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Chapter 10

Cold Ischaemic Injury in Kidney Transplantation

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Additional information is available at the end of the chapter

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1. Introduction

Kidney transplantation is considered the best treatment for end stage renal failure (ESRF) with longer life expectancy and superior quality of life compared to dialysis therapy [1-3]. However, a major constraint to transplantation is the lack of suitable organ donors. To increase the number of available organs there has been an incentive to use 'marginal' donors such as donation after cardiac death (DCD) and expanded criteria donors (ECD), in addition to kidneys from the traditional living and deceased donors [4,5]. Although an important source of organs for transplantation, once transplanted a significant proportion of these kidneys have early graft dysfunction.

There are many attributing factors that influence the outcome of the transplanted graft. Donor and recipient age, creatinine clearance, history of hypertension, poor human leukocyte antigen (HLA) matching, cause of death, ethnicity, the cold ischaemic (CI) time and in the case of DCD donors the warm ischaemic insult have all been described as major determinants of graft function and graft survival [6]. The CI time is perhaps the only modifiable factor that significantly affects graft outcome.

Since the 1970s organ preservation has relied on hypothermic conditions to allow an organ to be preserved outside the body from the time of retrieval until transplantation. This allows the organ to be allocated nationally, to the most suitable and immunologically matched recipient. Nonetheless, hypothermic preservation has its limitations and viability cannot be sustained for an indefinite period of time. Hypothermic preservation has been described as 'a compromise between the benefits and detriments of cooling' [7].

2. Standard criteria donor (SCD)

Deceased organ donors fall into three categories. A standard criteria donor is a deceased donor who is declared brain dead after a stroke or other brain injury. Brain death means that there is the irreversible loss of function of the brain.

3. Donation after cardiac death (DCD) donor

Donation after cardiac death donors (DCD) are donors from which the organs are retrieved after the cessation of circulation due to a cardiac arrest. These organs are regarded as marginal organs due to the warm ischaemic (WI) insult that they receive before the onset of preservation. This WI interval causes a degree of injury that can lead to irreversible damage, resulting in an unfavourable outcome after transplantation. Four classifications of DCD donors have been categorised depending on the circumstances of death and when the organs are retrieved [8,9] (Table 1).

Category	Definition	Type
1	Dead on arrival	Uncontrolled
2	Unsuccessful resuscitation	Uncontrolled
3	Awaiting cardiac arrest	Controlled
4	Cardiac arrest while brain death	Controlled/uncontrolled

Table 1. Maastricht categories of donation after cardiac death donors.

Maastricht type 1 and 2 donors are patients who have died suddenly from a cardiac event or trauma and therefore are usually based in the Accident & Emergency department. After a failed resuscitation, the patient is pronounced dead and a 5 minute 'hands off' period allowed to lapse. The organs are perfused *in-situ* through aortic cannulas inserted through the femoral artery [10].

Maastricht type 3 and 4 are patients who are based on an intensive care unit after a severe brain injury. The patient does not meet the criteria for brain stem death and will maintain spontaneous ventilation. Under controlled conditions with no possibility of recovery withdrawal of treatment is planned. After the cessation of the heartbeat the patient is transferred to the operating theatre and the kidneys retrieved after *in-situ* cooling. In the uncontrolled situation an unexpected cardiac arrest follows brain stem death. The WI time is usually within the region of 15 minutes for controlled donors but can be considerably longer in the uncontrolled situation.

4. Expanded criteria donors (ECD)

Expanded criteria donors (ECD) are defined as any brain dead donor aged ≥ 60 years or over 50 years with ≥ 2 of the following conditions; Hypertension, terminal serum creatinine equal or greater than 132μmol/L or death resulting from an intracranial haemorrhage.

5. Cold ischaemic injury

Hypothermic preservation is based on the principle that cooling an organ inhibits the enzymatic processes. There is a 2-3 fold decrease in metabolism for every 10°C reduction in temperature [11,12]. This slows the depletion of adenosine triphosphate (ATP) and also inhibits the degrading processes (phospholipid hydrolysis). Nonetheless, under

hypothermic conditions the metabolic rate remains at about 10% and therefore over time, the hypoxic conditions cause substantial injury [12] this is termed CI injury.

