
1	0.41	4.24	0.10
2	6.26	45.93	0.14
3	6.78	59.35	0.11
4	0.8	11.54	0.07
5	2.94	13.40	0.22
6	12.57	47.77	0.26
7	4.52	27.85	0.16

a GFR and ERPF values were calculated in ml/min/100 kidney weight.

Figure IV,5: In group II (ex-vivo perfused) all kidneys had low filtration fractions (FF) at one hour after implantation (*). For comparison the FF values of the control group are also given (o).

Histology: In this group all dogs were sacrificed three weeks after implantation. Biopsies of these kidneys showed normal morphology. However, signs of pyelonephritis were present to a certain amount in all kidneys due to ascending infection, which is inherent to the implantation in the neck (168).

Normothermic ex-vivo perfusion: On the third preservation day the kidneys were connected ex-vivo to the circulation of the anaesthetised donor dog. All kidneys regained their normal colour as soon as the three hour normothermic blood perfusion started. Within five minutes the kidneys produced urine. During the ex-vivo perfusion the mean arterial pressure delivered by the donor dog was between 90 and 100 mm Hg. The mean blood flow through the kidney amounted to 110 ± 17 ml/min/100 g kidney weight and remained constant during each separate perfusion. In six out of seven experiments GFR and ERPF determinations were performed during the three hours ex-vivo perfusion (in one case the urine collection failed). In five out of six measurements the GFR and ERPF increased to a multiple of the first hour values during the perfusion (Fig. IV,6). In one case the first hour values of GFR and ERPF were relatively high; the ERPF showed a moderate decrease in the third hour whereas no change in GFR was observed. In this perfusion the urine production decreased in contrast to the other experiments, in which the low diuresis remained unchanged throughout the ex-vivo perfusion. During each perfusion a low filtration fraction (<0.28) was measured.

Discussion:

In this series experiments (group II) the preservation time of dog kidneys could be extended successfully up to six days by three hour ex-vivo perfusion halfway the hypothermic pre-

Figure IV,6: Perfusion parameters of the ex-vivo perfusion halfway the six day preservation period in group II. The renal blood flow is presented in mean values $\pm$ S.D. In six out of seven experiments GFR and ERPF determinations were performed during the three hours perfusion (in one experiment the urine collection failed). The urine production, GFR and ERPF are presented for each perfusion. The filtration fraction (GFR/ERPF) showed no variation and was low throughout each perfusion (<0.28). FF values are not presented in the figure.

servation period. In a consecutive series seven out of seven dogs survived in a healthy condition after implantation (172).

A normothermic ex-vivo perfusion of kidneys as applied in group II has been used by Baitz (173) and Lavender (174) in the sixties for preservation and viability tests. Lavender demonstrated that it was technical feasible to preserve a kidney ex-vivo for long time. In a 35 year old male patient with terminal renal failure, a kidney was anastomosed to the vessels in the forearm. The renal artery was cannulated, the renal vein drained freely into an organ container and hence into the vein of the forearm. The venous reservoir comprised the entire organ container with the kidney. A blood pump was not included in the circuit. Urine was collected through a cannula in the ureter. Good renal function was measured until rejection occurred after 25 days. Lateron Carter et al. (175) have described an ex-vivo perfusion model for rabbit kidneys to evaluate hypothermic storage with new flush solutions.

Normothermic ex-vivo perfusion in combination with hypothermic continuous perfusion has been described by Pauşescu et al. (176). They found that dog hearts perfused hypothermically for 24 hours and thereafter functionally tested ex-vivo for six to seven hours could be subjected again to hypothermic preservation and subsequent ex-vivo perfusion for another period, without loss of viability and function. In their studies the aim of the ex-vivo perfusion was to evaluate an amino-acid containing perfusion fluid, which was used as perfusate in hypothermic heart preservation.

The results of six day kidney preservation in group II are in accordance with the data of Pauşescu et al. However, the good results are likely due to a beneficial effect of the normothermic ex-vivo perfusion itself and not to the special composition of the perfusate in the hypothermic preservation, as stated by Pauşescu. The ischaemic damage to the kidney induced by hypothermic perfusion was restored by

the normothermic ex-vivo perfusion, which allowed the organ to sustain another three days of hypothermic preservation.

It must be noticed that the composition of the perfusate in the Gambro[®]-machine was altered by the flushing with Eurocollins prior to the hypothermic perfusion. Against the successful preservation model the objection can be raised that in group II, the composition of the perfusate was influenced twice by the wash-out with Eurocollins solution, whereas this occurred only once in the control group. However, it is not likely that a single Eurocollins perfusion halfway the preservation period should account for the excellent results obtained in group II. Belzer and Southard (160) reported disappointing results in experiments with cold storage and intermittent perfusion. In each experiment kidneys treated with this procedure did noticeable worse than kidneys either cold stored or perfused separately for the same preservation period.

The concept of the ex-vivo perfusion in combination with hypothermic continuous perfusion was established by Kootstra (164) and van der Wijk (165), who obtained good results in four and six day preservation experiments. The aim of the experiments in group II was to study the kidney function during the ex-vivo perfusion of three hours, which was the optimal time to improve the six day preservation results (177). In all ex-vivo perfusions in group II low blood flow values were measured which was likely due to the low arterial perfusion pressure delivered by the donor dog. First the arterial pressure was reduced by the height of the organ chamber, which was necessary to keep the venous pressure between 0 and 5 mm Hg. Secondly, the perfusion pressure was lowered by the narrow cannulae fixed in the wall of the organ chamber.

In comparison to the total renal blood flow, very low values for GFR were measured during the ex-vivo perfusion. These low values might be explained by interstitial oedema, arisen during the preceding hypothermic perfusion. The ERPF

values were four to 20 times higher than the GFR values, which indicate that a concentration gradient of ^{131}I -hippurate could be created between the tubular lumen and the peritubular capillaries by active functioning tubular cells.

During the ex-vivo perfusion a marked increase in GFR and ERPF was observed in five out of six cases, total blood flow being constant. The increase in ERPF was greater than the rise in GFR. This finding may indicate an improving extraction of ^{131}I -hippurate, a phenomenon also described in human renal allotransplantation during the first 24 hours (178). As opposed to the increase in GFR the urine production remained relatively low. It is of interest that in the only dog with initial high and constant GFR and ERPF values, the urine production decreased. So in all experiments in group II the water reabsorption improved during the ex-vivo perfusion.

In most kidneys of group II subnormal filtration fractions were measured after six days preservation. In successful human allotransplantation subnormal filtration fractions have also been reported (179,180,181), although the total ischaemic time of those kidneys varied between 16 and 55 minutes. Herdman et al. (179) explained this phenomenon by dilatation of glomerular efferent arterioles in the presence of normal or partially closed afferent arterioles. Hereby the lowered filtration pressure caused a decrease in GFR while the blood flow along the proximal tubules continued undisturbed.

In these preservation experiments the FF, measured at one hour after implantation, seemed to have a predictive value for the viability of the kidney. As shown in figure IV,5 all kidneys with life-sustaining function had low FF values, whereas the non-viable kidneys of the control group had a high FF, which obviously indicates absence of tubular function.

The experiments described in this chapter illustrate the beneficial effect of an intermediate normothermic ex-vivo

perfusion once more. Although a preservation time of six days was reached, there was no question of true preservation in group II since the kidneys were connected again to the blood circulation of the donor dog. However, the data gave valuable information useful for the replacement of the ex-vivo perfusion by an artificial heart-lung circuit, which was the next step in the preservation experiments.

CHAPTER V HYPOTHERMIC PERFUSION COMBINED WITH ISOLATED BLOOD PERFUSION

The aim of the experiments described in this chapter was to investigate whether the benefit of normothermic perfusion halfway the preservation period was an intrinsic effect of the ex-vivo situation, or whether the same results could be achieved using a heart-lung machine.

