Assessment of the Viability of Renal Cells Following an Ischaemic Insult

A Thesis Submitted in Fulfilment of the Regulations for the Degree of Doctor of Medicine, MD.

Paul Charles Butterworth

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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Renal transplantation is the best treatment for end-stage renal failure. The relative shortage of organs may be addressed by transplanting kidneys from non heart-beating donors (NHBD). This thesis addresses the results of transplantation from NHBD and how they may be improved, with emphasis on the search for a pre-transplant test of viability.

In Chapter 1 the role of NHBD kidney transplantation in addressing the organ shortage is reviewed.

Chapter 2 presents the results of the Leicester kidney transplant programme. Kidneys from NHBD show a higher rate of primary non-function than cadaveric donor kidneys but there is no difference in graft survival. A reliable viability test would prevent the transplantation of kidneys severely damaged by ischaemia and so improve results.

In Chapter 3 viability testing in renal transplantation is reviewed.

Chapter 4 describes experiments resulting in the development and testing of an in vitro model of warm ischaemia. Cultured pig and human tubular epithelial cells were found to be tolerant of anoxia in this model.

In Chapter 5 the anaerobic metabolism of these cells is studied. Lactate is produced by glycolysis even in oxygenated conditions. Differences between the metabolism of tubular cells in vivo and in vitro, prevent in vitro modeling of warm ischaemia.

In Chapter 6 the utility of detection of apoptosis in pre-transplant biopsies in predicting transplant function is investigated. Apoptosis is seen in the juxta-cortical medulla of NHBD kidneys but not in the cortex. Cadaveric donor kidneys show no apoptosis. The presence of apoptosis does not predict transplant function.

In Chapter 7 the utility of histomorphometric analysis of interstitial fibrosis in the assessment of kidney quality is studied. Kidneys from NHBD have more severe interstitial fibrosis than cadaveric kidneys but the difference is small and correlation with transplant function is poor.

In Chapter 8 the findings of these experiments are summarized and the future of viability testing in renal transplantation is discussed.

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Publications

Research work presented in this thesis has resulted in the publication of the following papers in the peer reviewed literature:

Butterworth PC, Taub N, Doughman TM, Veitch PS, Bell PRF, Nicholson ML.
Are kidneys from non heart beating donors second class organs?
Transplantation Proceedings 1997; 29: 3567-3568

Butterworth PC, Mistry NS, Horsburgh T, Nicholson ML.
Renal tubular epithelial cells tolerate prolonged warm ischaemia in vitro.
Transplantation Proceedings 2000; 32: 161-162

Butterworth PC, Horsburgh T, Nicholson ML.
Distribution and predictive value of apoptosis in biopsies from non heart beating donor kidneys.
Transplantation Proceedings 2000; 32: 163

Butterworth PC, Bailey E, Horsburgh T, Nicholson ML.
Interstitial fibrosis in the cortex of donor kidneys; relationship to donor type and post transplant function.
Transplantation Proceedings 2000; 32: 185

Contents

CHAPTER 1

ADDRESSING THE ORGAN SHORTAGE: RENAL TRANSPLANTATION FROM NON HEART-BEATING DONORS

ADDRESSING THE ORGAN SHORTAGE: RENAL TRANSPLANTATION FROM NON HEART-BEATING DONORS

Introduction

Kidney transplantation is now recognised as the optimal treatment for end stage renal failure. It provides the best quality of life for patients and is cheaper in the long term than any form of dialysis. Continued improvement in the results, in particular with respect to recipient morbidity and mortality, has meant that transplantation is a viable option for increasing numbers of patients. The ranks of patients waiting for transplantation are further swelled by existing transplant patients whose allografts fail. However, the number of donor organs available for transplantation has failed to rise at a rate comparable to that at which patients are added to the waiting list. This relative shortage of organs has resulted in an expansion of waiting lists worldwide. In the UK, at the end of 1999, there were 5731 patients waiting for renal transplantation and this number is growing at a rate of 2% per year. The number of donor organs available is currently static and shows signs of contracting (UKTSSA, 1995). In the United States there has been a modest rise in the number of available organs but demand continues to outstrip supply and the waiting list continues to grow (UNOS, 1996).

Underlying the shortage of donor organs is a variety of medical and social factors. In the UK the number of trauma victims who become potential organ donors has declined

significantly following the introduction of legislation governing the wearing of rear seat belts in 1990 (UKTSSA, 1996). In addition, over the last 20 years there has been a dramatic decline in the number of deaths from intracranial haemorrhage, thus reducing the number of organs potentially available from this source. However, it is by no means clear that the sole reason for the organ shortage is a lack of potential donors. Not all potential organ donors are identified as such by medical personnel and some physicians may be reluctant to discuss organ donation with relatives (Nathan et al, 1991). The most common reason for failure to realise potential organ donation is lack of consent from families. Refusal of consent is associated with low levels of educational achievement and socio-economic and cultural background (Cutler et al, 1993), which present significant obstacles to improving donation rates. Efforts to increase donation rates from the existing donor pool will depend on education of physicians and the general public aided, perhaps, by changes in consent laws to a policy of presumed consent as exists in Spain where organ donation rates are the highest in Europe. The benefits of these efforts will only be realised in the long term. In the short term the only ways of tackling the organ shortage are by promoting live donation, which is likely to have only a limited impact and by expansion of the donor pool to include potential donors who would previously have been considered unsuitable. These so called marginal donors or expanded criteria donors fall into two major groups:

(i) patients with beating hearts who are declared brain stem dead i.e. conventional cadaveric donors who have characteristics previously considered disadvantageous to organ donation such as advanced age, hypertension, diabetes mellitus or abnormal organ function

(ii) patients who have suffered cardio respiratory arrest from which they are not resuscitated: non heart-beating donors (NHBD). Ischaemic injury to the organs begins as soon as the heart stops beating and probably earlier during any prodromal period of hypotension. However, if the ischaemic injury is not too prolonged and cortical necrosis does not occur, the kidneys may recover function once reperfused by transplantation.

The concept of using organs from non heart-beating donors is not new. Brain stem death criteria were only defined in 1968 (Beecher, 1968) and indeed legal recognition of brain stem death criteria for the purposes of organ retrieval has only recently reached the statute in some in countries: in India, 1994 and Japan, 1997 for example. Prior to this, all organs for transplantation were procured from patients who had suffered cardiorespiratory arrest. However, the relative shortage of cadaveric (brain stem dead) donors in the western world has renewed interest in non heart-beating donors, resulting in the various presentations of potential donors being carefully described. Four donor categories are now recognised (Kootstra, 1995).

Category 1:Dead on Arrival

Patients who die outside of a hospital, such as at the scene of an accident or at home following a myocardial infarction may be brought to the emergency room and declared *dead on arrival.* If the period of cardiac standstill is not prolonged then the kidneys may be viable. At present many of these cadavers are taken straight to the mortuary and obtaining organs from these potential donors would require a change in current practice and the role of the coroner.

Category 2:Unsuccessful Cardiopulmonary Resuscitation

Patients in hospital who suffer cardiorespiratory arrest from which they cannot be resuscitated.

Category 3: Awaiting Cardiac Arrest

A group of patients exist who do not meet the brain stem death criteria but in whom elective withdrawal of support is very likely to result in their death. These patients, for whom organ donation should already have been discussed with their relatives, are awaiting cardiac arrest.

Category 4: Cardiac Arrest Whilst Brain Dead

There are a number of patients in whom consent for multiple organ donation has already been obtained, who suffer cardiac arrest after having been declared brain dead but prior to organ retrieval surgery. In this situation resuscitative efforts should be made but if these are unsuccessful then conversion to a non heart-beating procedure can be made.

The largest number of potential donors are likely to come from categories 2 or 3. However, a number of ethical issues need to be addressed before proceeding with organ procurement. The point at which resuscitation becomes unsuccessful must be decided by the attending physician without the involvement of the transplant team (Kootstra, 1996). Some countries have very strict guidelines on the abandonment of resuscitative efforts and this precludes non heart-beating donation in some circumstances (Schlumpf et al, 1996). In addition, the 'dead donor' rule has long been recognised in transplantation but the diagnosis of

cardiac death, which must be made for category 3 donors, has been the subject of some debate. The original Pittsburgh protocol waited for only two minutes of cardiac standstill before proceeding with organ procurement (Casavilla et al, 1995). However, after circulatory standstill the electroencephalogram may continue to register brain activity for several minutes. Obtaining an electroencephalogram in the surroundings of potential non heart-beating organ donation is not a practical proposition and so a period of ten minutes between cardiac standstill and the commencement of the organ procurement procedure has been recommended to ensure irretrievable brain damage analogous to brain death (Kootstra, 1995).