The depletion of ATP due to the inhibition of oxidative metabolism increases levels of adenosine, inosine and hypoxanthine within the cell leading to the formation of lactic acid [13]. This lowers the intracellular pH causing lysosomal instability and the activation of lytic enzymes [14,15]. The depletion of ATP also reduces a large number of cellular processes. Inactivation of the Na+/K+ ATPase pump allows the accumulation of calcium, sodium and water within the cell causing cellular swelling [15]. The binding of transition metals such as iron to their carrier proteins (transferrin, ferritin) is also inhibited which increases the intracellular concentration of free iron [16,17]. This is a strong catalyst for the generation of oxygen free radicals which promotes the production of other free radicals [14]. The impact of CI injury is evident immediately after transplantation when oxygenated blood is re-introduced into the kidney. The downstream effects of ischaemia reperfusion (I/R) injury results in tubular and vascular damage with the impairment of blood flow to the kidney and reduced urine output after transplantation. The kidney can withstand CI times up to 48 hours. Nonetheless, attempts have been made to reduce CI injury and on average the CI time now falls below 24 hours in most transplant centres.

6. Impact

6.1. Delayed graft function

Renal graft function after transplantation is typically measured as incidence of delayed graft function (DGF). There are several definitions of DGF however the majority of centres define DGF as the requirement for dialysis within the first week after transplantation. The diagnosis is based on low urine output, slow decline in serum creatinine levels and increased metabolic instability. Acute tubular injury, otherwise termed acute tubular necrosis (ATN) caused by ischaemic injury is the main cause of DGF after transplantation [18]. DGF is associated with complications such as acute rejection, increased fibrosis and the risk of poorer long term graft survival. It also has a significant economic cost, can complicate patient treatment and prolong hospital stay [19]. Rates of DGF typically range from 5 to 40% in deceased donor kidney transplants [20]. Rates of DGF in live donor transplantation are significantly less (2-5%) due to the short CI time and healthy younger donors [21].

Many experimental studies have shown that the duration of CI directly influences graft function. Several studies suggest that even after 6 hours of CI, significant injury occurs [22,23]. Clinically, the CI time has been clearly shown as an independent risk factor for DGF and reducing the CI time can reduce the incidence of DGF. In an analysis of a series of DBD transplants the risk of DGF was found to increase by 23% for every 6 hours of CI [24] and Locke *et al* found that limiting the CI time to less than 12 hours reduced the risk of DGF by 15% [25]. Other studies have shown that the risk of DGF is increased by 3.3 and 4.4 fold by increasing the CI time by 5 and 10 hours [26].

6.2. Graft survival

The CI time is regarded as an independent risk factor for DGF and DGF is associated with reduced graft survival [27,28]. However, recent evidence suggests that the association of CI time and DGF may have less of an impact on graft survival than previously thought. A multicentre analysis of kidney preservation found that only when the preservation period exceeded 18 hours was the CI time associated with reduced graft survival [29]. A large analysis of registry data of paired deceased donor kidneys found that DGF induced by CI injury had a limited impact on the long term outcome. Nonetheless, in other studies the CI time has been found to independently influence graft survival even in live donor transplantation and in young deceased donors [30,31].

The disparity between DGF and survival is perhaps due to the lack of sensitivity of DGF in determining the severity of kidney injury. DGF is a simple and standard method of reporting early graft dysfunction. However, dialysis within the first week after transplantation can be used to correct metabolic instability without the presence of significant kidney injury. As such, it is difficult to determine the impact of DGF. DGF due to CI can be reversible and therefore have no effect on long term outcome [32]. However, in severe cases, DGF can lead to incomplete recovery and reduced graft survival due to the loss of nephron mass [33]. Giral-Classe *et al* reported that rather than the incidence of DGF, the duration of DGF was the important factor with DGF over six days associated with reduced long term graft survival [34]. More recently, urinary biomarkers have been used to determine the severity of acute kidney injury with cystatin C, neutrophil gelatinase-associated lipocalin (NGAL), interleukin-18 (IL-18] and kidney injury molecule-1 (KIM-1) emerging as the most reliable and sensitive markers of injury [35-37]. Although, not readily used as a diagnostic tool in clinical practice, they may be applied more frequently in the future.

7. Acute rejection

Acute rejection (AR) following renal transplantation can be split into two categories, cell mediated rejection and antibody mediated rejection (also termed vascular rejection). Acute cellular rejection is the more common of the two types and with the introduction of modern immunosuppressive agents rates have dropped from 50% a decade ago to 15-20% today. The typical stimulus for cellular rejection is the presence of so-called 'passenger leucocytes' which are immune cells carried within the blood vessels and tissues of the donor organ. Following transplantation they are exposed to the recipient immune system which recognises them as foreign and results in activation of host lymphocytes which attack the donor kidney. Antibody mediated rejection is less common and usually more severe and if left untreated can rapidly destroy the graft.