Materials and methods:

In eight dogs (Group III) kidneys were preserved by hypothermic perfusion for six days. On the third day of the preservation period the kidneys were normothermically perfused in a heart-lung machine. The experimental model is shown in figure IV,1. The heart-lung machine was filled with fresh alloblood (10 ml/g kidney weight) drawn from the carotid artery of a blood donor dog which was anaesthetised with nembutal (30 mg/kg intravenously) and treated with 400 IU heparine/kg and 1 g amoxicilline. The perfusion of the preserved kidney started within two minutes after priming of the circuit. After three hours blood perfusion the kidney was flushed with Eurocollins at 4°C and perfused in the Gambro® machine with the same perfusate for another three days. The heart-lung machine applied in group III was designed and constructed in the laboratories of Prof. Nizet (Liege, Belgium) (11) (Fig. V,1, V,2). The oxygenator was made of two concentric plexiglass cylinders fixed on the same base. The venous blood was spread over the outer surface of the inner cylinder and the inner surface of the outer cylinder by a slowly turning disc. The blood, flowing down the cylinder was oxygenated by a humidified mixture of 95% O₂ and 5% CO₂ which was blown between the cylinders. The oxygenated blood was pumped upwards from the bottom of the oxygenator to the renal artery. In the arterial line a blood filter (250 µm)

Figure V,1: Scheme of the heart-lung machine applied for the intermediate perfusion in group III. The perfusion circuit included a film oxygenator and a hydraulic balloon pump (Dale Schuster type). Halfway the six day preservation period the kidneys were perfused for three hours with fresh heparinised alloblood. After three hours blood perfusion the kidneys were placed back on the Gambro[®] machine.

Figure V,2: Heart-lung machine for blood perfusion in group III. The kidney lay in a shallow plexiglass bowl and was covered with a plastic bag to maintain sterility and to prevent dehydration (see figure V,1).

and a pulse damper were included. The Dale Schuster type (13) blood pump was made as follows: within a small plexi-glass cylinder with two light in- and outlet stainless steel valves, a balloon was constructed. This balloon - the finger tip of an operating glove - was filled with saline solution and connected with a pressure tubing to a piston pump. The piston of the pump regulated the volume of the balloon by hydraulic transmission. By varying the deflection of the piston the stroke volume of the blood pump was altered (11). The blood pressure was measured at the end of the arterial line by a mercury manometre. The renal blood flow was determined by intermittent collection of venous blood in a calibrated pipet. The kidney lay in a shallow plexiglass bowl and was covered with a plastic bag to maintain sterility and to prevent dehydration. Blood leaking from the renal capsule was added continuously to the venous blood. The oxygenator and the blood pump were surrounded by a thermostatic water bath. Finally an infrared lamp prevented cooling down of the kidney. Throughout the blood perfusion a perfusion pressure of 120 mm Hg was maintained. At the start of the perfusion 40 mg promethazine* per liter perfusate was added. At hourly intervals 5 ml glucose solution (5 g/100 ml) was given to maintain normal blood glucose levels.

Before each experiment the whole perfusion circuit was sterilised with ethylen-oxyde gas. The ethylen-oxyde was removed by ventilation of the perfusion circuit with sterile air during 10 days.

As in group II, GFR and ERPF measurements were done by clearance calculations. At the start of the isolated blood perfusions in the heart-lung machine a single dose of 0.005 mCi ^{125}I othalamate and 0.002 mCi ^{131}I -hippurate was added to the perfusate. Urine and blood samples were taken at hourly intervals. After implantation and contralateral nephrectomy kidney function was assessed by daily measurements of serum

* The action of promethazine is discussed in chapter IX.

creatinine concentration. One hour after implantation GFR and ERPF were measured as in the control group (Chapter III). Histological examination of the kidneys was performed at autopsy three weeks after implantation or after uraemic death. This was done in the same way as described in the control group (Chapter III).

Results:

During the hypothermic perfusion the same perfusion characteristics were found as in group II (Chapter IV). After implantation all kidneys regained normal colour upon revascularisation. The mean diuresis measured at one hour after implantation was 5 ml/h/100 g (range 2-15 ml), which was significantly lower ($p < 0.01$) than in the ex-vivo perfused group (II). Although the diuresis was significantly higher ($p < 0.05$) than in the control group (Chapter III), the urine production decreased to anuria within a few hours after implantation. In three out of eight dogs the diuresis recovered two days after implantation. Only these three dogs had life-sustaining kidney function. In the three surviving dogs the mean serum creatinine concentration reached a maximum value of 890 $\mu\text{mol/l}$ on day three (Fig. V,3).

Most GFR and ERPF values, measured one hour after implantation, were lower than in group II and as in this group, no correlation with the serum creatinine concentration values could be demonstrated either (Table V,1).

As in the control and ex-vivo perfused group, the three surviving dogs had low filtration fractions after implantation (Fig. V,4). However, low filtration fractions (0.11 - 0.50) were also measured in the five dogs which did not prove to have life-sustaining kidney function (see discussion).

Histology: In this group all non-viable kidneys showed a typical feature at autopsy. On bisecting the kidneys a hae-

Figure V,3: Serum creatinine concentration in group III (mean values $\pm$ S.D.). In the isolated blood perfused group 3 out of 8 kidneys had life-sustaining kidney function. The results of the control group (I) with hypothermic perfusion only are also presented.

Table V,1: GLOMERULAR FILTRATION RATE, EFFECTIVE PLASMA FLOW AND FILTRATION FRACTION IN SIX DAYS PRESERVED KIDNEYS ONE HOUR AFTER IMPLANTATION

GROUP III (heart-lung perfused)

<u>dog</u>	<u>GFR^a</u>	<u>ERPF^a</u>	<u>FF</u>
1 ^b	0.12	0.73	0.16
2 ^b	0.86	3.34	0.26
3	0.11	0.98	0.11
4	0.04	0.08	0.50
5 ^b	2.07	40.00	0.05
6	0.51	4.38	0.12
7	0.07	0.16	0.44
8	0.08	0.41	0.20

a GFR and ERPF values were calculated in ml/min/100 g kidney weight.

b These dogs had life-sustaining function.

Figure V,4: In group III (isolated blood perfused) the 3 surviving dogs had low filtration fractions after implantation (●). However, low filtration fractions were also measured in the 5 dogs which did not prove to have life-sustaining function (see discussion). For comparison the FF values of the control group (o) and the ex-vivo perfused group (*) are also presented.

Figure V,5: In group III all non-viable kidneys showed a typical feature at autopsy. On bisecting the kidneys a haemorrhagic zone was observed between the cortex and the medulla. In histological sections of these kidneys, bleeding around vessels was associated with glomerular and tubular damage. In some places unaffected blood vessels together with normal glomeruli and tubules were seen (Hematoxillin/Eosin ca. 250 x).

morrhagic zone was observed between the cortex and the medulla. In histological sections of these kidneys bleeding around vessels was associated with glomerular and tubular damage. In some places unaffected blood vessels together with normal glomeruli and tubulus were seen (Fig. V,5). At autopsy of the surviving dogs, three weeks after implantation, small traces of the typical haemorrhagic zone were observed on bisecting the kidneys. Apart from this the histological sections showed normal morphology as in group II.

Isolated blood perfusion. During the intermediate blood perfusion in the heart-lung machine the mean arterial pressure was kept at 120 mm Hg. In all kidneys the renal blood flow increased steadily from 165 ± 21 ml/min/100 g in the first hour to 260 ± 49 ml/min in the third hour (Fig. V,6). After three hours perfusion macroscopic haemolysis was detected in the perfusate of the heart-lung machine. During the first hour of blood perfusion, GFR and ERPF values were higher than those in group II. However, after a slight increase in the second hour all values dropped to the same level or even lower levels than the first hour values (Fig. V,6). No correlation could be demonstrated between the values of GFR and ERPF and life-sustaining function after implantation. As in group II a low filtration fraction was measured during the blood perfusion. In most experiments the urine production decreased slowly throughout the perfusion (Fig. V,6).

Discussion:

In this series experiments successful six day kidney preservation was not achieved. Only three out of eight dogs survived after autotransplantation. The intermediate blood perfusion performed in a heart-lung machine, including a film oxygenator and a hydrolic balloon-pump, had not the same beneficial effect as the normothermic ex-vivo perfusion

Figure V,6: Perfusion parameters of the blood perfusion halfway the six day preservation period in group III. The renal blood flow is presented in mean values $\pm$ S.D. The urine production, the glomerular filtration rate and the effective renal plasma flow are presented for each perfusion experiment. The filtration fraction showed no variation and was low throughout each perfusion (range 0.06-0.25). FF values are not presented in the figure.

in group II.