After Declaration of Death

When the arrested patient has been declared dead, cardiopulmonary resuscitative efforts cease. From this point onwards the kidneys will suffer injury due to warm ischaemia as a result of circulatory standstill. Warm ischaemia causes reversible 'acute tubular necrosis' or irreversible cortical necrosis if prolonged. In an attempt to prevent the damage caused by warm ischaemia, commencement of external cardiac massage and artificial ventilation until the kidneys can be cooled or procured has been advocated. (Note that this is not resuscitation because death has already been declared). However, the value of continued cardiac massage and artificial ventilation is by no means clear. Early studies on the haemodynamic effects of cardiopulmonary resuscitation demonstrated that the cardiac output achieved is usually poor (Del Guercio et al, 1963; MacKenzie et al, 1964). The kidneys suffer a profound decrease in their blood flow and this is made worse by the administration of adrenergic drugs during attempted resuscitation of the victim (Holmes et al, 1980) and despite good oxygenation of the

arterial blood, acid base disturbances are usually severe (Steedman et al, 1992). Nevertheless, not all resuscitated victims of cardiac arrest suffer acute renal failure, especially if the duration of arrest and resuscitation is short (Mattana et al, 1993). The majority of groups who retrieve kidneys from non heart-beating donors employ cardiac massage and artificial ventilation, akin to cardiopulmonary resuscitation in the arrested patient, in an effort to limit the warm ischaemic injury prior to cold perfusion of the organs, but good evidence for its efficacy is lacking.

In Situ Perfusion

The primary aim of the transplant team on their arrival at a potential non heart-beating donor is to limit the injury caused by warm ischaemia. The principal actions of organ preservation i.e. flushing of blood, cooling and the prevention of cell swelling (Pegg, 1996) need to be performed as quickly as possible after cardiorespiratory arrest. In situ preservation was first reported successfully in dogs using a catheter passed into the aorta (Banowsky et al, 1971). Although successful transplants were achieved, the need for fluoroscopy to accurately site the catheter rendered the technique too cumbersome for use in the emergency room. Around the world different groups have performed in situ cooling and perfusion in the emergency room in a number of different ways. A group in Madrid use a portable cardiopulmonary bypass machine to achieve whole body hypothermia prior to the retrieval (Arias et al, 1991). A group in Barcelona take the donor to the operating theatre whilst cardiac massage is being performed and cannulate the aorta before massage is stopped (Castelao et al, 1993). However, the most important development has, without doubt, been the invention of the Double Balloon Triple Lumen (DBTL) catheter (Garcia-Rinaldi et al, 1975). This device,

which is inserted via a cut down onto the iliac or common femoral artery, isolates a distal segment of aorta from which the renal arteries arise and facilitates perfusion of the kidneys with preservation fluid at 4°C. Researchers in Maastricht began using this technique in the emergency room situation in 1980 (Kootstra et al, 1986; Booster et al, 1993a) and have been followed by others in Switzerland and the UK. In the United States a group in Rochester, New York have described in situ cooling by irrigating the peritoneal cavity with lactated Ringers solution at 4°C using a chest drain for infusion and a Foley catheter to vent (Orloff et al, 1994). Japanese workers have also used a combination of intraperitoneal cooling and in situ perfusion with a double balloon catheter (Matsuno et al, 1993). These systems allow the kidneys to be preserved before consent is gained from the relatives and enable the discussion of donation with the relatives to take place in a more controlled fashion. The ethical issues regarding operating on the cadaver in the absence of formal written consent need to be addressed locally but a policy of presumed consent would certainly permit preparatory handling of the potential organ donor, such as the insertion of the DBTL catheter, prior to the discussion of donation with the relatives. In the United States, in situ perfusion is permitted if 'performed with due respect' on the grounds that it avoids depriving the relatives of their option to consent to organ donation and is not a mutilating procedure (Anaise, 1993).

Not all dead or dying patients will be suitable organ donors. The expanded criteria for heart beating cadaveric organ donors (Alexander, 1992) do not apply in full to the non heart-beating situation because of the additional insult to the organs of a period of warm ischaemia. Older kidneys may have increased susceptibility to the effects of warm ischaemia because of advanced vascular disease and so the upper age limit of non heart-beating kidney donation is

set at 60 years (Booster et al, 1993b; Nicholson, 1996a). Furthermore a history of uncontrolled hypertension or complicated diabetes contraindicates kidney transplantation from these donors. Other potential donors will be excluded because, as with cadaveric donors, consent for organ donation cannot be obtained. If the donor is suitable and consent is obtained, having secured in situ perfusion, retrieval of the organs proceeds in the usual manner.

Preservation of Kidneys from Non Heart-Beating Donors

There are two important factors in the storage of cadaveric kidneys for transplantation: (i) the perfusion fluid and (ii) the method of storage. The most commonly used perfusion fluids are HTK (Custodiol), Euro-Collins Solution, Marshall's solution and the University of Wisconsin solution (UW). There are two methods of storage: simple cold storage, the standard method in conventional cadaveric transplantation, where the perfused kidney is kept in ice until transplanted and machine preservation, where the kidney is perfused in a pulsatile fashion with perfusion fluid at 4°C. Prolonged preservation of several days, followed by successful transplantation has been reported in dogs using continuous pulsatile machine perfusion (Belzer et al, 1967) but this duration of preservation is not required in man. A number of centres procuring kidneys from non heart-beating donors advocate machine perfusion because of the ischaemic insult which the kidney has already suffered. However, much of the evidence for the beneficial effects of this practice is only experimental (Booster et al, 1993c and d) and is confounded by the fact that simple cold storage in one preservation medium has been compared with pulsatile machine perfusion using a different preservation medium. Using pulsatile machine preservation, the Maastricht group have demonstrated an improvement in

early function compared with simple cold storage (Daemen et al, 1997). However, no advantage of machine perfusion has yet been demonstrated with respect to primary non-function (allografts which never work sufficiently to render the recipient dialysis independent) or long-term graft survival.

Post Transplant Management of the Transplanted NHBD Kidney

Transplant surgery proceeds in exactly the same fashion for kidneys retrieved from non heart-beating donors as for conventional cadaveric kidneys. The major difficulty in management occurs in the immediate post-operative period and is delayed graft function. This is a clinical diagnosis best defined as temporary failure to produce life sustaining function during which time dialysis continues to be required. The overwhelming majority of kidneys from non heart- beating donors suffer delayed graft function when transplanted as a consequence of acute tubular necrosis. Delayed graft function impacts negatively on the psyche of the recipient and the cost of the transplant procedure is raised by a prolonged hospital in-patient stay but there are also physical consequences. The most important effects are on the incidence of acute rejection and on the long-term outcome in terms of graft and patient survival. The issue of whether or not delayed graft function is an independent negative influence on graft survival is debated (Troppmann et al, 1996; Shoskes et al, 1996) but grafts which suffer delayed graft function and acute rejection certainly have worse graft survival times than those which suffer either event alone (Nicholson et al, 1996(b)).

Ischaemic damage may increase the immunogenicity of the allograft. In a mouse model of unilateral renal ischaemia, warm ischaemia and reperfusion results in an increased expression of Major Histocompatibility Complex (MHC) Class 1 antigens on tubular cells and MHC class II antigens on interstitial cells (Shoskes et al, 1990). This effect has also been found in the rat kidney (Shackleton et al, 1990). Local tissue injury may also stimulate antigen presentation by dendritic cells (Ibrahim et al, 1995). The clinical significance of this experimental data remains unclear but an increased incidence of acute rejection is reported in patients with delayed graft function (Howard et al, 1994). Acute rejection cannot be reliably diagnosed clinically whilst the graft is not functioning. The gold standard for the diagnosis of rejection is needle core biopsy and weekly biopsies have been proposed to exclude concomitant rejection during periods of delayed graft function (Jain et al, 2000). Whilst generally safe this procedure is unpleasant for the patients and carries a small risk of significant haemorrhage.