Acute rejection is an important factor in early outcomes of transplantation and is closely associated with delayed graft function (DGF) [38-41]. The precise link between DGF, acute rejection and CI time is difficult to fully elucidate. Prolonged CI has been shown to be one of the main risk factors for DGF and DGF is an independent risk factor for AR [42]. However, DGF is a result of a number of factors and it is over simplistic to ascribe acute rejection to

just one of those factors. Nonetheless there is evidence that the CI time, alongside other factors, including duration of dialysis, number of HLA mismatches, panel reactive antibodies more than 5% are independent predictors of AR. A large retrospective analysis of 611 transplants demonstrated that CI time was the strongest predictor of DGF [42]. The risk of DGF increased from 9.6% with 12 hours CI time to 21.5% with 24 hours CI time. In the same analysis the risk of AR was increased by 4% for each additional hour of CI time and the risk of rejection in patients receiving kidneys with less than 24 hours CI time was 14.1% compared to 29.3% in kidneys with greater than 24 hours CI time. Furthermore, death-censored graft survival is significantly reduced in patients in whom AR complicates DGF. In addition CI duration of greater than 24 hours has a significantly reduced death-censored graft survival in comparison with durations of less than 24 hours [42].

8. Donor specific effects

Kidneys from DCD and ECD donors commonly present with high rates of DGF compared to SCD and live donors. [43]. DGF typically ranges from 22% to 84% in DCD kidneys compared to 14% to 40% in DBD donors [25, 44-47]. Evidence suggests that the outcome of kidneys from uncontrolled DCDs is poorer when compared to the controlled DCDs with significantly higher rates of DGF, as a response to the longer duration of warm ischaemic (WI) injury under the uncontrolled situation [48].

Kidneys from ECD have a 70% increased risk of graft loss and higher rates of DGF [25,49,50]. The prognosis is even poorer in DCD kidneys from older donors (over 50 years) with the risk of graft failure rising to 80% [25].

In addition to DGF, a small but significant proportion of kidneys from DCD donors also have primary non function (PNF) with rates reported to range from 4 to 19% amongst transplant centres over the last 30 years [51,52]. PNF is particularly detrimental as the patient is exposed to surgery and immunosuppressive therapies without benefit. Furthermore, they may become sensitized to donor antigens, reducing the opportunity for future transplants.

The WI insult in DCD kidneys and the reduced capacity of kidneys from ECDs to recover and regenerate are certainly major contributing factors for early graft dysfunction. Experimental evidence suggests that the combined effect of WI and CI injury exacerbates the injury during reperfusion and the duration of CI has been found to have a strong influence on graft outcome [53]. However, the impact of CI in clinical transplantation is again varied. It appears that as in SCDs, long term graft survival is not necessarily affected by DGF and CI not necessarily an independent predictor of graft survival. Recent evidence from clinical DCD and DBD programmes have reported similar rates of graft survival after 5 and 10 years [45,54-57]. In a series of 112 uncontrolled DCD kidneys, DGF rates were 84% compared to 22% in DBD donors [54]. Nevertheless, the graft survival rates were similar in both groups of patients, 69.3% versus 75.5% at 5 years and 50.3% versus 57.9% at 10 years, respectively. The link between WI, CI and graft survival is not well documented. However, it appears that prolonged CI after a period WI may not be as detrimental to graft survival as previously thought and that kidneys can recover from ischaemic injury with no long term effects [58].

9. Preservation techniques

Organ preservation was first introduced into clinical transplantation in the 1960s. Until this time without proper preservation conditions, kidneys were transplanted as soon as possible after retrieval to minimize the injury. It was then recognized that in order to improve the outcome of transplantation, better methods of preservation were required. Experimental studies in the 1950s by Lapchinsky [59] in the Soviet Union and the early work by Carrel and Lindbergh, showed that ischaemic injury could be minimized by reducing the temperature [60]. In 1963, Calne *et al* used the concept of hypothermic temperatures to extend the preservation time and successfully transplant canine kidneys after 12 hours of storage [61]. This led to the application and development of preservation techniques and solutions that are used today.

10. Static cold storage

Static cold storage (CS) is undoubtedly the simplest and most widely utilised method of hypothermic preservation. The kidney is flushed with cold preservation solution to remove the blood and cool the organ. The kidney is then stored in solution surrounded by crushed ice. Preservation solutions have been designed to counteract the detrimental effects of CI injury. There are a number of commercially available preservation solution, which all contain the same basic formula. This includes an impermeant to minimise swelling and provide stability to the ultra-structure of the cell. A buffer and a balanced electrolyte composition with either a high or low Na+ / K+ ratio to prevent the build up of intracellular acidosis and further minimize cellular swelling (Table 2). Solutions with a high potassium concentration are classified as intracellular and those with a high sodium concentration extracellular solutions.