Isolated blood perfusions of kidneys as applied in the experiments of group III have been used by several authors but mainly for physiological and pharmacological studies (20,21) (see Chapter I,1). Few authors have reported attempts to preserve kidneys for transplantation with normothermic preservation techniques (16,30,33). Their data indicate that the functional integrity of such isolated kidneys can be preserved for approximately eight hours. Factors which might be responsible for organ failure at longer perfusion periods are lack of substrates and hormones, accumulation of waste products and damage caused by the mechanical way of perfusion (30). In the experiments of group III these drawbacks of normothermic preservation were limited by the shorter time of blood perfusion. Moreover the damage induced by the mechanical perfusion was reduced (Nizet et al.) by the application of a Dale-Schuster type hydraulic balloon pump instead of a conventional roller pump.

In the heart-lung machine a mean arterial pressure of 120 mm Hg was used throughout the blood perfusion. This pressure was routinely used in the perfusion circuit of Prof. Nizet. Although GFR and ERPF were higher than in the ex-vivo perfused group, the values were still low in proportion to the total renal blood flow (Chapter IV). The higher perfusion pressure applied in the isolated blood perfusion accounted for the higher initial GFR and ERPF values. However, the GFR and ERPF decreased during the third hour, while a steady increase in blood flow was measured. This suggests that the extraction of ^{131}I -hippurate was impaired even more. In addition the macroscopic haemolysis that occurred at the end of the blood perfusion had a detrimental effect on the tubular fraction.

Other investigators have described a similar decrease in GFR and tubular function in isolated blood perfusion of not-preserved kidneys (15,17,20). Several explanations for the impaired renal function were given. Nizet et al. (12) stated

that during the course of the perfusion the release of vasodilatating factors in the heparinised blood reduced the resistance of pre- and postglomerular arterioles, leading to oedema and increased tubular pressure. Thereby a decrease in GFR and hippurate extraction might occur. Waugh and Kubo (20) emphasized that microembolisation by fat globules was responsible for the decline in GFR, since addition of the fat emulsifying agent Pluronic-F68 significantly reduced the decline in GFR. Berkowitz et al. (182) and lateron Gagnon et al. (22) also reported decline in GFR and tubular function in spite of increasing blood flow. Dilatation of efferent arterioles, which modulate the hydrostatic pressure in the glomeruli, and an increased medullary blood flow were thought to be responsible for the functional alterations after several hours of perfusion.

In this study the filtration fraction measured at one hour after implantation did not have such a predictive value for the viability of the kidney as in the other groups. Figure V,4 shows that kidneys in group III with low filtration fractions did not prove to have life-sustaining function after implantation. An explanation for this phenomenon was found in kidney histology at autopsy. Haemorrhagic infarction, which was especially present in the outer medullary zone, was considered to be responsible for the tubular and glomerular damage. These data suggest that the heart-lung perfusion in group III had been beneficial to the tubules but on the other hand, had caused endothelial damage.

These findings might explain the low filtration fractions after implantation, indicating the presence of active functioning tubules, and the anuria that occurred afterwards caused by the vascular damage. The endothelial damage was probably caused by the higher perfusion pressure applied during the mechanical blood perfusion (120 versus 90-100 mmHg in group II).

In conclusion it was found that the intermediate blood perfusion had to be improved and that the experiments des-

cribed here could not answer the question whether the normothermic ex-vivo perfusion could be replaced by an artificial blood perfusion. The experiments were useful for the design of a new perfusion circuit especially adapted to the requirements of the intermediate normothermic blood perfusion.

Based on the experience obtained with the perfusion circuit of Prof. Nizet (Liège, Belgium), a modified heart-lung machine was built.

To reduce the blood damaging effect of the mechanical way of perfusion, the design of the perfusion circuit had to come up to the following demands:

- a) the length of tubing should be kept as short as possible and,
- b) the blood gas interface should be avoided or reduced to a minimum (183,184).

As in the perfusion circuit of Prof. Nizet a Dale-Schuster type hydraulic balloon pump was used instead of a roller pump. Besides the less traumatic effect on the blood, this pump had another advantage: it could be placed in a water-bath. Hereby the use of a heat-exchanger could be avoided.

For the intermediate blood perfusion in these experiments some other specific requirements were necessary:

- a) it should be possible to sterilise the circuit,
- b) blood (heparinised) leaking from the capsule of the perfused kidney should be re-added continuously to the circulating blood volume,
- c) the priming (filling) of the circuit should be performed quickly and it should be able to read the priming volume,
- d) it should be possible to measure both the perfusion pressure and the renal blood flow, and finally to take blood samples and to collect the produced urine.

Perfusion circuit: As shown in figure VI,1 the actual perfusion circuit was composed of a disposable reservoir (polyvinylchloride, RV 500-1, Scimed, Minneapolis, Mn), the hydraulic balloon pump, a disposable membrane oxygenator (Kolobow 400 2A, Scimed, Mn), a cone-shaped plexiglass filter with a single-layer nylon filter (20 cm^2 , $200 \text{ } \mu\text{m}$ pore size) and an

Figure VI,1: Circuit for normothermic blood perfusion of canine kidneys. The perfusion circuit included a membrane oxygenator (Kolobow 400, 2A, Scimed), a hydraulic balloon pump (Dale Schuster type), a single-layer nylon blood filter, a venous reservoir and an organ chamber. The organ chamber was made of a disposable venous reservoir (RV 500-1, Scimed) opened on its long side, spread out check-formed and fixed in an aluminium frame.

organ chamber.

The balloon in the blood pump was made of a fingertip of an operating glove and it was replaced after each perfusion. The nylon filter in the blood filter was also replaced after each perfusion. The organ chamber was made of a disposable venous reservoir (RV 500-1, Scimed) which was opened on its long side, spread out check-formed and fixed in an aluminium frame. Hereby a kidney-shaped space was created, under which blood from the renal vein and from leakage from the renal capsule could be collected. The ureter cannula was led out through a separate hole in the organ chamber. Humidity was maintained by covering the upper side of the kidney with a small piece of plastic and by folding up the organ chamber.

By this construction the blood gas interface was limited to a few square centimeters just below the kidney. The parts of the perfusion circuit were connected with silicone tubing with a total length of one meter. The arterial line was provided with a sample line and a connection for a mercury manometer. The hydraulic balloon pump, which was the same as described in chapter V, was provided with a flow indicator; the amplitude of the pump-piston, which was proportional to the stroke volume of the pump, was indicated by a needle on a calibrated scale.

Preparation for the blood perfusion: The perfusion circuit as shown in figure VI,1 was constructed under sterile conditions and fixed on a heavy frame of iron and plexiglass. The venous reservoir was fixed on a plexiglass plate on which the priming volume roughly was indicated. The balloon of the pump was filled with fluid and connected via a pressure tubing with the piston-pump. Next the iron frame with the perfusion circuit was suspended from the ceiling above a thermostatic water bath (38°C). The perfusion circuit was kept in position by a counterweight connected via a pulley with the iron frame.

Figure VI,2: Scheme of the modified heart-lung machine for isolated blood perfusion of canine kidneys.

An aquarium was used as waterbath: (I used the aquarium of my wife and so I prevented her from keeping turtles in it, for I hate those creatures). The sample line and the tubing to the mercury manometer were filled with sterile saline.

Isolated blood perfusion: As described in chapter V the heparinised blood was drawn from the carotid artery of a donor dog. The venous reservoir of the heart-lung machine was rapidly filled with donor blood. The perfusion of the preserved kidney started within two minutes after priming of the circuit.

The blood pump was adjusted to a perfusion pressure of 100 mm Hg. The blood was oxygenated in the membrane oxygenator by air to which 20% O_2 and 5% CO_2 was added to maintain an oxygen saturation of about 95% and a pH of 7.4. At the start of the perfusion 20 ml of a solution containing 0.21 g sodiumcarbonate and 0.75 g glucose was added per liter perfusate. Now the whole circuit (except the organ chamber) was submerged in the thermostatic waterbath. Hereby the air in the venous reservoir was removed. A scheme of this situation is shown in figure VI,2. Next the priming valve of the venous reservoir was closed, and by a short firm push on the venous reservoir the air-bubbles in venous line were pressed back into the organ chamber. Hereby the level of the blood in the organ chamber was the same as that of the fluid in the water bath. Finally an infra-red lamp directed to the kidney maintained normothermia of the kidney. A picture of the isolated blood perfusion is shown in figure VI,3.

Results of isolated blood perfusions with not-preserved kidneys: To examine the qualities of this newly built perfusion system four kidneys were perfused during three hours. A warm ischaemia time of two minutes was needed for nephrectomy, insertion of an arterial cannula and transportation of the

Figure VI,3: Modified heart-lung machine for blood perfusion. The whole circuit except the organ chamber was placed in a thermostatic waterbath (38°C). The blood/gas interface was limited to only a few square centimeters just below the kidney (see Fig. VI,2).