All published series report a high incidence of delayed graft function in non heart-beating donor allografts. Since delayed graft function may have a negative impact on long term outcome a number of strategies have been used in an attempt to limit the number of grafts affected. Cyclosporine nephrotoxicity is a well recognised cause of delayed graft function since therapeutic levels cause renal vasospasm and toxic levels cause oliguria. Avoidance of Cyclosporine is thus to be considered beneficial until function is established. Induction protocols, using anti-lymphocyte antibodies without Cyclosporine, have been recommended in some situations where delayed graft function might be anticipated because rejection may be prevented without exacerbating renal vasospasm (Abramowicz et al, 1996). However,

treatment with these agents may need to be discontinued before the graft functions if their use is complicated by the development of recipient antibodies or potentially life threatening infection with Cytomegalovirus or Pneumocystis carinii. In this event Cyclosporine needs to be introduced prior to the establishment of graft function. Data to support these protocols is limited and in the non heart-beating situation, induction of immunosuppression with anti-lymphocyte antibodies has been complicated by severe neurological side effects (Kehinde et al, 1994).

Results of Renal Transplantation from NHBD

In those institutions that have started programmes, there is no doubt that the use of kidneys from non heart-beating donors has had a significant impact on the number of transplants performed. The number of transplants from non heart-beating donors may represent as much as 40% of transplant activity (Daemen et al, 1996). However, some transplant centres remain reluctant to use these organs because of concerns regarding both the short term and more particularly the long term results. The total number of transplants performed worldwide using kidneys from non heart-beating donors is relatively small compared with the experience with conventional cadaveric organs. The Japanese have the largest experience because the concept of brain stem death is not widely accepted in that culture. The group in Nagoya found five year graft survival rates of 72% (Yokoyama et al, 1993) which is very encouraging but these results are difficult to extrapolate to other countries because of the technique used to procure the organs: in situ perfusion was performed after cardiac arrest but the catheters were sited pre mortem. The absolute numbers of kidneys from non heart-beating donors that have

been transplanted outside Japan remains relatively small. No increase in the incidence of vascular or urological problems in the immediate post operative period has been reported. However, the warm ischaemic insult to which these organs have been exposed has two consequences. Most series report an increased incidence of primary non-function compared with conventional cadaveric heart beating kidneys (Wijnen et al, 1995; Nicholson et al, 1996a). Primary non-function is a catastrophic outcome for the psyche of the patient and has both financial and immunological consequences. Similarly, the rates of delayed graft function are reported to be 36-100% in NHBD renal transplantation compared with 25-30% in heart beating donor transplants. Despite the association between delayed graft function and increased rates of acute rejection no published study has shown an increased rate of rejection for non heart-beating donor kidneys compared with conventional cadaveric heart beating kidneys. Differences in graft function between NHBD and heart beating donor transplants at three months simply reflect the higher rate of delayed graft function in the NHBD transplants. One year graft survival and graft function has been reported to be not significantly different to that in heart beating donor transplants (Wijnen et al, 1995). Three year graft survival is reported not to be different when comparing NHBD transplants and heart beating donor transplants from kidney only donors (Koffman et al, 1993), though both sets of results are somewhat disappointing in this study. No study has yet shown a significant difference in graft survival between conventional cadaveric heart beating and non heart-beating donor kidneys to five years.

Conclusions

Organ procurement from non heart-beating donors is one approach to addressing the organ shortage. The use of rapid minimally invasive techniques for in situ preservation allows requests for organ donation to be made in a more calm and less time pressured atmosphere. The high reported incidence of primary non-function in NHBD transplants remains an obstacle to their widespread acceptance throughout the transplanting community. It remains to be seen whether the negative influence of delayed graft function on long term graft survival, which is seen in conventional cadaveric heart beating transplants, will also be seen in non heart-beating donor organs with longer follow up. If this is the case then potential recipients of kidneys from NHBD need to be aware of the 'quality' of the organ they are going to receive. It is currently unclear if kidneys from NHBD are 'second class' organs compared to conventional cadaveric kidneys. In the following chapter the results of transplantation from NHBD in the Leicester transplant unit are presented and compared with the results of transplantation from contemporary conventional cadaveric donors.

CHAPTER 2

RESULTS OF RENAL TRANSPLANTATION FROM NON HEART-BEATING DONORS: COMPARISON WITH CONVENTIONAL CADAVERIC DONORS.

RESULTS OF RENAL TRANSPLANTATION FROM NON HEART-BEATING DONORS: COMPARISON WITH CONVENTIONAL CADAVERIC DONORS

Introduction

Despite offering a means for addressing the shortage of donor organs, NHBD transplant programmes are currently few in number for two main reasons. Firstly, relatively few transplants from NHBD have been performed and the follow up is relatively short, so convincing renal transplant surgeons of the merits of such a programme is difficult. Secondly, the workload for the transplant team in establishing a NHBD organ retrieval programme is considerable (Nicholson et al, 1996c). Issues regarding ownership of the organs of the recently deceased 'donor' present further obstacles, as do ethical issues surrounding in situ perfusion of donors where a policy of presumed consent does not exist (Anaise et al, 1993; Younger et al, 1993). If greater use of NHBD organs is to be made, the transplant community must be convinced that these organs provide good function and long-term graft survival. Furthermore, the potential recipients of such organs need some information as to what they can expect from an organ that carries a considerable immunogenic load irrespective of its function, which may make achieving a beneficial match for any subsequent conventional cadaveric transplant more difficult.

The issues are thus:

(i) Do NHBD kidneys reliably provide sufficient function to render the patient dialysis independent?

(ii) For how long will they continue to provide adequate function?

(iii) How do the results compare with transplantation of conventional cadaveric donor kidneys?

Several groups around the world have now published short term results of NHBD renal transplant series (Dunlop et al, 1995; Casavilla et al, 1995; Wijnen et al, 1995; Sclumpf et al, 1996; Gonzalez Segura et al, 1995). The results of transplantation in Japan, where kidneys are often retrieved using a similar in situ perfusion technique in the asystolic donor, also suggest that adequate renal function can be achieved in the recipients of NHBD kidneys (Yokoyama et al, 1993). However, there is little data as to whether the results of transplantation with NHBD kidneys are comparable to the results of transplantation with conventional cadaveric donor kidneys. This question is unlikely to be answered by the usual route of randomised controlled trial because neither class of organ is freely available. Depriving a potential recipient of a transplant because they are in the wrong arm of such a trial is not ethical and it is difficult to blind the retrieving and transplanting team to the origin of the organ. In addition there are many independent prognostic variables which impact upon the outcome of renal transplantation and these would be difficult to control for in a randomised trial where the numbers would be inevitably limited.

Prognostic Factors for Graft Function and Survival in Renal Transplantation

Multivariate analysis of the databases of the large transplant registries in the United States and Eurotransplant region have established a number of parameters of independent prognostic significance for graft function and survival after renal transplantation. These parameters include variables relating to the donor, the HLA mismatch, the transplant process, the recipient and the post transplant course. The relative significance of these variables in any particular situation is much debated and many published papers are contradictory but any comparison of transplant series that does not control in some way for these parameters is likely to have considerable problems with confounding. The key variables are as follows:

(i) Donor Factors

The impact of donor age is clear with kidneys from donors older than 55 having poorer outcomes both in terms of the quality of early function and long term graft survival than kidneys from donors under 55 (Alexander et al, 1991). However, the primary concerns are the nephron mass in the donor kidney and the innate ability of the kidney to repair injury (Halloran, 1999). The age of 55 is a somewhat artificial cut off because glomerular filtration rate declines in a more or less linear fashion with age over 30 years (Davies et al, 1950). The impact of donor sex is seen chiefly in relation to the level of the serum creatinine after transplantation: women have smaller kidneys than men. The ratio of donor kidney weight to recipient bodyweight is a determinant of post transplant function (Kim et al, 2001). Improved long-term graft survival of kidneys from male donors is found by some authors (Neugarten et al, 1996).

(ii) Recipient Factors

The closeness of the HLA antigen match between recipient and the donor organ is of fundamental importance as it determines the severity of the recipient's immunological attack on the graft: best results of all are seen in transplants between monozygotic twins and best results in cadaveric transplants when the donor and recipient are fully matched at HLA DR, A and B loci (Opelz et al, 1991). Mismatches at the HLA DR locus are most important especially for the risk of acute rejection in the first six months after transplantation. However, mismatches at HLA B and A loci are also important prognostic factors variables for long term graft survival in the Cyclosporine era (Thorogood et al, 1992). Similarly the relative sensitisation of the recipient to a variety of immunogenic antigens may contribute to the immunological 'attack' on the transplanted organ. All registries show that first transplants have better graft survival than subsequent or retransplants. Furthermore, recipients with a high level of antibodies against a panel of antigens have poor outcomes compared with relatively unsensitised recipients.