Components	
Impermeants	glucose, lactobionate, mannitol, raffinose, sucrose
Colloid	hydroxyethyl starch (HES), polyethylene glycol (PEG)
Buffers	citrate, histidine, phosphate
Electrolytes	calcium, chloride, magnesium, magnesium sulphate, potassium, sodium
Anti-oxidants	allopurinol, glutathione, mannitol, trytophan
Additives	adenosine, glutamic acid, ketoglutarate

Table 2. Components commonly used in preservation solutions

11. Static cold storage solutions

11.1. Euro Collins

In 1969 Geoffrey Collins developed the first acellular preservation solution (Collins solution) containing a high concentration of potassium and glucose [62]. Collins solution was later modified omitting some of the ingredients such as magnesium, heparin, procain and replacing glucose with mannitol to provide better osmotic properties and lower the viscosity [63-65]. It was renamed Euro Collins solution and was widely used amongst the transplant community.

11.2. Hyperosmolar citrate

Hyperosmolar citrate (HOC) or more commonly known as Soltran or Marshall's solution was first developed in the 1970s as an alternative to Collins solution [66,67]. It is has a high potassium content and contains basic ingredients using citrate as a buffer. Its hypertonicity is designed to prevent fluid entry into cells. It is a relatively inexpensive, non-viscose solution that is still commonly used throughout the UK in kidney transplantation. It is not recommended for DCD or marginal kidneys despite the fact that there is little evidence to support this view.

12. University of Wisconsin solution

University of Wisconsin (UW) solution has a high potassium concentration to maintain the intracellular ionic balance. It is a more complex preservation solution compared to Euro Collin and HOC, containing trisaccharide raffinose and the anion lactobionate as osmotic impermeants, a phosphate buffer, anti-oxidants (glutathione) to scavenge oxygen free radicals, allopurinol to block the activity of xanthine oxidase and adenosine, an ATP precursor. It also contains the colloid hydroxyethyl starch (HES), to prevent cellular swelling [68]. However, it is debatable whether this is it necessary in a static storage solution and there is some evidence showing that HES can increase tubular damage and cause red blood cell aggregation. Another potential disadvantage of UW solution is the high concentrations of potassium. Although thought important in the prevention of the build up of intracellular calcium, potassium can induce cellular depolarization, reduce cellular 5'-triphosphate content and activate voltage-dependent channels, such as calcium channels [69]. Nonetheless, due to its composition UW solution had, and still has, a significant advantage over other preservation solutions enabling kidneys to be stored for longer periods with better function and less histological injury after transplantation. It is still considered the 'gold standard' preservation solution today.

13. Histidine-Tryptophan-Ketoglutarate (HTK)

HTK was originally developed as a cardioplegic solution but because of its low viscosity was quickly adopted for clinical preservation of the kidney, pancreas and liver [70-72]. It is an extracellular solution and uses the impermeant mannitol and histidine as a buffer. It also contains 2 amino acids, tryptophan, to stabilize cellular membranes and prevent oxidant damage and ketoglutararate, a substrate to support anaerobic metabolism. Recent concerns have been raised regarding its use for ECD and DCD kidneys or for kidneys with prolonged storage times [73]. Some clinical studies have associated its use with the increased risk of PNF and early graft loss [74]. Nonetheless, it is a popular preservation solution widely used throughout Europe and the UK.

14. Celsior solution

Celsior is an extracellular solution and was initially designed for heart transplantation. It contains a high sodium concentration with histidine as a buffer, lactobionate and mannitol

to prevent oedema and glutathione as an antioxidant. The solution has proved beneficial in heart, liver, pancreas and in kidney transplantation [75-78].

15. Outcome

An abundance of experimental studies have investigated the efficacy of one solution over another with the majority of studies labelling UW solution as the most superior. However, clinically the evidence is sparse. UW, HTK and Celsior appear to be the better preservation solutions with little difference in rates of DGF between the solutions its usage. Euro Collin solution is not widely used and is regarded as inferior with the suggestion of increasing the risk of DGF [79]. The outcome of individual preservation solutions is more apparent when the CI time is extended beyond 24 hours with UW fairing significantly better than other solutions.

16. Hypothermic machine perfusion

Since the introduction of CS techniques in the 1970s there has been much debate about whether CS or hypothermic machine perfusion (HMP) is the best method of kidney preservation. Undoubtedly, the simplicity of CS has a significant advantage over HMP. However, HMP is it thought to be a better method of preservation in that it allows a continual flush of the microcirculation, prevents the accumulation of waste products, sustains a higher metabolic rate, protects against depolarization of the endothelial cell membrane and reduces free radical formation [80].