TABLE VI, I PERFUSION PARAMETERS OF NOT-PRESERVED KIDNEYS (N=4) PERFUSED FOR THREE HOURS WITH FRESH, HEPARINIZED BLOOD IN THE MODIFIED HEAR-LUNG MACHINE.

Time min.	Blood flow ^a	GFR ^a	ERPF ^a	FF	Urine ^a	U/P ^b (Iothalamate)
0'	356 ± 96	29 ± 23	203 ± 131	0.14 ± 0.08	0.25 ± 0.11	138 ± 47
30'	450 ± 35	38 ± 27	223 ± 136	0.16 ± 0.07	0.35 ± 0.16	157 ± 75
60'	506 ± 83	53 ± 19	295 ± 39	0.18 ± 0.06	0.82 ± 0.47	132 ± 62
90'	579 ± 117	47 ± 18	221 ± 52	0.24 ± 0.05	1.33 ± 0.90	67 ± 38
120'	590 ± 114	44 ± 16	180 ± 35	0.24 ± 0.05	1.57 ± 0.95	60 ± 27
150'	600 ± 109	40 ± 16	151 ± 39	0.27 ± 0.10	1.92 ± 1.28	32 ± 15
180'						

a Mean values ml/min/100 g ± S.D.

b U/P Iothalamate (GFR/U vol.) indicates the water reabsorption rate.

kidney to the perfusion machine. During the perfusion blood flow was measured and GFR and ERPF values were determined as described in chapter V. The urine and blood samples were taken at 30 minute intervals.

The results are summarized in Table VI,1., which gives an impression of the functional capacity of the isolated perfused kidneys. The results are similar to those reported by other investigators (15, 20). They described supranormal blood flow and increasing urine production which might be due to a progressive loss of antidiuretic hormone. During the second part of the perfusion GFR and the ERPF decreased while a steady increase of blood flow was measured. As discussed in chapter V this might be explained by a reduced resistance of pre- and postglomerular arterioles, leading to oedema and increased tubular pressures (12).

It was concluded that the new perfusion machine, as described in this chapter, was well adapted to the special requirements of a normothermic kidney perfusion halfway a hypothermic preservation period.

CHAPTER VII HYPOTHERMIC PERFUSION COMBINED WITH ISOLATED
BLOOD PERFUSION IN A MODIFIED HEART LUNG MA-
CHINE: PILOT STUDIES

In this chapter the first experiences with the newly built heart-lung machine in the six day preservation model are described. The pilot studies are divided in four small groups.

- a. In four dogs the right kidney was preserved by hypothermic perfusion in the Gambro[®] machine for six days. On the third day of the preservation period the kidney was normothermically perfused with whole blood from an unrelated donor dog. The experimental model is shown in figure IV,1. The intermediate blood perfusion in the modified heart-lung machine was performed as described in chapter VI. After three hours blood perfusion the kidneys were flushed with Eurocollins at 4°C and perfused in the Gambro[®] machine for another three days. In these pilot studies the viability of the preserved kidney was only assessed by survival after implantation and serum creatinine measurements.

In this group only one out of four dogs had life-sustaining kidney function after implantation and contralateral nephrectomy. The only survivor had a maximum serum creatinine concentration of 490 $\mu\text{mol/l}$ on the third day after implantation. At the start of the intermediate blood perfusion the kidneys regained their normal colour and produced urine within five minutes. However in this group the perfusion characteristics differed strongly from those described in chapter V and VI. After 15 to 20 minutes perfusion a marked decrease in blood flow was observed (Fig. VII,1, continuous line). During the second and third hour of the perfusion the blood flow increased slowly to supranormal values. The urine production, GFR and ERPF values followed the blood flow

Figure VII,1: Perfusion parameters of the blood perfusion halfway the 6 day preservation period in the pilot studies. During the first part of the blood perfusion, a severe vasoconstriction was measured (continuous line). Addition of phenoxybenzamine (n=2) did not reduce the decrease in blood flow (dotted line). In 3 experiments mannitol was given after one hour perfusion (broken line). This resulted in an increase in the renal blood flow. In all experiments the other parameters followed the blood flow.

pattern; after a decrease they increased slowly to their initial values at the end of the perfusion (Fig. VII,1). The blood perfusions were technically well performed without any macroscopic haemolysis. The unexpected decrease in blood flow was probably due to vasoconstriction and was thought to be responsible for the poor results after implantation. So if there had been any beneficial effect by the blood perfusion itself, this was masked by the vasoconstriction during the first part of the perfusion. The possible mechanism of the vasoconstriction will be discussed in chapter IX.

- b. In other pilot studies attempts were made to counteract the vasoconstriction during the intermediate blood perfusion. In two experiments 20 mg phenoxybenzamine (Dibenzylamine[®]) per liter perfusate was added at the start of the three hours normothermic perfusion. However this vasodilating drug did not influence the marked decrease in blood flow during the first hour of the blood perfusion. After implantation of these kidneys anuria occurred and the dogs died uraemic. As in the former four experiments the glomerular filtration rate and the effective renal plasma flow decreased in proportion with the blood flow. These values remained low during the second and third hour of the blood perfusion (Fig. VII,1, dotted line).
- c. In the next experiments the effect of hyperosmolar mannitol on the decreased blood flow was examined. It has been demonstrated by other investigators (185) that hyperosmolar mannitol induces a decrease in renal vascular resistance in ischaemic damaged kidneys by reducing tissue- and endothelial cell swelling. According to other authors (186) mannitol would also have a vasodilator effect in ischaemic kidneys, mediated mainly by increased prostacyclin (PGI₂) activity. In three preservation ex-

periments 20 ml mannitol solution (20 g/100 ml) per liter perfusate was given after the first hour of the normothermic blood perfusion. In these perfusions the blood flow increased upon infusion of the mannitol solution (Fig. VII,1, broken line). Initially the glomerular filtration rate and the effective renal plasma flow increased in proportion with the blood flow. In two experiments however, these values decreased during the third hour of the perfusion and in one case they remained constant.

In the last mentioned experiment the dog survived after the six day preservation period with a maximum serum creatinine of 400 $\mu\text{mol/l}$ on day two. After implantation the other two kidneys had a weak consistency despite firm pulsations of the renal artery. These kidneys never produced urine. These experiments gave evidence that mannitol was effective in reducing the vascular resistance of poorly perfused kidneys. On the other hand mannitol seemed to have a detrimental effect on the kidney during prolonged perfusion in the heart-lung machine. The latter effect might be explained by a progressive dehydration of kidney cells by the hyperosmolar perfusate.

- d. Finally in some pilot experiments the vasoconstriction during the intermediate blood perfusion was influenced by the addition of carbachol i.e. a vasodilating drug with direct action on the vessel wall. Mannitol was given just during the last minutes of the perfusion. In these experiments the vasoconstriction was functionally antagonised and the dogs survived after implantation. This pilot study was extended to a series experiments. The results are presented in chapter VIII.

In this chapter a series of 11 experiments (group IV) is described in which dog kidneys were preserved by hypothermic perfusion and 3 hours isolated blood perfusion halfway the preservation period (see experimental model, Fig. IV,1). As in the control group the hypothermic perfusions were performed in the Gambro[®] machine (Chapter III). The isolated normothermic blood perfusion was performed according to the technique described in chapter VI. Based on the experiences in the pilot studies (Chapter VII) an attempt was made to improve the blood flow during the blood perfusion.

At the start of the blood perfusion 200 μ g carbachol per liter perfusate was added and during the last five minutes of the perfusion 20 ml mannitol solution (20 g/100 ml) per liter perfusate was administered. The perfusion pressure in the heart-lung machine was 100 mm Hg. During the blood perfusion GFR and ERPF values were determined as in group III. Urine and blood samples were taken at 30 minute intervals. After the preservation period of six days implantation followed by contralateral nephrectomy was performed. As in the other experimental groups the kidney function was assessed by daily serum creatinine measurements. GFR and ERPF were also measured one hour after implantation as described in chapter III. In the same way follow up determinations of GFR and ERPF were performed at three, ten and 21 days after implantation in the last five dogs of this group (IV). Three weeks after implantation the dogs were sacrificed. After autopsy histological examination of the kidney biopsies was performed.