(iii) The Transplant Process

The duration of cold ischaemia is an independent risk factor in transplant outcome. Cold ischaemia causes renal injury and is an independent risk factor for both short-term function (a strong association with delayed graft function) and long-term graft survival. Transplantation with more than 24 hours of cold ischaemia confers worse graft survival than grafts transplanted with less than 24 hours of cold ischaemia (Tesi et al, 1993). However, in a manner similar to the situation with donor age, it seems likely that this is a continuous change rather than a step wise one. Similarly the kidney does not tolerate prolonged warm ischaemia

during transplantation and prolonged anastomosis times may be linked to delayed graft function.

Case / Control Study of the Results of Renal Transplantation from NHBD in Leicester.

To investigate how the results of NHBD transplantation compare with the results of transplantation from cadaveric donors, a case / control analysis has been performed. The analysis is retrospective but all the data was collected prospectively. All of the NHBD transplants form the case group; a subset of contemporary cadaveric transplants form the matched control group.

Subjects: The Transplant Programme

Cadaveric donor kidneys were retrieved locally or supplied via the United Kingdom Transplant Support Service because of a beneficial match. Non heart-beating donor kidneys were all retrieved locally by in situ perfusion and cooling using Marshall's hyperosmolar citrate at 4°C infused under gravity through a double balloon triple lumen catheter. All kidneys were preserved by static ice storage. The transplant procedure was performed by any one of a number of surgeons in the Leicester transplant team. Immunosuppression was by 0.5g of methylprednisolone peroperatively and postoperatively by a steroid and Cyclosporine based regime in both groups, except for the first 16 NHBD transplants in which an induction regime of OKT3 was used. This regimen was subsequently abandoned due to the high incidence of

respiratory and central nervous system side effects (Kehinde et al, 1994). Recipients of cadaveric donor kidneys received Cyclosporine at a dose of 15mg/kg/day and recipients of NHBD kidneys received 7mg/kg/day, the aim being to reduce calcineurin inhibitor nephrotoxicity in the already ischaemically more damaged NHBD kidneys. Cyclosporine was tailed to 5mg/kg/day in both groups over 6 weeks and 3 weeks respectively. Azathioprine, 1-2mg/kg/day adjusted for leucopenia, was given to all NHBD recipients and retransplanted recipients of cadaveric kidneys. All transplants were biopsied at one week and in the case of delayed graft function at weekly intervals to exclude subclinical acute rejection.

Selection of Matched Cadaveric Donor Controls

A number of variables may influence the outcome of renal transplantation as described above. In order to minimise the confounding effect of these variables in the case / control analysis, for each NHBD transplant a number of cadaveric transplants were selected as the controls based on matching of parameters known to influence outcome. For a cadaveric transplant to be matched to a NHBD transplant the criteria were as follows;

(i) Complete match on all of 4 major criteria

Donor Age - within five years

HLA DR mismatches

Cold Ischaemic Time - ≤24 hours or >24 hours

First or Retransplant

(ii) At least 2 matches on 4 minor criteria

Donor Sex

HLA mismatch at A and B loci

Anastomosis Time (Warm Ischaemia) - ≤30 minutes or >30 minutes

Acute Panel Reactive Antibodies - ≤10% or >10%

Each one of 39 consecutive NHBD was assessed for matching with every one of 234 consecutive cadaveric transplants performed between 1990 and July 1996. In this way 91 cadaveric transplants were matched with the 39 NHBD transplants with the number of cadaveric transplants matched to a single NHBD transplant ranging from 1 to 6.

The outcome measures studied were:

1. Incidence of primary non function - defined as failure to provide life sustaining renal function at any stage after transplantation.
2. Incidence of delayed graft function - defined as dialysis dependence during the first 7 days after transplantation, with the exception of a single dialysis for the treatment of hyperkalaemia or fluid overload, in recipients whose grafts go on to give life-sustaining function.
3. Incidence of acute rejection occurring in the first six months post transplant (proven by biopsy).
4. Graft Function as measured by the serum creatinine and by the estimated creatinine clearance which was calculated using the Jelliffe formula (Jeliffe et al, 1973)
5. Graft survival.

Statistical analysis was performed using the Mann Whitney U test for continuous variables and Fisher's Exact test for categorical variables. Graft survival was measured using the Kaplan

Meier technique, censoring for death with a functioning graft and using the log rank test for comparison.

Results

1) Incidence of Primary Non-Function

Non Heart-Beating Donors	4/39 = 10%
Cadaveric Donors	1/91 = 1%
Fisher's Exact Test	p= 0.03

2) Incidence of Delayed Graft Function

Non Heart-Beating Donors	35/39 = 90%
Cadaveric Donors	25/91 = 27%
Fisher's Exact Test	$p < 0.0001$

3) Incidence of Acute Rejection

Non Heart-Beating Donors	10/39 = 26%
Cadaveric Donors	33/91 = 36%
Fisher's Exact Test	p = 0.31

4) Graft Function

There was no difference in graft function, as measured by serum creatinine, between the groups at 6 months. However, function was significantly worse in the NHBD group than in the conventional cadaveric group at 2 years post transplant; at 3 years the difference is of marginal statistical significance (Figure 2.1 and Table 2.1). Similarly estimated creatinine clearance shows no consistent differences between the groups from 6 months post transplantation (Table 2.2)

5) Graft Survival

Cumulative graft survival rates by Kaplan Meier estimate were lower at 6 months, 1 year, 2 years and 3 years for NHBD transplants than for conventional cadaveric transplants (Figure 2.2). However, whilst a trend may be suggested by examination of the survival curves, analysis of the data by the log rank test shows no significant difference ($p=0.2$).

Figure 2.1: Box and Whisker Plot Showing the Median, Quartiles and Range of Values of Serum Creatinine after Transplantation with Kidneys from Conventional Cadaveric and Non Heart-Beating Donors.

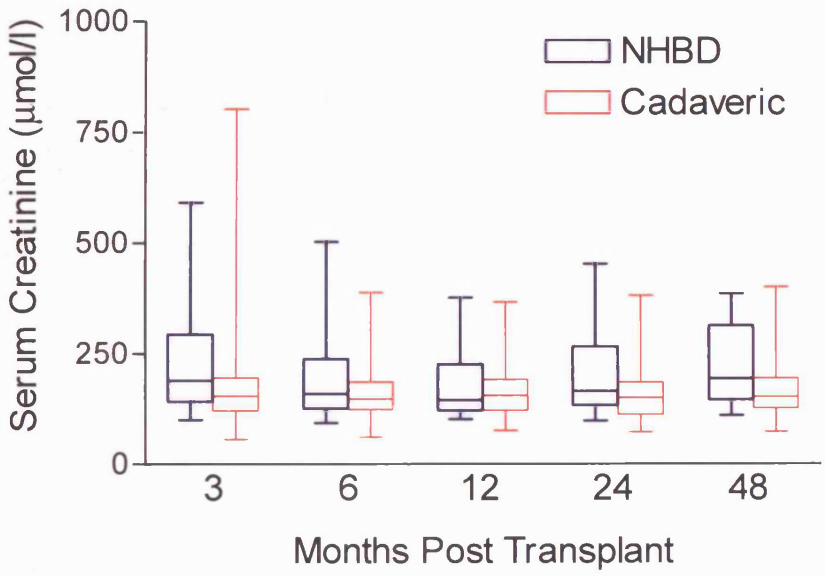


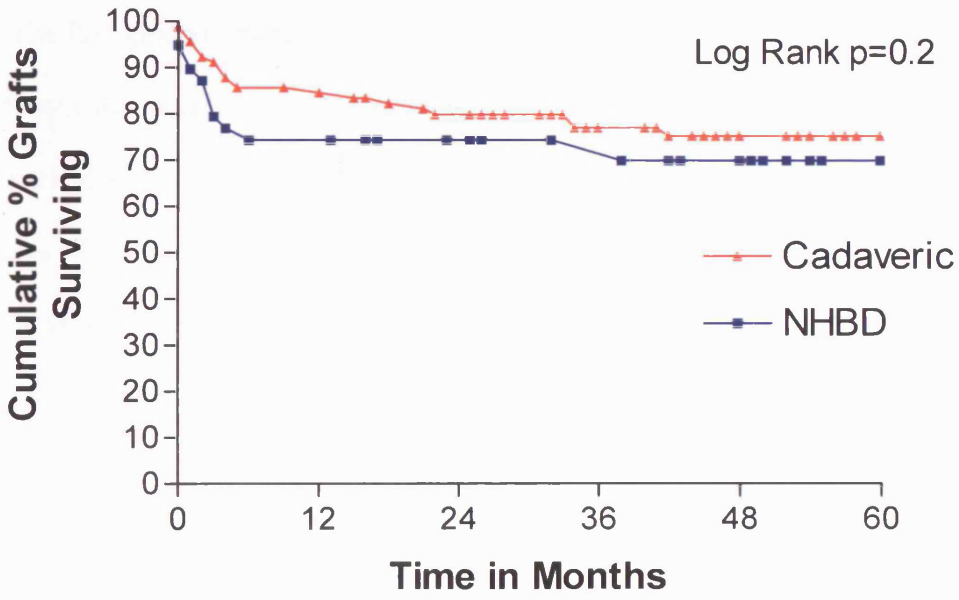
Table 2.1: Results of Renal Transplantation from Conventional Cadaveric and Non Heart-Beating Donors (NHBD) Expressed as Median Serum Creatinine (µmol/l) and Comparison of the Results using the Mann Whitney U Test.