Folkert O Belzer was the first to develop a portable HMP system [81,82] in the 1960s. However, with the introduction and success of CS in the 1970s there was little development of this technique in subsequent decades. Nonetheless, with the increasing use of DCD and ECD kidneys over the last decade, there has been renewed interest into the use of HMP. New simpler and portable systems have been developed such as the Lifeport Kidney Transporter (Organ Recovery System, US) which has encouraged the use of this technology. Many experimental studies have found HMP to improve preservation [7,12] and the quality of the kidney. The largest multicentre clinical trial conducted in Europe comparing CS and HMP in deceased donors found that HMP reduced the risk of DGF compared to CS (adjusted odds ratio, 0.57; P=0.01] and improved 1 and 3 year graft survival [83,84]. Although the overall rate of DGF was only reduced by 6%.

The evidence suggests that HMP may be more beneficial in reducing DGF rates in marginal kidneys. In a sub-analysis of 82 pairs of DCD kidneys from the European trial, the DGF rate in the HMP group was 53.7% compared to 69.5% in kidneys that were statically stored [85]. However, there was no significant difference in graft survival at 1 or 3 years. In a further sub-analysis of ECD donors in this trial, HMP reduced rates of DGF from 29.7% to 22% and also improved 1 and 3 year graft survival in ECD kidneys [84,86]. In contrast to this support for HMP, a multicentre UK trial found no beneficial effects of HMP. 45 pairs of controlled DCD kidneys were randomized to HMP or CS [87]. The DGF rates were 58% vs 56% in the HMP and CS groups respectively. However, this trial has been criticised for the sequential design and the small number of patients [88].

HMP techniques are still open to criticism with the suggestion of increased endothelial injury, as found in a recent study of porcine livers [89], risk of trauma to the vessels and the question of cost effectiveness compared to static storage techniques [90]. Nonetheless, it appears that HMP may hold a significant advantage in reducing CI injury compared to CS techniques. The experimental evidence is strong and there is a growing abundance of evidence from clinical studies to suggest an advantage. However, the evidence is not conclusive and there is a need for more clinical trials to determine the superior method of preservation.

17. Normothermic machine perfusion

Maintaining an organ under normothermic conditions is an alternative technique of preservation. Continuous perfusion of the kidney at warmer temperatures with the delivery of nutrients and oxygen has the advantage of avoiding hypothermic injury and hypoxia. In addition, it also may aid recovery and prevent further injury.

Early attempts at normothermic preservation were generally unsuccessful due to the inability to maintain cellular integrity and support renal metabolism. However, advances have been made over the last few decades with the use of technology borrowed from cardiac surgery. The development of less traumatic perfusion pumps and the recognition of the necessity for the delivery of nutrients and oxygen to achieve successful perfusion has made normothermic preservation a realistic contender in clinical transplantation.

Normothermic perfusion can be applied in various ways. The concept of extracorporeal membrane oxygenation (ECMO) to maintain extracorporeal circulation at normal room or body temperature with hyperoxygenated blood can be used to maintain tissue perfusion after the heart has stopped. Normothermic recirculation has proved beneficial in the retrieval of hearts, lungs and abdominal organs. Valero *et al* assessed the effects of implementing this technique in clinical practice in small group of DCD donors [91]. Circulation was maintained for 60 minutes before total body cooling. The incidence of DGF and PNF was reduced after normothermic recirculation compared to standard *in situ* or total body cooling. Gravel *et al* described a DGF rate of 11% in controlled DCD donors [92] and Lee *et al* found similar 5 year graft survival rates to DBD and living donors [93]. Maintaining circulation before retrieval is thought to condition the organs by up-regulating adenosine receptors which may protect against preservation injury [91]. Reznik *et al*, recently reported the application of extracorporeal normothermic recirculation in uncontrolled DCD donors using leukocyte depleted blood [94,95]. Initial graft function was achieved in 6 out of the 16 patients. In the kidney, more evidence is needed to determine how normothermic recirculation before retrieval correlates with early and longer term graft function.

In consideration of the logistical problems of prolonged preservation a great deal of research has focused on using normothermic preservation in combination with hypothermic techniques. Experimentally, intermediate periods of normothermic preservation have been used to restored energy metabolism with replenishment of adenosine levels, effectively

'resuscitating' the organ and retaining viability compared to kidneys stored under hypothermic conditions [96,97].

