Preclinical evaluation of a new organ preservation solution
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Chapter 1

General introduction and outline of the thesis
General introduction

History of organ transplantation

The capability to transplant solid organs today is based on the development of the vascular anastomosis in 1902 by the French surgeon Alexis Carrel1. Half a century later, on December 23rd, 1954, the first successful transplantation of human organs was performed in Boston, under the direction of Dr. Joseph Murray. The surgical team removed a kidney from a healthy donor and transplanted it into his identical twin, who was suffering from chronic glomerulonephritis. The organ functioned immediately and the recipient survived for nine years, at which time his allograft failed from recurrent glomerulonephritis2. This milestone in the field of transplantation led to the development of successful kidney transplant programs worldwide.

Following the success of kidney transplantation, in the late 1960s, transplantation of heart and liver became a reality. In 1967, Dr. Christian Barnard performed the first human heart transplantation in South-Africa and in that same year in the USA, Thomas Starzl performed the first successful liver transplantation3,4.

In the years that followed, kidney transplantation became an accepted treatment for end-stage renal failure. Rejection of the donor graft by the immune system of the recipient, however, limited clinical success to transplantation of kidneys between identical twins5. In order to extend solid organ transplantation beyond identical twins, approaches to suppress the recipient’s immune system were pursued. Except for a few successful kidney transplantsations between non-identical twins, first attempts failed to defy the rejection process6,7. With the identification of the immunosuppressive effect of ciclosporin in 1972, a major breakthrough in this field was realized8. The efficacy of ciclosporin in preventing organ rejection was first demonstrated in liver transplants performed by Starzl9. Unequivocally, organ transplantation can be recognized as one of the true medical success stories of the 20th century. Over the past decades however, only moderate improvement of the quality of organs for transplantation was achieved10. Nevertheless, transplantation of solid organs has become a victim of its own success because the demand for donor organs has far exceeded its supply. Moreover, indications for transplantation have broadened substantially and the availability of donor organs could not be increased sufficiently to prevent the development of a severe discrepancy between supply and demand. The persistent organ shortage today necessitates employment of suboptimal donor organs for transplantation.
Donor types

Whereas the first clinical accomplishments in organ transplantation were achieved with living donors, the successful introduction of immunosuppressive drugs extended the application of solid organ transplantation to organs from deceased donors. Before the concept of brain death was introduced in the Harvard criteria in 1968, all deceased donor grafts were retrieved from donation after cardiac death. In hindsight, this can be considered as the predecessor of non-heart-beating donation.

Basically, for postmortem organ donation, three main donor types can be distinguished: the heart-beating donor, the non-heart-beating donor and the marginal donor (e.g. older donors, steatotic liver donors). The distinction between heart-beating and non-heart-beating donors lies in the criteria used to define death. For donation of heart-beating donors, criteria for brain-stem death are used, whereas cardiac criteria are applied for donation of non-heart-beating donors.

With the establishment of a legal definition for brain death, most transplant centers worldwide initiated programs based on organ retrieval from heart-beating, brain dead donors in order to avoid the complications associated with warm ischemic damage that organs from non-heart-beating donors by definition have sustained. In 1994, during the First Workshop on Non-Heart-Beating Donation in Maastricht, this donor category was reintroduced by Kootstra in an attempt to substantially enlarge the donor pool. In 2008, over 40% of all deceased organ donors in the Netherlands was associated with circulatory arrest and can therefore be classified as non-heart-beating donors. With increasing numbers of grafts sustaining warm ischaemic damage, maintenance of organ viability during preservation has once again become an important factor to preserve current high standards for functional outcome and long-term survival after transplantation. From this perspective, improvement of preservation methods is of vital importance.

History of organ preservation

Organ preservation is defined as the process starting from organ procurement to transplantation into the recipient. At the time transplantation of cadaveric kidneys had been introduced, extracorporeal preservation of the organ was not implemented. The donor graft was rushed from the donor to the recipient to keep the time of ischemia to a minimum in order to prevent perishing of the isolated organ from lack of blood and oxygen. Over the past four decades, the value of organ preservation has been become evident to the field of organ donation and transplantation. Without efficient preservation, it is impossible to maintain
organs viable for transplantation outside the body for the time needed to transport the graft between the center of procurement and the center where the transplantation can be performed.