Time	NHBD		Cadaveric		Mann Whitney
	µmol/l	(n)	µmol/l	(n)	
3 Months	189	32	154	84	p=0.01
6 Months	159	29	148	78	p= 0.15
1 Year	145	27	155	74	p=0.41
2 Years	165	21	150	65	p=0.04
3 Years	193	16	153	46	p=0.07

Table 2.2: Results of Renal Transplantation from Conventional Cadaveric and Non Heart-Beating Donors (NHBD) Expressed as Median Estimated Creatinine Clearance (mls /min) and Comparison of the Results using the Mann Whitney U Test.

Time	NHBD		Cadaveric		Mann Whitney
	mls/min	(n)	mls/min	(n)	
3 Months	34.8	32	40.9	84	p=0.04
6 Months	44.9	29	43.9	78	p= 0.26
1 Year	44.4	27	43.5	74	p=0.26
2 Years	34.9	21	44.1	65	p=0.04
3 Years	37.8	16	40.2	46	p=0.45

Figure 2.2: Kaplan Meier Graph Showing the Survival of Renal Transplants from Conventional Cadaveric and Non Heart Beating Donors (NHBD).



Actuarial Survival %				
NHBD	74	74	74	70
Cadaveric	83	79	77	75
Numbers at Risk				
NHBD	30	23	19	14
Cadaveric	77	68	49	36

Discussion

The first group to publish a comparison of the results of transplantation using NHBD and cadaveric donor kidneys, which included some control for these important variables was the Maastricht group. Using a case / control approach they 'matched' a number of NHBD transplants with a number of cadaveric donor transplants having similar profiles of the important variables outlined above (Wijnen et al, 1995). There were no differences in the survival of the two types of graft. These results of the Leicester series are broadly comparable with the Maastricht data.

In cadaveric renal transplantation it is well established that acute rejection is an important predictor of poor long-term graft survival (Ferguson, 1994). Delayed graft function is also associated with decreased allograft survival in many series (Sanfilippo et al, 1984; Yokoyama et al, 1994; Shoskes et al, 1996) and an increased incidence of rejection in transplants with delayed graft function is reported (Howard et al, 1994). Some authors suggest that in the absence of rejection, delayed graft function is not deleterious (Troppmann et al, 1996) and prolonged delayed graft function was seen to have little impact in a large Spanish series (Fontan et al, 1996). However, the combination of delayed graft function and rejection is considered particularly deleterious and these transplants have particularly poor survival (McLaren et al, 1999; Nicholson et al 1996b).

It is therefore reassuring that these results show no excess of acute rejection for the NHBD transplants despite the higher rate of delayed graft function and the lower doses of

Cyclosporine. The effect of the induction OKT3 given to a subset of the NHBD as a confounding variable is uncertain. Subset analysis on NHBD grafts not receiving induction therapy has not been performed because the numbers are small. However, there is as yet no evidence that delayed graft function is of prognostic significance in NHBD transplantation and this data is unlikely to be available in the near future because the number of NHBD transplants showing primary function (for comparison) is small.

Serum creatinine is not the ideal measure of true renal function in transplanted kidneys but data on glomerular filtration rate are not available. The quality of function in terms of serum creatinine shows a trend to be worse in NHBD transplants. The data for creatinine and estimated creatinine clearance at three months is a reflection of more delayed graft function in the NHBD group than in the cadaveric group. As the delayed graft function resolves serum creatinine in the NHBD group improves so that at six months there is no significant difference between the groups.

Many formulae exist for the estimation of creatinine clearance from the serum creatinine and characteristics of the patient. However, these formulae have been derived from a number of different study populations, none of which were renal transplant recipients. The utility of the Cockroft and Gault formula in the analysis of the results of the Leicester transplant unit are published (Butterworth et al, 1997): correlation between the estimated creatinine clearance and the clinically measured value was poor. The accuracy of this formula is improved in transplant recipients by administration of cimetidine, which inhibits tubular creatinine secretion (Kemperman et al, 2002), but this approach cannot be applied

retrospectively. In a comparative study of the predictive performance of a variety of estimating equations in renal transplant recipients, the Jelliffe formula was found to be the most precise, but quite wide differences between true and estimated value still occur (Goerdt et al, 1997). When this formula is applied to the Leicester data no consistent differences in graft function are seen after three months but interpretation of this data requires great caution because of the inherent inaccuracies in the technique.

The data and statistical analysis suggest that NHBD kidneys may not give quite as good function as the cadaveric kidneys with increasing duration of follow up, but at the time intervals studied the suggested differences would not be clinically significant to an individual patient. Certainly serum creatinine in the NHBD group compares satisfactorily with the cadaveric group at 3 years of follow up from a purely clinical standpoint. Actuarial graft survival is also shown to be comparable but further analysis of renal function data with larger numbers and longer follow up may be disappointing.

The major area of concern in the transplantation of NHBD kidneys is the incidence of primary non-function, which is significantly higher than in transplantation of cadaveric organs. The key factors involved are those causing irreversible renal damage prior to donor nephrectomy, the most important of which is warm ischaemia caused by inadequate renal perfusion during cardiorespiratory arrest, inadequate in situ flushing and inadequate cooling. The Leicester approach to the assessment of viability at the time of procurement has simply been to assess the macroscopic appearance of the kidney for evidence of inadequate cold perfusion. If a more sensitive test of viability were available it might be possible to reduce the

primary non-function rate and to decrease the discard of kidneys considered macroscopically inadequate, but in fact viable. The next chapter reviews viability testing in cell and organ systems with reference to their application in the setting of renal transplantation.

Addendum

This study has been extended to involve more NHBD transplants, matched in the same way to a contemporary series of cadaveric transplants, with longer follow up, to a maximum of 7 years. Published recently, (Metcalfe et al, 2001) the extended study showed no statistically significant difference in graft survival between the NHBD and cadaveric donor groups. It confirms the slightly worse function in terms of serum creatinine for NHBD kidneys but it is unlikely that this is of clinical significance for the recipient. Furthermore there was no difference in this larger series in the incidence of primary non-function. This may be explained by reference to a learning curve in the opinion of the retrieving surgeon concerning the viability of the in situ perfused kidneys. Early enthusiasm for the technique may have resulted in transplantation of more ischaemically damaged kidneys and thus a higher rate of primary non-function. Tempering of this enthusiasm by experience may result in only kidneys with ideal appearances being transplanted. The possible corollary being that some potentially viable kidneys may have been discarded.

It appears that doubts about the relative merits of transplantation with a NHBD kidney can be firmly put aside.

CHAPTER 3

REVIEW OF VIABILITY TESTS AS APPLIED TO RENAL TRANSPLANTATION.

REVIEW OF VIABILITY TESTS AS APPLIED TO RENAL TRANSPLANTATION.

Introduction

The early results of the Leicester NHBD renal transplant programme are concordant with all of the other published series of NHBD transplant programmes with regard to one key outcome. The incidence of primary non-function is high compared to conventional cadaveric donor series despite those kidneys that are considered poorly perfused at the time of retrieval being discarded. This is considered to be the result of the unquantifiable period of warm ischaemia to which kidneys from NHBD have been subjected. Primary non-function of a renal transplant carries a significant physical, psychological and immunological burden for the patient and a high rate of primary non-function discourages transplant surgeons from setting up NHBD programmes. In seeking to minimise the rate of primary non-function it is essential to ensure that the kidney is viable at the time of transplantation. The search for a pretransplant test of kidney viability has been ongoing since the earliest days of renal transplantation but until the advent of NHBD programmes interest in renal viability had waned because of the success of cadaveric kidney transplantation. This chapter reviews the state of the art in viability testing by outlining the known effects of ischaemia on the kidney, the current techniques available for its measurement and the limitations of these techniques when applied to the transplant situation.