Brasile *et al* found that a period of warm *ex-vivo* perfusion at the end of the preservation period could resuscitate the kidney after warm and cold ischaemic injury [98,99]. More prolonged normothermic preservation periods have also been more beneficial than hypothermic techniques [100,101]. The only report of a normothermic kidney perfusion technique in clinical practice is by Hosgood and Nicholson [102]. In this single case report of a short period of normothermic perfusion of a marginal kidney with an oxygenated packed red blood cell based solution, the recipient had immediate graft function compared to DGF in the recipient of the paired CS kidney. Further results of the ongoing series at Leicester are awaited. Nonetheless, despite the potential benefits, normothermic preservation is logistically difficult to carry out requiring technical support and expensive perfusion systems.

18. Biomarkers

Measuring the amount of ischaemic injury during preservation would be advantageous as the quality of the kidney could be assessed and a decision made upon its viability. This would be particularly beneficial for marginal kidneys to reduce the likelihood of PNF. Viability is normally assessed by numerous factors including donor history, duration of cardiac arrest, the quality of in-situ perfusion, CI interval and visual inspection of the kidney. Ultimately this relies on the judgement of an experienced surgeon. To avoid PNF, surgeons are typically cautious and therefore many kidneys are deemed unsuitable for transplantation and are discarded [57]. HMP has been used to assess viability. Two aspects can be measured; Firstly, the continuous recirculation of preservation solution through the kidney allows the perfusate flow to be measured and intra-renal resistance can be calculated. Secondly, the perfusate can be sampled to measure cellular injury.

Clinically, the perfusion flow index (PFI) has been used as a measure of flow and resistance [103,104]. This is based on a minimum flow being obtained for a given pressure. The Transplant Group at Newcastle, UK recommend that a PFI of greater than 0.6ml/min/mmHg/100 gram of kidney is needed for a kidney to be deemed suitable for transplantation [105]. However, the ability of these parameters to predict DGF or PNF in clinical practice is limited. Jochman *et al* recently reported that although renal resistance (RR) at the end of HMP was an independent risk factor for DGF and 1 year graft survival, it had a low predictive power and could not be relied on as a sole measure of viability [106]. This is in agreement with other small clinical studies by Sonnenday [107] and Guarrera [108] *et al* that showed that kidneys with poor perfusion parameters had a similar outcome to those with good parameters.

Viability can also be measured by sampling the perfusate for biomarkers of cellular injury. Markers such as redox free iron, glutathione S-transferase (GST), total glutathione S-transferase (tGST), lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAG), heart-type fatty acid binding protein (H-FABP) and alanine aminopeptidase (Ala-AP) have

all been used to determine injury [104-106,109]. There is little information on their predictive value. However, Jochman *et al* recently published the results from the European HMP trial in which perfusate samples were taken for the assessment of biomarkers at the end of HMP [106]. GST, NAG, and H-FABP were found to be independent predictors for DGF but not for graft survival in the first year after transplantation. LDH, ASAT, and Ala-AP were found to have no predictive potential for post transplant outcome. Furthermore, the biomarkers did not correlate with intra renal resistance. The evidence suggests that viability assessment during HMP cannot be used independently but may be used collectively with the kidney characteristics and donor demographics to determine the suitability of a kidney for transplantation.

Normothermic preservation techniques may hold more promise in the assessment of viability compared to HMP techniques. During normothermic perfusion renal function and metabolism are restored. In experimental models, low levels of blood flow, reduced renal function and low oxygen consumption have been associated with increased ischaemic injury. Furthermore, these functional measures could be combined with injury biomarkers to assess the quality of the kidney.

19. Experimental studies

19.1. Oxygenation

There is a growing body of evidence in support of recovering ischaemically damaged organs with oxygenated preservation techniques at low temperatures. Historically, oxygenation was considered an essential component of hypothermic kidney preservation in order to support mitochondrial resynthesis of ATP and to delay the injury process. However, with the introduction of the modern day preservation solutions, and the rapid adoption of simple CS techniques, oxygen was not thought to be a vital ingredient and as such is not commonly applied in the clinical setting. Various techniques have been used to apply oxygen under CS and HMP conditions.

Retrograde oxygen persufflation is a simple technique whereby filtered and humidified oxygen is bubbled directly through the renal vasculature during CS. The gas is then allowed to escape through small perforations in the surface of the organ. Reports of its application date back to the 1970s [110,111]. Experimentally, there has been renewed interest in this technique showing a beneficial effect on graft function when compared to CS and HMP techniques [112,113].