In 1907, Carrel first described the use of hypothermia to preserve an artery for several days with a chilled perfusate. In cooperation with the aviator and inventor Charles Lindbergh, Carrel later perfused organs with large volumes of oxygenated plasma under normothermic conditions, thereby successfully maintaining tissue viability for several days. Not until the 1960s, significant progress in the area of organ preservation was achieved. In a preclinical study, Sir Roy Calne and David Pegg demonstrated that surface cooling and ice storage at temperatures between 0°C and 5°C could provide reliable protection for several hours. In clinical renal preservation, storage periods for up to 12 hours using surface cooling could thereafter be achieved successfully. The addition of a cold intravascular flush further improved the preservation quality of the grafts. In the same period, Starzl used the technique of extracorporeal perfusion to preserve canine kidneys and livers followed by successful transplantation. In the late 1960s, Belzer and Collins each published landmark papers describing advances in organ preservation which changed clinical transplantation of deceased kidneys from an emergency procedure to a semi-elective procedure. Belzer showed in 1967 that canine kidney preservation was possible for 72 hours by continuous hypothermic machine perfusion with cryoprecipitated plasma. In that same year, he successfully applied machine perfusion for the first time for the preservation of a human kidney. Cryoprecipitated plasma, derived from human plasma, became the standard perfusion solution for human kidneys. A disadvantage of this human product was its potential for spreading diseases. Therefore, the need for a synthetic perfusate was well recognized.

In 1969, Collins introduced a solution for cold static storage. With this new preservation solution he applied a brief washout of the graft followed by immersion in the solution and storage on melting ice for up to 30 hours. The unique feature of this solution was that it resembled the composition of the intracellular fluid instead of the fluid present extracellularly. During the ischemic preservation period, migration of intracellular components to the extracellular space was reduced and cell swelling prevented.

As a result of the success of this simple and inexpensive technique, the main focus of preservation research shifted from machine perfusion to cold storage. The achievements made by Collins accelerated research into improvement of solutions, culminating in development of the University of Wisconsin (UW) solution by Belzer. For more than two decades, the UW solution has been the accepted standard for preservation of heart-beating abdominal organs.

Initially introduced by Bretschneider in 1980 as a cardioplegic solution for open-heart surgery, the low-viscosity Histidine-Tryptophan-Ketoglutarate (HTK) solution has gained wide acceptance for the preservation of abdominal organs for transplantation. Throughout the years, HTK has been
applied with mixed success in kidney, liver and pancreas transplantation. Today, HTK is advocated as the solution of choice for preservation of non-heart-beating donor grafts due to its low viscosity which enables a more effective washout of blood remnants than the high viscosity UW solution. In 1994, Celsior solution, a low-viscosity extracellular solution with a high buffering capacity, was introduced for cold storage preservation of thoracic organs. Celsior proved to be as effective as UW in preserving abdominal organs thus creating a universal solution applicable for multiorgan procurement procedures. Despite this advantage, Celsior has not gained wide acceptance worldwide. Today, following positive results from preclinical and clinical studies, hypothermic machine perfusion is again gaining ground as the method of choice for the preservation of kidney grafts and its application for preservation of liver and pancreas grafts appears promising.

**Principles of organ preservation**

Organ preservation is the “supply line” for organ transplantation. It allows time to transport the organ from the donor hospital to the best tissue typed and matched recipient or to the patient in critical condition, desperately in need of an organ transplant. The principles of organ preservation methods currently applied are based on suppression of cell metabolism by hypothermia. When the temperature of the organ is reduced from 37°C to below 4°C, the cell metabolism is decreased to approximately 5% to 8%. To prepare the organ for hypothermia, blood is washed out and replaced with a preservation solution designated for hypothermic storage. The composition of the solution appears to be a critical determinant for the tolerance of the organ to hypothermic storage. Belzer suggested that the solution used for washout and preservation should counteract the detrimental side effects of hypothermia. Cell swelling, interstitial edema, acidosis and the production of free radical oxygen species should be kept to a minimum. **Cell swelling** during hypothermia is caused by impairment of the activity of the sodium pumps (Na⁺/K⁺ ATPase), which normally maintain a high sodium and low potassium concentration in the extracellular compartment. As a result, sodium enters passively into the cell, thereby creating a hyperosmolar intracellular environment leading to influx of water. The resulting phenomenon of cell swelling can be counteracted by adding impermeants to the preservation solution. Extravasation of the solution during washout, creating an expansion of the interstitial space, can compress the capillary system and may lead to an uneven distribution of the washout solution throughout the organ. **Interstitial edema** can be prevented by incorporation of a colloid into the solution, which allows free exchange of constituents of the washout solution without expansion of the interstitial space. Prevention of **intracellular acidosis** is also
a prerequisite for maintaining the viability of the organ during the preservation period. As metabolic activity is present, although reduced, at temperatures between 0°C and 5°C, anaerobic cellular metabolism perseveres. As a result, the cellular adenosine triphosphate (ATP) content is rapidly depleted, accompanied by formation of lactic acid and hydrogen ions. Severe acidosis activates phospholipases and proteases causing lysosomal damage and eventually cell death. Therefore, potent buffers to prevent acidosis are essential components of preservation solutions.