Effects of Acute Renal Ischaemia

The kidney is relatively tolerant of acute ischaemic insults compared with the brain and heart. This is seen every day in clinical medicine by virtue of the fact that so few patients who suffer circulatory standstill as a consequence of cardiac arrest suffer severe renal impairment (Mattana et al, 1993). The clinical entities that may follow acute renal ischaemia depend on its severity, its duration and sometimes its cause.

Prolonged severe ischaemia causes cells to die by the process of necrosis. Lack of oxygen causes cessation of oxidative phosphorylation and cellular adenosine triphosphate (ATP) is rapidly depleted. Classically this is said to result in failure of the sodium potassium ATPase, an influx of sodium and water into the cell, subsequent rupture of intracellular membranes with release of lysosomal enzymes and finally plasmalemma rupture. These changes affect the most metabolically active cells i.e. the proximal tubular epithelial cells, most profoundly and result in the pathological phenomenon of cortical necrosis which has readily identifiable histopathological features.

Lesser ischaemic insults result in the clinical picture of acute renal dysfunction known as 'Acute Tubular Necrosis' (ATN). However, this entity, which is the usual cause of delayed graft function after transplantation, is not always associated with abnormal morphology and is perhaps better considered as acute Intrinsic Renal Failure. There is an abrupt and sustained decrease in renal excretory function (Glomerular Filtration Rate) occurring within minutes to days of an acute ischaemic (or nephrotoxic) insult which is not immediately reversed when the

initiating insult is eliminated. The mechanisms by which this decrease in excretory capacity occurs remain controversial but include:

i) changes in glomerular haemodynamics- renal blood flow may remain reduced or rapidly return to normal in established ATN: the interlobar arteries have been identified radiographically as a site of increased vascular resistance (Oken, 1984) and pre glomerular arteriole vasoconstriction reduces the pressure in the glomerular capillaries resulting in low GFR. However, GFR does not respond to vasodilatation, a phenomenon which is probably the result of concurrent reduction in the surface area and permeability of the glomerular capillaries (Baylis et al, 1976).

ii) back leakage of filtrate from damaged tubules - injury to the tubular cells may result in loss of tight junction integrity, or with more severe injury, cellular dysfunction with loss of barrier functions. Tubular filtrate could then diffuse freely back into the parenchymal interstitium. This phenomenon has been correlated with the severity of the ischaemic injury in animals (Donohoe et al, 1978) and has been identified in man (Moran et al, 1986).

iii) obstruction of the renal tubules - tubules may be obstructed by simple cell swelling or by intratubular protein precipitates and cellular debris. Detached cells may adhere to other tubular cells or form intratubular aggregates.

iv) tubulo-glomerular feedback- decreased absorption of sodium in the proximal nephron results in increased sodium delivery to the macula densa and consequently preglomerular arteriolar vasoconstriction.

Morphology of Acute Tubular Necrosis

In cases of clinical Acute Tubular Necrosis caused by nephrotoxins, especially the heavy metals, histopathological changes may be dramatic. In contrast, when the precipitating insult is renal hypoperfusion, morphological changes are subtle especially early in the history of the disease. Abnormalities are generally evident on electron microscopy (Jones, 1982) but under the light microscope necrosis of tubular cells is not necessarily seen (Solez et al, 1979) and indeed the histological appearances may be absolutely normal. When present the histopathological appearances are as follows: the glomeruli show no significant changes; the proximal tubular cells show early loss of the brush border of microvilli with later tubular dilatation and flattening of the epithelium as a result of cell loss; the intratubular space contains cellular debris and hyaline casts. Interstitial oedema may be seen and can be recognized by the widening of the distance between the tubules (Solez, 1983). However, it is important to remember that there may be established clinical ATN and a biopsy of renal cortex may be correctly reported to be normal. No correlation can reliably be found between morphological appearance and function.

Viability Testing in Renal Transplantation

It is possible that primary non-function of a renal transplant is a result of cortical necrosis occurring prior to transplantation. However, this is likely to be an over-simplification as it takes no account of the haemodynamic and immunological environment into which the kidney is transplanted. Fundamentally the kidney must not be infarcted before transplantation but the frankly infarcted or non-perfused kidney is obvious at retrieval and would not be transplanted. In addition a kidney may be considered to have suffered a significant ischaemic insult and subsequently show the phenomenon of acute tubular necrosis but may recover to give excellent renal function as a transplant. The aim of a viability test is to prevent the transplantation of kidneys that will never function because of injury occurring before retrieval or during preservation and to prevent the discard of kidneys which may appear damaged but have the potential for functional recovery. Both of these situations apply in NHBD renal transplantation. The techniques which have been applied to try to differentiate between kidneys which will function and those which will not function, i.e. proposed viability tests, will now be outlined.

i) Histopathological Assessment

Traditional histopathologic changes do not correlate well with transplant function and the light microscopic findings after renal hypoperfusion are variable as described above. In addition traditional histopathological preparations are not particularly suited to viability testing because of the time required to fix, embed, cut, stain and mount the specimens for assessment.

Andrews has reported the use of a novel microscope technology to assess the real time histopathological status of living tissue. Tandem Scanning Confocal Microscopy (TSCM) generates 120 images per second from a thick object (such as a tissue slice) by eliminating all the reflected light from above and below the plane of focus - no fixation of the tissue is required so this is microscopy of the living tissue or 'vital microscopy'. Changes in tubular microvillous brush border and epithelial swelling could be followed over 72 hours in harvested, cold stored rat kidneys (Andrews, 1994) and in an in vivo model where rat kidneys were subjected to ischaemia by arterial cross clamping (Andrews et al, 1991). However these anatomical changes are qualitative and could only be measured by semi quantitative grading at best. Further, as the author points out, the logistics of mounting the larger human kidney, still cold stored, on the microscope stage may be insurmountable. No human clinical studies of TSCM in human renal transplantation are reported and indeed the technique is not widely reported from other centres.

ii) Quantitation of Tissue Energetics

Since anoxia causes oxidative phosphorylation to cease, there is a rapid decrease in the amount of high energy phosphates in the form of adenine nucleotides within the cell. Measurement of cellular ATP might then reasonably be considered a way to assess the severity of prior and current ischaemia. ATP, total adenine nucleotides (TAN) and nucleotide degradation products (hypoxanthine) have all been studied as potential predictors of organ viability.

Measurement of ATP alone has not been shown to be useful because the amount of ATP in a cell at any one time is necessarily very small even in health and turnover is high. The ATP content of renal tissue is almost completely depleted within a few minutes of the onset of warm ischaemia (Bore et al, 1979) and clinical experience shows that 1 hour of warm ischaemia may still result in a viable transplanted kidney. Measurement of total adenine nucleotides i.e. ATP, Adenosine diphosphate (ADP) and adenosine monophosphate (AMP) in a model of in situ warm ischaemia in the rat shows a closer relationship to duration of warm ischaemia (Calman, 1974) than ATP alone, but there remains a wide scatter in the data.

During static cold storage of human kidneys the amount of ATP and ADP falls and the amount of AMP and adenine nucleotide degradation products rises (Maessen et al, 1988a; Vigues et al, 1993). Hypoxanthine is quantitatively the most important degradation product of adenine nucleotides in the preserved kidney (Buhl et al, 1976; Maessen et al 1988a). The ratio of degradation products, chiefly hypoxanthine, to total adenine nucleotides has been shown to reflect the period of warm ischaemia in a canine model of renal ischaemia caused by renal artery clamping (Maessen et al, 1988b). A set of 'standard' index curves were developed by these authors with the aim of estimating the duration of warm ischaemia suffered by a kidney showing a particular purine catabolic index (ratio of degradation products to adenine nucleotides). To date these curves have not been validated by retransplant experiments and no comparable study in humans exists in the literature.

The major problem in the utilisation of these tests in a clinical setting is a logistical one. Firstly in situ flushing with perfusate is likely to wash out metabolites and degradation

products from the kidney making their post retrieval assessment impossible. Secondly the quantitative assessment of adenine nucleotides is very difficult. High pressure liquid chromatography has been used extensively in the experimental setting but it is unlikely that this can be performed sufficiently swiftly to be of value in the clinical setting. Commercial kits are available to assay ATP, ADP and AMP separately but they have not been widely utilised in this context.