Hyperbaric oxygenation is the delivery of oxygen under increased atmospheric pressure. Hyperbaric oxygenation is normally used to treat decompression sickness, carbon monoxide poisoning, gas embolism, circulatory disorders and to promote wound healing [114-116]. However, it has been used in organ preservation. Under normal atmospheric pressure there is a limit to the amount of oxygen that can be carried in the blood. Increasing the atmospheric pressure at which it is delivered, increases the amount of dissolved oxygen in

the plasma allowing deeper penetration into the tissue (Henry's Law). Therefore, tissues can be adequately oxygenated in the absence of a blood flow, a particular advantage in organ preservation [114,115]. Although an interesting concept and benefits have been demonstrated in liver and bowel transplantation, there has been little evidence of its use in kidney preservation in recent times.

Oxygen can also be added during HMP. At present, HMP is not supplemented with oxygen based on the presumption that air equilibration in perfusates sufficiently supports energy metabolism and that oxygen consumption at 4ºC is around 5% of that found at body temperature [117]. However, ATP can be restored in part, with the addition of oxygen and energy substrates during perfusion [118]. Short periods of oxygenated perfusion after CS have also been used to resuscitate and condition organs, correcting ATP loss, reducing levels of oxidative stress and improving organ viability [119]. The addition of free radial scavengers such as superoxide dismutase (SOD) to the preservation solution has been found to be beneficial [119,120] in preventing the generation of oxygen free radicals in this highly oxygenated environment.

20. Oxygenated solutions

Oxygen can also be effectively administered during preservation by the use of artificial oxygen carriers. Perfluorocarbons (PFC) are inert solutions that have a high capacity for dissolving oxygen. They release oxygen down a concentration gradient creating a highly oxygenated environment which is not affected by temperature [121,122]. They can be added simply during CS in a technique called the two layer method (TLM). The density of the PFC allows two layers to be formed, PFC on the bottom and the preservation solution on top. The organ is placed in the solution and remains between the two layers. Oxygen can be continuously added allowing adequate diffusion through the organ. TLM has been particularly beneficial for pancreas preservation, allowing a sufficient amount of ATP to be generated to improve organ viability [121,123]. The use of TLM has shown potential in other organs but has failed to gain much support as the ability of oxygen to penetrate deep into tissue in more densely capsulated organs has been questioned. In the kidney its beneficial effect was found in a rat model, however, when applied in a porcine model the results showed no advantage [121,124-126].

PFC can also be formulated as an emulsion for continuous perfusion and was applied during early attempts at machine perfusion [126-129]. However, the instability and adverse effects of the emulsions at that time prevented their continued application [121].

Other novel oxygen carriers have recently been applied experimentally in kidney preservation. Hemarina-M101 (M101] is a respiratory pigment derived from a marine invertebrate, *Arenicola marina* [130]. It has an extremely high affinity for oxygen and functions over a large range of temperatures (4-37ºC) releasing oxygen against a gradient. Using a porcine kidney model Thuiller *et al* recently showed in that adding M101 to UW or HTK solution during CS for 24 hours improved renal function and reduced fibrosis after

transplantation. Micro-bubbles derived from Dodecafluoropentane (DDFPe) are also being investigated as oxygen replacement therapies and may in the future be applied during organ preservation [131,132].

In addition to hypothermic conditions, perfluorochemical and haemoglobin solutions can also be used to deliver oxygen at normothermic temperatures [133]. Brasile *et al* originally developed an acellular normothermic solution based on a modified cell culture medium and PFC emulsion (Perflubron) [134]. The perfusate was made up of a highly enriched tissue culture-like medium containing essential and non-essential amino acids, lipids, carbohydrates.

Historically, haemoglobin based solutions such as Stroma-free haemoglobin failed to demonstrate benefit experimentally because of toxic effects on the kidney. However, a newly developed solution, pyridoxalated haemoglobin-polyoxyethylene (PHP) has been deemed to be a more stable solution [133]. New more stable 2nd and 3rd generation PFCs are being developed and several are undergoing clinical trials to assess their safety. Humphreys *et al* recently used a commercially made PFC 'Oxygent' to provide oxygenation and reduced ischaemic injury to the kidney during a period of warm ischaemia by retrograde infusion through the urinary collecting system [135].

Other solutions such as Lifor, a new artificial preservation medium containing a non protein oxygen carrier that can be used at room temperature may also be used for preservation [136, 137]. These new solutions may hold more promise for future development of normothermic preservation perfusates. Nonetheless, the use of these normothermic perfusates in clinical practice is still awaited.