Figure VIII,1: Serum creatinine concentration in group IV (mean values $\pm$ S.D.). In this group all dogs had life-sustaining kidney function. Two dogs had to be sacrificed for technical reasons. The results in the control group (I) with hypothermic perfusion only, are also presented.

Results:

Hypothermic perfusion: During the hypothermic perfusion the same values of perfusion pressure, perfusate flow and pH were measured as in the control group (I) (Fig. III,3). After the normothermic ex-vivo perfusion the same perfusion parameters were measured as during the first part of the hypothermic perfusion.

Survival after implantation: Upon revascularisation all kidneys regained normal colour and produced immediately urine. At one hour after implantation the urine production was significantly higher ($p < 0.01$ Wilcoxon test) than in the control group and than in group III (Chapter III, V). The urine production averaged 24 ml/hour (range 4-51 ml/hour) and was not significantly different from that in group II (Chapter IV). In this group nine out of 11 dogs survived in a healthy condition. Unfortunately two dogs had to be sacrificed at respectively three and seven days after implantation because of wound dehiscence and urine leakage (technical failures). The serum creatinine values followed the pattern of the other dogs. In this group the mean serum creatinine concentration returned to normal within two weeks after implantation and had a maximum value of 533 $\mu\text{mol/l}$ (range 279-885 $\mu\text{mol/l}$) on day two (Fig. VIII,1). No significant difference (Wilcoxon test) was observed between the mean serum creatinine concentration values after implantation in this group (IV) and group II (Fig. IV,4).

Kidney function after implantation: GFR and ERPF values measured at one hour after implantation are shown in Table VIII,1. As in the ex vivo perfused group (Table IV,1) much variation was found among these values and the individual values did not correlate with the serum creatinine concentration level after implantation. As shown in figure VIII,2 all kidneys had low filtration fractions after implantation

Table VIII,1: GLOMERULAR FILTRATION RATE, EFFECTIVE RENAL PLASMA FLOW AND FILTRATION FRACTION MEASURED IN SIX DAYS PRESERVED KIDNEYS ONE HOUR AFTER IMPLANTATION

GROUP IV (modified heart-lung perfused)

dog	GFR ^a	ERPF ^a	FF
1	2.85	26.40	0.11
2	0.53	3.95	0.13
3	14.45	77.52	0.19
4	5.47	49.66	0.11
5	3.48	19.05	0.18
6	1.17	10.71	0.11
7	4.52	30.64	0.15
8	3.40	13.73	0.25
9	1.74	7.65	0.23
10	1.72	8.67	0.20
11	0.15	0.55	0.27

a GFR and ERPF values were calculated in ml/min/100 g kidney weight.

Figure VIII,2: In group IV (modified blood perfused; +) all dogs had low filtration fractions at one hour after implantation. For comparison the FF values of the other groups are also presented. (o:controls; *:ex-vivo perfused; •:isolated blood perfused in heart-lung machine with film oxygenator).

Table VIII,2: FOLLOW-UP OF GLOMERULAR FILTRATION RATE, EFFECTIVE RENAL PLASMA FLOW AND FILTRATION FRACTION IN SIX DAYS PRESERVED BLOOD-PERFUSED KIDNEYS AFTER IMPLANTATION.^a
(GROUP IV)

day	GFR ^b	ERPF ^b	FF
0	2.31 $\pm$ 1.69	12.25 $\pm$ 11.31	0.22 $\pm$ 0.05
3	21.80 $\pm$ 6.02	78.05 $\pm$ 11.98	0.28 $\pm$ 0.05
10	57.57 $\pm$ 8.81	196.92 $\pm$ 15.06	0.29 $\pm$ 0.04
21	61.77 $\pm$ 22.57	238.45 $\pm$ 78.99	0.26 $\pm$ 0.05

a Mean values $\pm$ S.D. of five dogs (no. 7-11) are presented.

b GFR and ERPF values are calculated in ml/min/100 g kidney weight.

(In figure VIII,2 the FF values after implantation of all four experimental groups are summarized).

In five dogs of group IV follow up clearance determinations were performed at three, ten and 21 days after implantation. The GFR and ERPF values returned to their normal range within ten days after implantation (Table VIII,2).

Histology: In this group all dogs were sacrificed three weeks after implantation. Biopsies of these kidneys showed normal morphology. However, signs of pyelonephritis were present to a certain amount in all kidneys due to ascending infection, which is inherent to the implantation in the neck (168).

In this group two dogs had to be sacrificed for technical reasons at respectively three and seven days after implantation. This offered the opportunity to compare their histology with that of the kidneys from group I and III which had no life-sustaining function. Histological sections of these two kidneys showed normal glomeruli and blood vessels. Signs of severe tubular damage were not seen in both kidneys; only a few casts were observed (Fig. VIII,3). On bisecting the kidneys at autopsy no haemorrhagic zone was observed between the cortex and the medulla as opposed to the kidneys of group III.

Isolated blood perfusion: During the blood perfusions the perfusion pressure was kept on 100 mm Hg. Initially in all kidneys a normal blood flow (266 ± 93 ml/min/100 g) was measured. After 20 minutes the renal blood flow decreased for about 30 minutes to increase finally to supranormal values (411 ± 68 ml/min/100 g) in the third hour of the perfusion (Fig. VIII,4).

The decrease in blood flow during the first part of the blood perfusion was less severe than in the pilot studies (Fig. VII,1). In this group the renal blood flow measured during the second part of the first hour was significantly

Figure VIII,3: In group IV, two dogs had to be sacrificed for technical reasons at respectively 3 and 7 days after implantation. This offered the opportunity to compare their histology with that of kidneys from group I and III which had no life-sustaining function. Histology of these 2 kidneys showed normal glomeruli and blood vessels. Signs of severe tubular damage were not seen in both kidneys; only a few casts were observed. (Histology 3 days after implantation, ca. 250 x Hematoxyllin/Eosin).

higher ($p < 0.05$ Wilcoxon test) than in six pilot experiments with and without phenoxybenzamine. During the blood perfusion different GFR and ERPF values were measured between the individual experiments. In each experiment the diuresis, GFR and ERPF decreased and increased in proportion with the blood flow. No further increase of these parameters was observed during the third hour of the perfusion (Fig. VIII,4). As in other groups a low filtration fraction was measured; since the GFR and ERPF both followed the same pattern, the filtration fraction remained unchanged throughout each individual perfusion.

During the last five minutes of the blood perfusion the hyperosmolar mannitol solution was added to the perfusate in the venous reservoir. This resulted in an abrupt increase in blood flow (20-30 ml/min/100 g kidney) and urine production (1-2 ml/min/100 g kidney).

Discussion:

Finally, in this group (IV) the preservation time of dog kidneys could be extended successfully to six days by three hours blood perfusion in a modified heart-lung machine half-way the hypothermic preservation period: nine out of 11 dogs survived in a healthy condition after implantation. Two dogs had to be sacrificed because of a technical failure.

In this group the serum creatinine concentration levels after implantation were similar to or even lower than those reported by other authors after three days hypothermic continuous perfusion (70). The good function of the kidneys in group IV preserved by this combined hypothermic and normothermic perfusion procedure was confirmed by the normal GFR, ERPF and FF values measured ten days after implantation.

During the three hours isolated blood perfusion the course of the GFR, ERPF and diuresis were influenced by the initial decrease in renal blood flow. As compared to the pi-

Figure VIII, 4: Perfusion parameters of the blood perfusion halfway the six day preservation period in the group IV. The renal blood flow is presented in mean values $\pm$ S.D. The urine production, the glomerular filtration rate and the effective renal plasma flow are presented for each perfusion experiment. The filtration fraction showed no variation and was low throughout each perfusion (range 0.06-0.25. FF values are not presented in the figure).

lot studies (Fig. VII,1) a better recovery of the GFR, ERPF and urine production was observed during the second hour, while these values remained stable during the third hour of the blood perfusion. The latter results obtained in this series experiments might be attributed to the improved blood flow conditions.

In comparison with the blood flow values in the pilot studies the blood flow decrease in this group was functionally antagonised by the addition of carbachol. The shorter and less severe vasoconstriction was considered of great importance to the recovery of the urine production, the GFR and ERPF (Fig. VIII,4). Evidence for this assertion was given by Conger et al. (187) who studied the effects of nor-epinephrine induced vasoconstriction in rats. In confirmation with others (188,189,190) they observed persistent low GFR values and oliguria after restoration of renal blood flow by addition of acetylcholine. They measured high tubular pressures due to intratubular obstruction, which was responsible for the marked reduction in glomerular filtration. With regard to these data it should be emphasized that the prevention of vasoconstriction is even more important in blood perfusion of preserved kidneys, since intratubular obstruction by "casts" is caused by prolonged hypothermic preservation itself (as shown in the histological sections of the control group) and by release of vasoconstrictive material by the kidney itself (Chapter IX).