However, it is possible to assay the cellular energetics of the whole organ using a non-invasive technique. The phenomenon of nuclear magnetic resonance is familiar to clinicians as a technique for imaging anatomy. A similar technique, known as magnetic resonance spectroscopy (MRS) utilises sensitive nuclei to investigate the composition of in vitro body fluids or function of ex vivo isolated organs. Measurement of the resonance of phosphorus-31 (^{31}P) can provide information about cellular bioenergetics in a wholly non-invasive way. The magnetic resonance spectrum of ^{31}P acquired from an isolated kidney contains up to seven peaks representing different phosphorus compounds. In cold stored kidneys there are no discernible peaks corresponding to high energy phosphorus compounds but the ratio of peaks corresponding to AMP and inorganic phosphate can be measured and provide a way of following the hydrolysis and regeneration of high energy phosphate nucleotides over time.

^{31}P MRS of the kidney was first studied in isolated cold stored and subsequently reperfused rat kidneys in 1979 (Sehr et al, 1979). Energy levels as measured by MRS have been shown to decay in a time dependent manner in the rat kidney (Bretan et al, 1986) and similar results are seen in a pig model of warm ischaemia followed by static cold storage.

(Cadrobbi R et al, 1997). In dogs the ability to regenerate high energy phosphates on reperfusion has been related to ^{31}P MRS parameters after ischaemia (Bretan et al, 1987).

Studies using ^{31}P MRS in clinical renal transplantation are few and contain limited numbers. A group in the Netherlands found MRS parameters to be related to whether the donor kidney was from a live donor, a cadaveric donor or a non heart-beating donor, which reflects the duration of warm ischaemia (Hene et al, 1994). The ratio of phosphate monoesters (AMP) to inorganic phosphate has been correlated with the incidence of delayed graft function and the adequacy of renal function (Bretan et al, 1989) but the attempting to describe cut off values to predict initial or delayed function only produces sensitivity of 0.75 and specificity of 0.87 with wide confidence intervals. A Japanese group showed MRS detection of high energy phosphate to be correlated with graft survival in live related and cadaveric kidneys, though overall graft survival in this data set is not as good as might be expected (Seto et al, 2001).

^{31}P MRS looks very attractive at first glance as a technique for not only assessing the severity of renal injury but perhaps also the potential for recovery. However, no attempt to describe cut off limits for the determination of the viable from the nonviable kidney has been published. Furthermore the technique has only been reported from a small number of centres, which suggests it is technically demanding and difficult to set up. Data concerning the reproducibility of the technique in renal transplantation are missing from the literature and in the last decade the number of reports has dwindled dramatically.

Efforts to improve adenine nucleotide status have not been shown to improve renal transplant viability in canine kidneys retransplanted after prolonged warm ischaemia (Pegg et al, 1984). Clearly there is more to viability testing than the assessment of tissue energetics.

iii) Quantitation of Cellular Necrosis

The process of necrotic cell death results in the loss of membrane integrity. As a consequence macromolecules, which are retained intracellularly or excluded by the cell membrane in health, may traverse the cell membrane of necrotic cells by diffusion. Permeability assays are widely used in tissue culture systems and in flow cytometry (Cook et al, 1989). The classic tests in this situation are dye exclusion tests- macromolecules which would normally be excluded from the cytoplasm of live cells by an intact cell membrane. Trypan blue is a large macromolecule which stains membrane damaged cells purple-violet under light microscopy whereas cells with an intact membrane exclude the dye and remain translucent. Propidium iodide, if able to enter the cell, binds to nucleic acids and becomes highly fluorescent and so is perfectly suited for the 'viability' assessment of large numbers of suspended cells by flow cytometry. However, the dye exclusion assays do not readily lend themselves to the assessment of tissue specimens.

Macromolecule release into the substrate (or perfusate of machine preserved kidneys) is an alternative form of permeability assay, which has the advantage of being a non invasive method of assessing membrane damage. This simple fact has provided a focal point for the assessment of renal viability from the earliest days of renal preservation up to today. The

macromolecules assessed and the size of the membrane defects required to release them may have changed in the hope of detecting earlier and less severe forms of ischaemic injury, but the basic tenet remains the same. The key targets for study have been lysosomal enzymes as these are strictly intracellular macromolecules. Lactate dehydrogenase (LDH) is the enzyme most extensively studied. Dehydrogenase activity is measured in cell or tissue supernatant fluids by the conversion of exogenously added lactate to pyruvate and assessment of the NADH that is a by-product. This approach was first reported in renal preservation by Belzer (Belzer, 1968). Canine kidneys subject to warm ischaemia were machine perfused and LDH release into the perfusate was measured. LDH release was greater in those kidneys subject to the longest period of warm ischaemia. However, it was later noted by other authors that whilst in general prolonging the duration of ischaemia does result in increased LDH release, the magnitude of the release varies between kidneys. Thus LDH release could not be used to predict the prior ischaemic period retrospectively in canine kidneys preserved by static storage on ice (Kohn et al, 1971). In a study of cadaveric donor kidneys preserved by pulsatile machine perfusion, increasing amounts of LDH were released with increasing periods of cold ischaemia but the amount released did not correlate with the duration of warm ischaemia or have value in predicting the adequacy of post transplant function (Horpacsy et al, 1979).

Many other enzymes have been investigated in the search for the compound that reliably reflects renal damage and also predicts post transplant function. The amount of 'Ligandin' released from machine perfused cadaver kidneys into the perfusate was found to be greater when the kidney showed delayed graft function than when the kidney showed initial function (Cho et al, 1981). Ligandin has since been renamed alpha glutathione S-transferase

(αGST). This is a relatively small cytosolic macromolecule (46000 daltons) and it was hoped that it would detect membrane permeability earlier in the process of tubular cell necrosis. In a study of NHBD kidneys preserved by pulsatile machine perfusion, the Maastricht group showed that there was a significant difference in the αGST released by the group of kidneys which functioned and the group of kidneys which did not function. However, the spread of the raw data shows that the release of this enzyme is not sufficiently reliable to discard kidneys in the certain knowledge that they will not function: the predictive value for discarding a nonviable kidney was only 56% (Daemen et al, 1997).

Proton magnetic resonance spectroscopy has been used to study low molecular weight compounds in fluids. Trimethylamine-N-oxide (TMAO) is an osmotically active molecule synthesized in the renal medulla. In health it is not detectable in the urine, but after ischaemia it is released into the urine presumably as a result of necrosis of medullary epithelial cells. MRS of perfusate has been used to study TMAO release during normothermic perfusion of pig kidneys after cold storage (Hauet, et al 1997) and release of TMAO into urine after transplantation also in a pig model (Richer et al, 2000). However, there are no reports of MRS analysis of the effluent from the primary flush after organ retrieval and quite what advantage the use of MRS has over a conventional biochemical assay is unclear. The technique has not been widely reported.

Electrolyte shifts also occur during necrosis but have been relatively poorly studied as measures of renal viability. In health potassium is the primary intracellular anion and sodium is primarily extracellular. Failure of the sodium potassium pump because of a lack of ATP

allows potassium to diffuse out of cells along its concentration gradient; rupture of necrotic cells releases large quantities of potassium into the interstitium. Biopsies of rat and human kidneys have been assessed for the intracellular potassium to sodium ratio, with high ratios correlating with better post transplant function (Sells et al, 1977). Extracellular ion shifts have more recently been studied in human transplants. Using a novel 'multisensor element' for non-invasive organ surface monitoring a German group has correlated post transplant function with extracellular potassium concentrations and changes on reperfusion at transplantation (Abendroth et al, 1993; Fenzlein et al, 1991). This approach has some problems as a viability test in the pretransplant setting because of the high potassium concentrations used in perfusion fluids but the simplicity is attractive.

Damage to the extracellular matrix may occur during cold storage. In orthotopic liver transplantation there is a correlation between levels of extracellular matrix components in the immediate pretransplant washout effluent and early graft function. Such a relationship is not seen in renal transplantation. (Rao et al, 1993)

iv) Functional Studies

During periods of ischaemia cells may rely on anaerobic metabolism for the production of high energy phosphates. Anaerobic metabolism results in the accumulation of lactate. The measurement of the accumulation of lactate in the kidney was one of the approaches taken by the pioneers of renal viability studies. Lactate release during the first hour of preservation of the kidney on the pulsatile perfusion machine was considered to reflect ischaemia prior to

retrieval and it was shown that the amount of lactate released correlated with whether there was delayed function or immediate function. However, there was some overlap in the data and no assessment of lactate release in kidneys showing primary non-function was made (Baxby et al, 1975). In contrast a German group could find no relationship at all between the amount of lactate washed out from human kidneys during the first hour of machine perfusion and either the duration of warm ischaemia or the adequacy of post transplant function (Horpacsy et al, 1979).