21. Experimental agents

I/R injury involves a cascade of events centralised by activated endothelial cells immediately after transplantation. One of the first inflammatory responses is the infiltration of neutrophils into the tissue. Cell adhesion molecules are recognised by leukocytes which interact with tissue cells to allow the movement of immune cells and mediators to the injury site [138,139]. This is mainly mediated through the up-regulation of endothelial adhesion molecules (ICAM-1, VCAM-1 and E-Selectin) [138]. The release of pro-inflammatory cytokines and chemokines, activation of the complement system and production of reactive oxygen species (ROS) [139] also cause significant cellular injury.

A vast number of therapies have been investigated to ameliorate the detrimental effects of I/R injury such as vasodilatory agents [140,141], antioxidants [142-144], anti-inflammatory agents [145,146] and growth factors [147] and in the experimental setting many of these have proved beneficial. Of particular interest are the therapies that collectively target several mechanisms of I/R injury, these include the endogenous gaseous molecules nitric oxide (NO) [148,149], carbon monoxide (CO) [150,151] and hydrogen sulphide (H_2S) [152,153]. Experimental models have shown their ability to reduce inflammation, oxidative damage,

apoptosis and promote smooth muscle relaxation causing vasodilation to enhance renal blood flow. However, their application into clinical practice is awaited.

There is no single agent used as standard clinical practice to treat I/R injury and reduce DGF. Nonetheless, there are several agents of interest that have recently been examined in clinical trials. Recombinant human erythropoietin (EPO) is a treatment for anaemia in renal patients however it also has cytoprotective properties and has been shown to protect against kidney injury in experimental models [154, 155]. However, the results from two clinical trials contradict the majority of animal studies and showed no benefit of EPO in reducing rates of DGF [156,157]. Furthermore, in one trial concerns of the increase in the incidence of graft thrombosis where raised [157]. Other trials to assess the effects of EPO are ongoing and the results are pending. It has been suggested that EPO mediates protection through a tissue receptor that is distinct from the classical EPO-receptor that is known to mediate erythropoiesis [158]. A new compound has been formulated, pyroglutamate helix B surface peptide (pHBSP) that has the tissue-protective properties similar to those of EPO but without causing erythropoiesis [158]. Early experimental models suggest that this agent is beneficial in reducing kidney injury and may hold promise for future clinical trials.

Several volatile anaesthetic agents sevoflurane and desflurane are also being trialled in clinical transplantation to reduce kidney injury. These agents are thought to have a conditioning effect that up-regulates protective mechanisms to reduce the I/R injury response [159]. The conditioning effect can also be applied by short intervals of ischaemia either directly to the organ or remotely to a limb [160]. It can be applied to the donor or recipient and again experimental models have shown the benefits of conditioning techniques. They are particularly attractive for clinical transplantation in that no pharmacological intervention is required and therefore the technique is expected to have a high safety profile. The results of several clinical trials are eagerly awaited. Propofol is another anaesthetic agent that may reduce I/R injury [161,162]. Experimental models have highlighted the anti-oxidant and anti-apoptotic properties of the agent [161,162].

There has been a great deal of emphasis on stem cell therapy to reduce kidney injury. The ability of stem cells to differentiate into multiple lineages with the capacity to stimulate the regeneration of renal tissue is particularly attractive in kidney transplantation. Bone marrow derived mesenchymal stem cells have been used in the rat kidney to reduce inflammation and oxidative damage [163-165]. However, there has been no clinical application of this therapy in kidney transplantation.

Immunosuppressant therapies used on induction can be used to reduce I/R injury and DGF. They suppress leukocyte infiltration and reduce endothelial injury. Anti-CD25 [166] and antithymocyte globulin (ATG) [167] are amongst some of the agents being currently being studied to reduce the incidence of DGF.

22. Conclusion

CI injury is detrimental to early graft function and as such early graft dysfunction is associated with reduced graft survival and complications after transplantation. However,

the direct impact of CI on long term graft survival is less clear. Clinical studies suggest that CI may not necessarily be an independent risk factor for reduced graft survival. Nonetheless, further evidence is needed to examine the relationship between CI injury and graft survival. Hypothermic preservation techniques are designed to counteract the detrimental effect of CI injury and hypothermic machine perfusion is emerging as a superior method of preservation compared with static cold storage. Other preservation techniques are being developed such as normothermic perfusion and the addition of oxygen and oxygen carriers during hypothermic preservation. These techniques may hold promise for the future to limit the damage caused by CI injury. Therapeutic agents administered to the recipient may also prove beneficial in reducing early graft dysfunction. Nonetheless, translation of these therapies from animal models to clinical practice remains difficult and the search for the optimal agent or therapy is ongoing.

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