Hyperosmolar mannitol solution was administered during the last five minutes of the isolated perfusion to counteract to some extent oedema which is inherent to isolated kidney perfusion, as described in chapter V. It has been demonstrated by other investigators (185) that hyperosmolar mannitol induces decrease in renal vascular resistance in ischaemic damaged kidneys by reducing tissue- and endothelial cell swelling. Indeed the infusion of hyperosmolar mannitol caused not only the expected osmotic diuresis but also an abrupt increase in renal blood flow during the last five

minutes of the blood perfusion. In this group the mannitol solution was given during the last minutes of the perfusion to avoid a possible detrimental effect on the kidney during prolonged perfusion. So a progressive dehydration of renal cells was prevented by short exposure to the hyperosmolar perfusate.

One hour after implantation all kidneys had low filtration fractions, indicating the presence of active functioning tubules. Histological examination of the kidneys of the two technical failures in this group reveals data which are comparable with the histology of non-viable kidneys of the control group and group III. In both kidneys normal glomeruli and blood vessels were seen without any signs of bleeding as in group III. These data suggest that vascular damage was prevented by the lower perfusion pressure (100 versus 120 mmHg) and the less complicated perfusion system applied in this group. In contrast with the control group few casts were observed in slices of both kidneys. This indicates that the intermediate blood perfusion had a beneficial effect especially on the tubular cells of the preserved kidney.

From the results presented in this chapter it can be concluded that irreversible damage induced by hypothermic preservation can be prevented by an artificial normothermic blood perfusion. By this method the preservation time can be extended up to six days without loss of viability of the kidney. The possible mechanism of the beneficial effect of the blood perfusion on the preserved kidney is discussed in chapter X.

In this chapter some remarks about the blood flow pattern during the normothermic blood perfusion are made. In the newly built perfusion apparatus, described in chapter VI, normal blood flow values were measured during perfusion of not-preserved kidneys (Table VI,1). In the course of the perfusion the blood flow increased steadily to supranormal values. This phenomenon, also described by others (20,21) might be due to depletion of vasotonic agents, e.g. the loss of renin substrate from the blood perfusate (19).

On the other hand the vasodilation may be explained by activation of plasma kinins induced by activation of the Hageman factor by exposure to the foreign surfaces of the perfusion apparatus. Evidence for the latter explanation of the vasodilation during the second part of the blood perfusion was given by Nizet et al. (12) who prevented the development of supranormal blood flow by addition of a soya bean trypsin inhibitor (Trypsin like enzymes induce the formation of bradykinin). Bradykinin like substances also enhance the renal permeability leading to oedema. Hereby the decrease in GFR in proportion to the renal blood flow can be explained.

During the isolated blood perfusion of preserved kidneys in the pilot studies with the new heart-lung machine, a strongly different blood flow pattern was observed. A marked vasoconstriction was measured during the first part of the perfusion (Fig. VII,1). Impaired renal blood flow during the first hour of isolated kidney perfusion has also been described by other investigators (17,20,21). According to Nizet et al. (21) this vasoconstriction is caused by a vasoactive material released mainly by erythrocytes in the perfusate. It has also been suggested that the vasoconstriction might be caused by catecholamines released in the blood donor during bleeding. However, in two pilot studies the addition of the alpha adrenergic blocking agent, phenoxybenzamine (191), failed to eli-

minate the vasoconstriction (Fig. VII,1). In three pilot experiments the infusion of hyperosmolar mannitol solution was effective in reducing the vasoconstriction (Fig. VII,1). It has been demonstrated by other investigators (185) that hyperosmolar mannitol induces a decrease in renal vascular resistance in ischaemic damaged kidneys, by reducing tissue and endothelial cell swelling. Johnston et al. (186) described that mannitol would also have a vasodilator effect in ischaemic kidneys, mediated mainly by increased prostacyclin (PGI_2) activity. Although mannitol was effective in reducing the renal vascular resistance in the pilot experiments, it seemed to have a detrimental effect as well during prolonged perfusion in the heart-lung machine. This might be explained by a progressive dehydration of the kidney cells by the hyperosmolar perfusate.

Finally in the successful series preservation experiments (group IV), the blood flow during the first part of the blood perfusion could be improved (Fig. VIII,4). As compared to the pilot experiments (Fig. VII,1) a significant ($p < 0.05$, Wilcoxon test) reduction of the decrease in renal blood flow could be obtained by the addition of carbachol. Figure VIII,4 shows that the vasoconstriction was not abolished but functionally antagonised. Blaine and Dunlay (192) measured vasodilatation after continuous infusion of acetylcholine in the renal artery. In the experiments of group IV satisfactory vasodilatation was brought about by a single dose of carbachol, which has a similar action on the vessel wall but which is broken down much less rapidly.

Although the vasoconstriction in group IV was reduced, the blood flow pattern still differed strikingly from the blood flow during the perfusion of not-preserved kidneys. As shown in Table VI,1 the blood flow in these kidneys did not decrease during the first hour of the blood perfusion. This suggests that preserved kidneys are more susceptible to vasoconstricting agents circulating in the perfusate. This might be due to either the accumulation of vasoconstrictive factors that cannot be inactivated rapidly enough by a preserved kidney, or by

a release of vasoconstrictive material by the kidney itself.

The blood flow difference between the not-preserved and the hypothermic perfused kidneys might be explained by the activation of the renin angiotensin system. Recently Derkx et al. (193) reported activation of (inactive) prorenin by exposure to cold.

It is suggested that cold-promoted activation of prorenin occurred during the hypothermic perfusion of the kidney. At the start of the normothermic isolated blood perfusion the release of activated renin would cause the formation of angiotensin I and subsequently angiotensin II, a potent vasoconstrictor. A typical finding was that the vasoconstriction in the preserved kidneys occurred after 20 minutes. McGiff and Itskovitz (194) reported the loss of vasoconstrictive activity of angiotensine II in ischaemic kidneys. Supposing that the renin angiotensine system is involved in the vasoconstriction during the blood perfusion, these observations may explain why the blood flow alterations occurred just after 20 minutes. Further experiments e.g. with the application of a converting enzyme inhibitor (Captopril), are necessary to elucidate these questions.

The proposed explanation makes it difficult to understand why the hypothermic perfused kidneys in group III did not show any vasoconstriction during the blood perfusion in the heart-lung machine with the film oxygenator. In the pilot studies and in group IV the only technical difference was the use of a membrane oxygenator instead of a film oxygenator. However, it is unlikely that this alteration could be responsible for the vasoconstriction. On the contrary the mechanical damage of the blood in group IV, which would cause vasoconstriction, was less than in group III since macroscopic haemolysis was not observed in group IV as opposed to group III. Probably the stable blood flow in group III was due to the addition of promethazine. In prior studies by Nizet et al. (12) the action of promethazine on the blood flow in isolated kidneys has been an empirical finding. Perhaps the antiserotonin (side) effects of

promethazine might explain why this antihistaminic drug prevented vasoconstriction in the heart-lung machine with the filmoxygenator. Promethazine was not added to blood perfusions in the heart-lung machine with the membrane oxygenator.

In this thesis the results of six day experimental kidney preservation are described. Successful preservation was achieved by a combination of hypothermic continuous perfusion and three hours normothermic blood perfusion, either "ex-vivo" or "isolated" in a heart-lung machine.

Successful experimental kidney preservation of three to four days by hypothermic continuous perfusion has been achieved by several investigators (72,75) (Chapter I,3). However, consistent good results have not been reported after longer preservation periods (69,75). In conformation with these data the results of the control group clearly showed that six day hypothermic perfusion is not likely to maintain the viability of a kidney: only one out of eight dogs survived.

It is suggested by several authors (195) that the non-viability of kidneys after prolonged hypothermic preservation is due to an impairment of energy metabolism. After preservation, suppression of ATP regeneration upon revascularisation will cause loss of viability of the kidney, since life supporting functions require rapid resynthesis of ATP. Southard et al. (130) found in slices of hypothermic preserved dog kidneys loss of adenosine nucleotides, reduction in respiration and ATP synthesis, and uncoupling of oxidation and phosphorylation in the mitochondria. A progressive fall in adenosine nucleotide content was also described by Buhl (196) in anaerobic perfused rabbit kidneys: a complete regeneration of adenosine nucleotides was obtained during one hour in vitro perfusion with resumed oxygen supply and addition of adenosine (196).