Upon reperfusion, the accumulation of metabolic waste products including breakdown products of ATP lead to the production of toxic substances and reactive oxygen species in particular. To minimize the detrimental effects of reactive oxygen species, antioxidants or free radical scavengers are important components. Also, at reperfusion, rapid regeneration of Na+ pump activity is necessary, as well as other energy-requiring metabolic processes that require ATP. Therefore, Belzer suggested that energy substrates (ATP precursors) are essential components for successful organ preservation.

Finally, in the development of preservation solutions, the electrolyte composition is an important topic. During the pioneering years in organ preservation, an intracellular type solution with high potassium and low sodium content was assumed necessary to prevent cell swelling. It was hypothesized that due to the inactivity of Na+/K+ ATPase enzymes during hypothermia, an intracellular sodium/potassium ratio in the extracellular fluid compartment could prevent sodium from entering into the cell. Intracellular type solutions like Euro-Collins, HTK and UW were long considered to be pivotal for preservation of cell and organ viability. Recent studies, however, have shown equal or better preservation quality using extracellular type solutions with a low potassium / high sodium ratio, such as Celsior and Institut Georges Lopez-1 solution (IGL-1). A low potassium content is known to facilitate the washout of blood remnants during procurement by prevention of potassium induced vasospasm.

Organ preservation methods

In current clinical practice, most organs are preserved by cold static storage prior to transplantation. However, since more suboptimal donor organs are employed, which require a more advanced preservation for maintaining viability, machine perfusion has regained clinical interest. Cold storage commences with a thorough washout of the vascular bed with cold preservation solution to allow rapid cooling of the organ, removal of blood remnants and equilibration of the preservation solution with the organ tissue. Subsequently, the organ immersed in cold preservation solution, is placed on melting ice until transplantation. Cold static storage is the preferred preservation method in most centers as it is less expensive and cumbersome than preservation using machine perfusion. However, the inability to remove metabolic waste products accumulating during...
cold storage as well as the inability to perform viability assessment prior to transplantation is considered to severely limit this technique. **Machine perfusion** is based on the principle of preserving the organ in a ‘better environment’. The perfusate is circulated by a pumping device generating a continuous or a pulsatile flow. Machine perfusion can provide a continuous supply of nutrients while metabolic waste products produced during cold storage can be removed from the organ and thereafter filtered from the recirculated perfusate. Furthermore, the possibility to provide oxygen to the organ through oxygenation of the perfusate is an important feature of this technique. Moreover, graft function may be predicted before transplantation based on parameters of graft perfusion characteristics and analysis of the perfusate.

**Organ preservation solutions**

Over the last decades, several preservation solutions have been developed with mixed success. Nowadays, in the vast majority of organ procurement procedures within the Eurotransplant region, the UW and HTK cold storage solutions are employed. The **University of Wisconsin solution** (UW) uses metabolic inert substrates such as lactobionic acid and raffinose to suppress hypothermia induced cell swelling. Lactobionate acts also as a chelator of calcium and iron, thereby reducing oxidative injury in cold stored grafts. It is suggested that lactobionate could be the key component in the UW solution. Other studies provided evidence that the colloid, hydroxyethyl-starch (HES), is the main component in UW. This high molecular weight molecule provides the required oncotic pressure to prevent extravasation of the perfusion solution which can result in interstitial edema and cell swelling. Other important constituents of UW include adenosine and glutathione. Adenosine was shown to effectively stimulate ATP synthesis in hypothermic preservation of kidney and liver. Glutathione and allopurinol were incorporated for their antioxidative capacity and have been shown to be beneficial in liver and kidney preservation. In UW, the buffer potassium phosphate is used to prevent acidosis.

**Histidine-Tryptophan-Ketoglutarate solution** (HTK), which was originally designed as a cardioplegic solution, consists of a large concentration of histidine, a potent buffer, combined with two amino acids. Tryptophan serves as membrane stabilizer while ketoglutarate acts as a substrate for anaerobic metabolism during preservation. HTK has a low viscosity, which allows for an effective washout of the microcirculation. HTK is considered to be an intracellular solution with an electrolyte concentration similar to the intracellular electrolytes ratio, i.e. low concentrations of sodium, calcium and magnesium. To date, only a single prospective multi-center randomized trial comparing the efficacy of UW solution and HTK in kidney transplantation has been...
described. Delayed graft function and 3-year graft survival did not differ between the two solutions\textsuperscript{59}. However, a recent retrospective multi-center study, comparing deceased donor kidney transplants preserved either with HTK or UW, showed that HTK preservation was independently associated with a 20\% increased risk of graft loss\textsuperscript{60}.

In 1983, Belzer and co-workers described their modification of the UW cold storage solution for application during machine perfusion preservation, which was introduced as the **Belzer Machine Perfusion solution** (Belzer MPS, currently known as KPS-1)\textsuperscript{61}. The composition of the perfusion solution is similar to that of UW cold storage except for the osmotically active impermeant gluconate instead of lactobionate, impermeants which prevent tissue swelling and the obstruction of blood flow. Lactobionate appeared to be ineffective when used for continuous machine perfusion for reasons that remain unclear. Belzer MPS was developed over 30 years ago and has not been modified since. Although the high molecular weight colloid hydroxyethyl-starch is regarded the main component, the high viscosity of UW solution is also considered a drawback; it prolongs the duration of perfusion while compromising the microcirculation\textsuperscript{62}. The high potassium level may cause vasoconstriction and may contribute to the hyperaggregation of hydroxylethyl-starch\textsuperscript{63,64}.

### Polysol

In 2002, during the development of a novel machine perfusion system, the need for improvement of the only commercially available perfusion preservation solution, Belzer MPS, emerged. In the years that followed, promising preliminary data was obtained with experimental machine perfusion preservation studies of rat and pig livers\textsuperscript{65-70}. Other groups also presented encouraging results with cold storage preservation studies of the rat liver and small bowel using Polysol compared with UW, HTK and Celsior\textsuperscript{71,72}.

In Polysol, the colloid polyethylene glycol (PEG)\textsuperscript{73-75} is used to prevent the disadvantages associated with HES, present in UW and Belzer MPS. Also, to maintain a near physiological oncotic pressure, negatively charged high molecular weight impermeants are incorporated. The colloid and impermeants present in Polysol did not affect the inherent viscosity of the solution to large extent. By using PEG, the Achilles heel of the Belzer MPS and UW solutions, the high viscosity and hyperaggregating effect on red blood cells, could be circumvented (Table 1).

The viscosity of HTK and Polysol is comparable. However, the absence of a colloid in HTK solution promotes extravasation of fluid into the interstitial space leading to interstitial edema and cell swelling and can thereby impair washout and cooling of the graft. Acknowledging the detrimental effect of acidosis, increasing with the length of preservation, Polysol contains a potent buffer system, comprising of HEPES, histidine and phosphate.
buffers. Also, to combat the, over time, increasing formation of reactive oxygen species, Polysol consists of the antioxidants glutathione, selenium, vitamin C and vitamin E.

Polysol has an extracellular type electrolyte composition i.e. a low potassium and high sodium concentration. This potassium/sodium ratio is known to limit the entry of calcium into the cell, thereby preventing calcium overload and avoiding a depolarization of smooth muscular cell membrane leading to vasoconstriction. This limitation of vasoconstriction favours a more homogeneous diffusion of the solution within the organ. Moreover, extracellular-type solutions maintain an equilibrium between the extracellular and intracellular compartment to prevent osmotic shifts and cell swelling\textsuperscript{76,77}.