These data might explain the mechanism of the beneficial effect of the blood perfusion on the preserved kidneys in the experiments described here. It is suggested that the metabolism was restored during the blood perfusion. Indeed the low filtration fractions measured throughout each blood per-

fusion indicated the presence of active energy metabolism in the proximal tubules. These data give evidence for the restoration of ATP-levels in tubular cells during the blood perfusion. However, the content of adenosine nucleotides was not measured in this study. Determination of these biochemical parameters after the normothermic blood perfusion would exclude the possibility of testing the viability of the kidneys "in-vivo" at the end of the preservation period. Another series of identical experiments will be needed to find out whether the blood perfusion does restore the adenosine nucleotide content and ATP-synthesis.

At this point a recently developed method to test the viability of an organ may be mentioned. Chan et al. (197) were able to measure the ATP-content of human kidneys in-vivo by ^{31}P nuclear magnetic resonance. This would enable the evaluation of the metabolic state of a preserved kidney with maintenance of the possibility to test the life-sustaining function of the organ "in-vivo". Certainly this might be a valuable method to obtain more information about the mechanism of the beneficial effect of the normothermic blood perfusion in these preservation experiments.

Although the exact mechanism of the blood perfusion on the hypothermic preserved kidney remains so far unknown, the histological data in group IV suggest a recovery of metabolism in tubular cells during the normothermic blood perfusion.

Explaining the mechanism of the blood perfusion in a simplified and trivial way, one could say that the preserved kidney is "recharged like a battery", before it becomes exhausted completely. However, a more interesting comparison can be made with the behaviour of hibernating mammals (198). For instance hibernating ground squirrels keep their body temperature at about $4-5^{\circ}\text{C}$ during dormancy. Regardless of ambient temperature or any other external stimulus, they wake up at seven to 12 days intervals. The animals rewarm rapidly from 4 to 36°C and exhibit full activity in eating,

drinking and excretory behaviour before they return to torpid again (C. Green, personal communication). The resemblance with this natural existing behaviour of hibernating mammals indicates that the kidney preservation model described here may be a logical approach to long-term organ preservation.

Conclusion

From this study it can be concluded that it is possible to prevent irreversible preservation damage in hypothermic continuous perfusion by three hours normothermic isolated blood perfusion, after which the organ can be preserved for another three days. The data suggest that by further improvement of renal blood flow during the first part of the blood perfusion, the beneficial effect can be obtained by a shorter perfusion period. Moreover this would limit the detrimental effects which are inherent to prolonged isolated blood perfusion of kidneys.

In addition further improvement of renal blood flow would make this preservation model suitable for viability monitoring of hypothermic preserved kidneys. Furthermore, if the same results can be obtained by using an artificial perfusate, it would be possible to use the model to find out which factors are responsible for the beneficial effect of the normothermic intermediate perfusion. This might also contribute to the development of improved hypothermic preservation techniques.

Finally, the good results of these six day preservation experiments suggest that the application of alternating hypothermic and normothermic perfusion might enable long-term preservation in the future.

SUMMARY

In this thesis a new kidney preservation method is described. Successful six day preservation of dog kidneys was achieved by hypothermic albumin perfusion combined with a short intermediate normothermic blood perfusion.

In chapter I a review is presented of the development and current status of various kidney preservation methods employed in the clinical and experimental situation: Preservation by normothermia is not suitable for transplantation surgery, although normothermic perfusion may be applied to assess the viability of a kidney before transplantation.

Next several flush solutions for cold storage preservation are discussed. Most of these solutions can be safely used up to 36 hours for kidney preservation. The effect of these preservation solutions is explained as follows: during hypothermic preservation osmotic cell swelling is caused by the decreased activity of the Na/K pump in cell membranes. The efficiency of the preservation solutions is due to the presence of impermeable anions and to the high osmolarity and not to the "intracellular" composition as stated formerly. The cold storage technique is less effective for preservation of kidneys which have suffered a considerable warm ischaemic time (non heartbeating donors). In these circumstances hypothermic continuous perfusion is preferable. Clinical and experimental data indicate that kidney preservation up to 72 hours can be obtained with hypothermic perfusion. The composition of several perfusates is discussed.

Kidney preservation by freezing has not been achieved as yet. On the other hand cryopreservation of cell suspensions and embryos can be performed without unacceptable loss of viability. Probably the characteristics in which organs distinguish from cell suspensions, namely the fixed geometry, the high density of various cells and a vascular system, are mainly responsible for the disappointing results so far ob-

tained in cryopreservation of organs.

Kidney preservation by combined hypothermic perfusion and cold storage does not result in prolonged preservation time. Kidneys preserved by intermittent cold storage and hypothermic perfusion would be less viable than kidneys preserved by one of these techniques alone for the same time.

In this thesis the results of combined hypothermic and normothermic perfusion are described. The study is a continuation of the research performed by Kootstra and van der Wijk. They have shown that the preservation time of dog kidneys could be prolonged by a combination of hypothermic and normothermic perfusion. Halfway a hypothermic and normothermic perfusion period of 4 to 6 days with an albumin perfusate the preserved kidney was connected with the circulation of the donor dog. After several hours normothermic perfusion the hypothermic preservation was continued with the same albumin perfusate. Normothermic, so-called "ex-vivo" perfusion resulted in a significantly better kidney function after autotransplantation and immediate contralateral nephrectomy.

In chapter II the materials and methods applied in the preservation experiments are described.

The experiments described in chapter III clearly show that a six day period of hypothermic perfusion only is not likely to maintain the viability of a kidney. In the control group (I) only one out of eight dogs survived after implantation.

The experiments discussed in chapter IV indicate that successful kidney preservation can be obtained by combination of hypothermic preservation and normothermic ex-vivo perfusion of three hours. In a consecutive series of seven experiments all animals survived with good kidney function (group II). During the three hour ex-vivo perfusion an increase in glomerular filtration and tubular secretion was measured. Although a preservation time of six days was

reached, there was no question of true preservation in group II since the kidneys were connected again with the donordog. In the next experiments an attempt was made to obtain similar results by replacement of the ex-vivo perfusion by an artificial perfusion in a heart-lung machine.

In eight experiments described in chapter V the normothermic perfusion was performed in a heart-lung machine including a film oxygenator and a hydraulic balloon-pump. Fresh heparinised blood was used as perfusate. Successful six day preservation was obtained in only three out of eight experiments (group III). Kidney function measured at one hour after implantation indicated that most kidneys did have active functioning tubules. The poor results in this series can be explained by endothelial damage which might be due to the high perfusion pressure during the blood perfusion. Evidence for this explanation is given by numerous haemorrhagic areas which were observed both macroscopically and microscopically at autopsy.

Chapter VI describes the construction of a modified heart-lung machine especially adapted to the requirements of the perfusion of a preserved kidney.

In chapter VII some pilot experiments are described in which the modified heart-lung machine was used in the six day preservation model. In these experiments an attempt was made to improve the renal blood flow during the heart-lung perfusion. By addition of carbachol and - at the end of the perfusion - mannitol, improvement of the results was obtained.

In a consecutive series of 11 experiments, described in chapter VIII, successful six day kidney preservation was achieved by three hour normothermic blood perfusion in the modified heart-lung machine (group IV).

In chapter IX special attention is paid to the blood flow pattern during the normothermic perfusion in the different preservation experiments.

Finally, in chapter X the mechanism of the beneficial effect of the normothermic blood perfusion is discussed. It is suggested that the energy metabolism in the cells is restored during the normothermic perfusion. The blood perfusion resulted in a better preservation of the tubules as shown by histological examination in group IV.

From this study can be concluded that it is possible to prevent irreversible preservation damage in hypothermic continuous perfusion by three hours normothermic isolated blood perfusion, after which the organ can be preserved for another three days. Further improvement of the renal blood flow during the normothermic perfusion would make this preservation model suitable for viability monitoring of hypothermic preserved kidneys. Finally, the good results of these six day preservation experiments suggest that application of alternating hypothermic and normothermic perfusion might enable long-term preservation in the future.

SAMENVATTING

In dit proefschrift wordt een nieuwe nierpreservatie methode beschreven. Succesvolle zesdaagse preservatie van hondenieren werd verkregen door middel van hypotherme perfusie met albumine gecombineerd met een korte tussentijdse normotherme bloedperfusie.

Hoofdstuk I geeft een overzicht van de ontwikkeling en huidige toepassing van verschillende nierpreservatie methoden in de klinische en experimentele situatie. Preservatie met behulp van alleen normotherme perfusie is niet geschikt voor transplantatie chirurgie, ofschoon normotherme perfusie kan worden toegepast om de vitaliteit van een nier te beoordelen voorafgaande aan transplantatie. Vervolgens wordt de samenstelling van verschillende preservatie vloeistoffen besproken die gebruikt worden voor de zgn. "cold storage" preservatie. De meeste van deze vloeistoffen kunnen veilig gebruikt worden voor nierpreservatie tot 36 uur. De werking van deze preservatie vloeistoffen wordt als volgt verklaard: tijdens hypotherme preservatie veroorzaakt de afgenomen activiteit van de Na/K pomp in de cel membraan osmotische zwelling van de cel. De werking van de preservatie vloeistoffen berust op de aanwezigheid van impermeabele anionen en de hoge osmolariteit, en niet, zoals vroeger werd beweerd, op de "intracellulaire" ionen samenstelling.

De "cold storage" techniek is minder effectief voor het conserveren van nieren die een aanzienlijk lange warme ischaemie tijd hebben ondergaan (bv. hart-dode donoren). In deze omstandigheden verdient hypotherme continue perfusie de voorkeur. Klinische en experimentele gegevens wijzen erop dat nierpreservatie tot 72 uur kan worden bereikt met behulp van hypotherme perfusie. De samenstelling van diverse perfusatievloeistoffen wordt besproken.

Nierpreservatie door middel van invriezen is tot nu toe nog niet gelukt. Daarentegen kan cryopreservatie van celsuspensies en embryo's worden verricht zonder inacceptabel

vitaliteits verlies. Waarschijnlijk zijn voornamelijk de eigenschappen waarin hele organen verschillen van cel-suspensies, te weten de vastliggende geometrische verhoudingen, de hoge dichtheid van verschillende cellen en een vaatstelsel, verantwoordelijk voor de teleurstellende resultaten die tot dusver zijn behaald met het invriezen van organen.

Door een combinatie van hypotherme perfusie met "cold storage" kan de duur van de nierpreservatie niet worden verlengd. Nieren die afwisselend met "cold storage" en hypotherme perfusie worden gepreserveerd zouden minder vitaliteit behouden dan nieren die even lang bewaard worden met één van de methoden afzonderlijk.

In dit proefschrift worden de resultaten van gecombineerde hypotherme en normotherme perfusie beschreven. Het onderzoek is een voortzetting van experimenteel werk verricht door Kootstra en van der Wijk. In dierexperimenten toonden zij aan dat de preservatieduur van hondenieren kon worden verlengd door een combinatie van hypotherme en normotherme perfusie. Halverwege een hypotherme perfusie periode van 4 à 6 dagen met een albumine oplossing werd de gepreserveerde nier aangesloten op de lichaamscirculatie van de donorhond. Na enkele uren normotherme perfusie werd de hypotherme preservatie voortgezet met hetzelfde albumine perfusaat. De normotherme zgn. "ex-vivo" perfusie leidde tot een significant betere nierfunctie na autotransplantatie en direkte contralaterale nefrectomie.

In hoofdstuk II worden de materialen en methoden beschreven welke werden toegepast bij de preservatie experimenten.

Uit de experimenten beschreven in hoofdstuk III blijkt dat een zesdaagse preservatie periode met alleen hypotherme perfusie resulteert in een irreversibel funktieverlies van de nier. In deze controle groep (I) overleefde slechts één van de acht honden na implantatie.

De experimenten besproken in hoofdstuk IV tonen aan dat succesvolle zesdaagse nierpreservatie kan worden bereikt

door de hypotherme preservatie te combineren met een drie uur durende normotherme ex-vivo perfusie. In zeven opeenvolgende experimenten overleefden alle proefdieren met een goede nierfunctie (groep II). Tijdens de drie uur durende ex-vivo perfusie werd een verbetering van de glomerulaire filtratie en de tubulus secretie gemeten. Bij deze experimenten was geen sprake van echte preservatie, aangezien de nier tijdelijk werd verbonden met de donorhond. In de volgende experimenten werd getracht dergelijke resultaten te bereiken door de ex-vivo perfusie te vervangen door kunstmatige perfusie in een hartlong machine.

In hoofdstuk V worden acht experimenten beschreven waarbij de normotherme perfusie werd uitgevoerd in een hart-long machine met een film-oxygenator en een hydraulische ballonpomp. Als perfusaat werd vers heparine bloed gebruikt. Van de acht experimenten werd in slechts drie gevallen succesvolle zesdaagse preservatie verkregen (groep III). De nierfunctie gemeten één uur na implantatie wees erop dat bij de meeste nieren wel actief secernerende tubuli aanwezig waren. De slechte resultaten in deze serie kunnen worden verklaard door endotheel beschadiging die zou zijn ontstaan door een hoge perfusie druk tijdens de bloedperfusie. Deze verklaring is aannemelijk door de vele haemorrhagische gebieden, welke zowel macroscopisch als microscopisch waarneembaar waren bij obductie.

In hoofdstuk VI wordt de constructie beschreven van een gewijzigde hart-long machine, die speciaal werd aangepast aan de eisen gesteld aan de perfusie van een gepreserveerde nier.

In hoofdstuk VII worden enkele pilot experimenten beschreven waarbij de gewijzigde hart-long machine werd gebruikt in het zesdaagse nierpreservatie model. In deze serie experimenten werd getracht de bloedflow door de nier tijdens de hartlong perfusie te verbeteren. Door toevoeging van carbachol en - aan het eind van de perfusie - mannitol werd verbetering van de resultaten bereikt.

In een serie van 11 opeenvolgende experimenten beschreven in hoofdstuk VIII, werd succesvolle zesdaagse nierpreservatie verkregen met behulp van drie uur normotherme bloedperfusie in de gewijzigde hart-long machine (groep IV).

In hoofdstuk IX wordt speciale aandacht besteed aan het verloop van de bloedflow tijdens de normotherme perfusie in de verschillende preservatie experimenten.

Tenslotte wordt in hoofdstuk X het mechanisme van het gunstig effect van de normotherme bloedperfusie besproken. Gesuggereerd wordt dat het energie metabolisme in de cel zich herstelt tijdens de normotherme perfusie. De bloedperfusie resulteerde met name in een betere preservatie van de tubuli, hetgeen werd aangetoond door histologisch onderzoek in groep IV.

De conclusie van dit proefschrift luidt dat het mogelijk is irreversibele preservatie schade ten gevolge van hypotherme perfusie te voorkomen door een drie uur durende geïsoleerde normotherme bloedperfusie; de nier kan hierna nog eens drie dagen worden gepreserveerd. Verdere verbetering van de bloedflow tijdens de normotherme perfusie zou dit preservatiemodel geschikt kunnen maken voor het bepalen van de vitaliteit van een hypotherm gepreserveerde nier.

Tenslotte suggereren de goede resultaten van deze zesdaagse preservatie experimenten dat in de toekomst de toepassing van afwisselend hypotherme en normotherme perfusie langdurige nierpreservatie mogelijk maakt.

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Bert Rijkmans werd geboren op 6 juni 1953 te Groningen. Van 1965 tot 1971 volgde hij zijn middelbare schoolopleiding aan het Praedinius Gymnasium te Groningen. Na het behalen van het eindexamen (B) ging hij medicijnen studeren aan de Rijksuniversiteit Groningen, waaraan hij in 1978 het arts-examen behaalde. Daarna was hij tot 1980 als wetenschappelijk medewerker verbonden aan deze universiteit bij de afdeling Experimentele Chirurgie (hoofd Prof. Dr. C.R.H. Wildevuur). Van 1980 tot 1981 was hij werkzaam als wetenschappelijk medewerker aan de Rijksuniversiteit Limburg te Maastricht bij de capaciteitsgroep Algemene Heelkunde. In de periode 1978 tot 1981 werd experimenteel nierpreservatie onderzoek verricht, gesubsidieerd door de Nier Stichting Nederland. Vanaf januari 1982 volgt hij de opleiding Algemene Chirurgie in het Ziekenhuis St. Annadal (opleider Prof. Dr. J.M. Greep).