Polysol was originally based on tissue culture media, which are known for their ability to maintain viability of isolated cells and tissue \textit{in vitro}. In contrast to the currently available preservation solutions, Polysol contains a variation of essential and non-essential amino acids. To date, fundamental research into the potential benefits of these substances has not been performed. Therefore, the individual value and interaction between the 60 constituents of Polysol remain unknown.

Following the promising preliminary data, the use of Polysol as cold storage as well as machine perfusion solution was evaluated in porcine autotransplantation models as described in this thesis. The pig is a model that is both rigorous (a lower tolerance of preservation injury than the human) and clinically relevant (pig organs are similar in size and anatomy to the human)\textsuperscript{46}. Thus, technology developed in the pig is likely to be suitable for clinical use\textsuperscript{78}. 
Outline of the thesis

Part I Preclinical cold storage preservation studies

The quality of organ preservation is generally considered to be a major determinant of graft function and survival. After retrieval from the donor, the temperature of the graft must be reduced as rapidly as possible washed out of blood remnants in order to prevent occlusion of the vascular bed before cold storage. Chapter 2 describes the effect of different preservation solutions on the washout of kidney grafts. Cooling capacity, kidney weight alteration, remaining blood remnants and histological evaluation after an ex vivo washout using 500 mL cold preservation solution at 4°C followed by 24 hours cold storage (CS) are discussed. Polysol, a new low viscosity preservation solution, was compared with the clinical standard for the preservation of heart-beating kidney grafts, the University of Wisconsin solution (UW) and Histidine-Tryptophane-Ketoglutarate solution (HTK), the clinical standard for CS of warm ischaemia-damaged kidney grafts. In Chapter 3, Polysol is compared with the UW solution for CS preservation of heart-beating kidney grafts. To this end, a porcine autotransplantation model was used to compare the effect of the solutions on the preservation quality. Chapter 4 describes the comparison between Polysol and HTK solution for the preservation of warm ischaemia-damaged kidney grafts. Using a porcine autotransplantation model, renal function and structural integrity of the grafts were assessed.

Part II Preclinical perfusion preservation studies

As machine perfusion has regained interest as a preservation method for kidney grafts, in this part, preclinical studies of the preservation of kidney grafts using machine perfusion are described. Also, after a pause of almost four decades, interest has recently been renewed in machine perfusion of the liver. In chapter 5, the literature is reviewed, describing experimental and clinical hypothermic machine perfusion studies of the liver. The techniques of machine perfusion, continuous or pulsatile flow, are discussed as well as the effects of oxygenation. In addition, developments in machine perfusion systems and machine perfusion solutions are described. Chapter 6 describes the effect of the perfusion pressure on the preservation quality of kidney grafts. Using a novel machine perfusion system for hypothermic oxygenated pulsatile perfusion, a mean perfusion pressure of 30 mmHg was compared with a mean perfusion pressure of 25 mmHg. The impact of the pressure applied during machine perfusion was assessed by using perfusion parameters, renal microcirculation, renal function and histological analysis.
In **chapter 7**, perfusion preservation of kidney grafts using the new machine perfusion system in combination with Polysol is compared with cold storage preservation using either UW or Polysol. To this end, parameters of renal microcirculation, renal function and structural integrity of heart-beating porcine kidney grafts using an autotransplantation model were compared.

**Chapter 8** describes hypothermic pulsatile perfusion preservation of warm ischaemia-damaged porcine kidney grafts using Polysol. Renal function and histological analysis of warm ischaemia-damaged grafts preserved using machine perfusion were compared with CS with either HTK or Polysol and non-ischaemic controls.

**Chapter 9** provides a summary of the chapters in this thesis as well as a discussion of the results.
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UW, University of Wisconsin solution, HTK, Histidine-Tryptophan-Ketoglutarate, PS, Polysol, cP, centi-Poise.
*The following amino acids are present in Polysol: alanine, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine.
†The following vitamins are present in Polysol: ascorbic acid, biotin, Ca-pantothenate, choline chloride, inositol, ergocalciferol, folic acid, menadione, nicotinamide, nicotinic acid, pyridoxal, riboflavin, thiamine, vitamin A, vitamin B12 and vitamin E.
References