The previous techniques for assessment of viability all measure the effects of the ischaemic insult. However, what is most important in the clinical setting is the ability of the kidney to recover from the insult in its new environment. Few tests of the residual function and so perhaps the potential for recovery have been described. However, the metabolic capacity of cells may be assessed using the tetrazolium reduction assay. Tetrazolium is reduced by functioning dehydrogenase enzyme systems in mitochondria to an insoluble formazan. This product is coloured and may be seen with the naked eye or assessed quantitatively by spectrophotometry after dissolution of the formazan. This technique was first reported using biopsy specimens from a variety of laboratory animals in 1967 (Terasaki et al, 1967). Time to perceptible colour change correlated well with the duration of warm ischaemia of $1mm^3$ biopsy specimens. These results could not be reproduced using biopsies of canine kidneys stored at 37°C with formazan product measured spectrophotometrically (Ham et al, 1969) but later work using real time photometry of colour change on tissue slices suggested that the rate of change of colour accurately reflected the previous warm or cold ischaemia to which the tissue slices had been subject (Yin et al, 1988). This work has particular relevance in the

NHBD setting where the true duration of warm ischaemia cannot be accurately determined. Human trials were suggested but none have been reported and indeed no replication of this exciting work has been reported either.

The perfusion characteristics of the non heart-beating donor kidney have been proclaimed as the most recent answer to the search for a functional test of viability. This technique of preservation is probably superior to static cold storage for ischaemically damaged kidneys (Matsuno et al, 1994) though this has not been established by randomised trials or long term graft survival data. Nevertheless machine perfusion facilitates the monitoring of flow and intrarenal resistance and the measurement of the release of substances from the kidney into the perfusate. Blood flow in the kidney immediately after transplantation does correlate with the presence of initial graft function and flow of perfusate through the kidney during preservation has also been correlated with graft function (Tesi et al, 1994). The data to support the enthusiasm for flow characteristics is not as clear as its supporters would suggest but it is certainly one avenue where further research is clearly needed and this will be explored further in the final chapter of this thesis.

What is clear is that currently there is no objective test of viability that allows some donor kidneys to be discarded in the confidence that they would not function after transplantation. Nor is there a test that provides reassurance that a kidney of dubious subjective viability, perhaps with a prolonged period of warm ischaemia will definitely function after transplantation.

In reviewing the viability tests currently available three areas remain largely uninvestigated:

1) No in vitro model of warm ischaemia exists in which tests of cell viability and perhaps even the protective effects of exogenous agents could be tested.

2) Little emphasis has been given to processes of cell injury other than necrosis: the role of apoptosis remains largely unexplored.

3) It has been largely assumed that all kidneys have the same tolerance to warm ischaemia and all that is important is the duration of warm ischaemia itself. No assessment has been made of the underlying quality of kidneys from NHBD.

These deficiencies in the literature will be addressed by the experimental work presented in the following chapters.

CHAPTER 4

IN VITRO MODELLING OF WARM ISCHAEMIA

IN VITRO MODELLING OF WARM ISCHAEMIA

Introduction

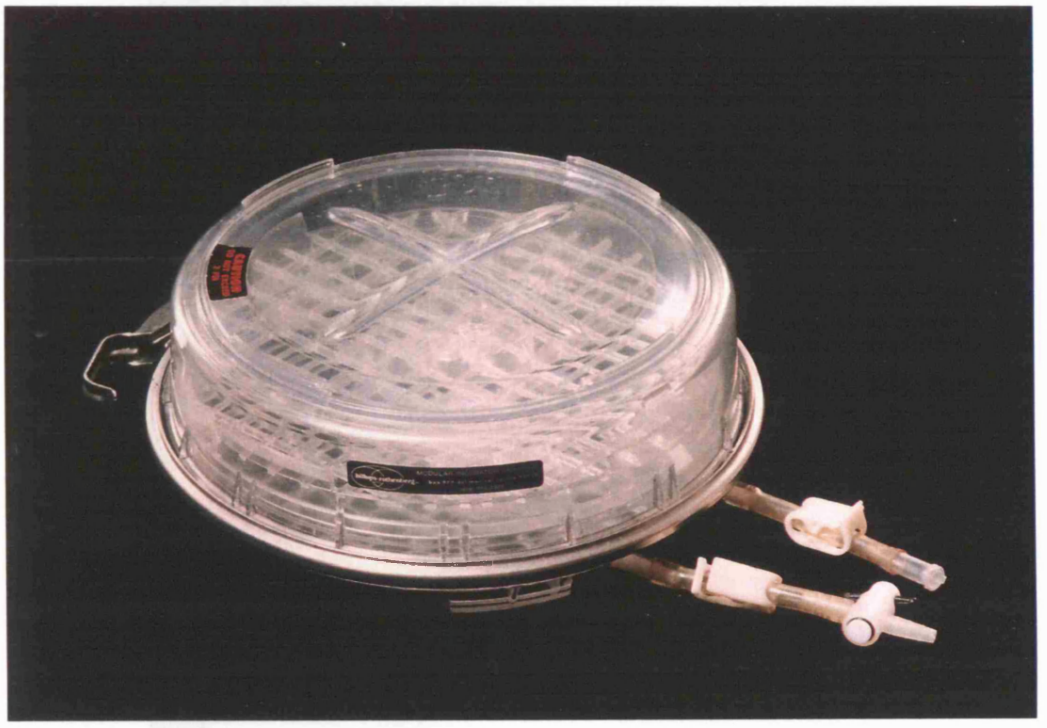
An in vitro model of warm ischaemia would significantly advance the search for a whole organ test of kidney viability. Perhaps more importantly it would facilitate the study of compounds or conditions that might protect the cells against the harmful effects of hypoxia. No in vitro model of this type currently exists in the transplantation literature. The freshly isolated nephron is commonly studied but tissue culture systems of a single cell type offer the advantage of reliably studying the same cell particularly if immortalised cell lines can be used. Poisons which arrest oxidative phosphorylation undoubtedly cause cellular Adenosine Tri–Phosphate (ATP) levels to fall in vivo, but a model which uses a deoxygenated environment might more accurately reflect the in vivo situation. Indeed such a model has been shown to have demonstrable effects on the metabolism of cultured pulmonary endothelial cells (North et al, 1994).

The first step in establishing an in vitro model is to develop a technique for rendering tissue culture medium deoxygenated/hypoxic. Some investigators simply describe gassing the medium with a hypoxic gas mixture (North et al, 1994). Two linked experiments were performed to investigate a gassing technique designed to deoxygenate culture medium and also to investigate how rapidly reoxygenation may occur and so how difficult it would be to manipulate deoxygenated medium in air.

1) Deoxygenation Experiment

The aim of the first experiment was to investigate the degree to which tissue culture medium becomes hypoxic when placed in an oxygen free environment and the rate at which this occurs. Three fluids used commonly in tissue culture were studied: phosphate buffered saline, RPMI tissue culture medium and RPMI with 10% foetal calf serum (FCS). Aliquots of 2ml of fluid (n=6) were pipetted into petri dishes so as to create as large as possible surface to volume ratio for equilibration of gases by diffusion. The petri dish was then placed in an airtight chamber (Figure 4.1) and flushed continuously with a gas mixture of 95% nitrogen/ 5% carbon dioxide. The chamber was then placed in an Atmosbag (Sigma Aldrich, UK) which was subsequently sealed then evacuated and refilled with a 95% nitrogen 5% carbon dioxide gas mixture three times (Figure 4.2). The medium was sampled using gas tight syringes at baseline (room air) and then at set time intervals the airtight chamber was opened in this environment of 95% nitrogen/ 5% carbon dioxide and the fluid was further sampled. Analysis for oxygen content was performed using a blood gas analyser (Ciba Corning).

Figure 4.1: Photograph of the Gas Tight Chamber Used in all of the In Vitro Modelling Experiments.



The chamber can be flushed with a gas mixture through the two lengths of tubing seen to the right of the picture. Access to the chamber is gained by opening the compressive circular metal lock: the spring loaded handle is seen to the left of the picture.

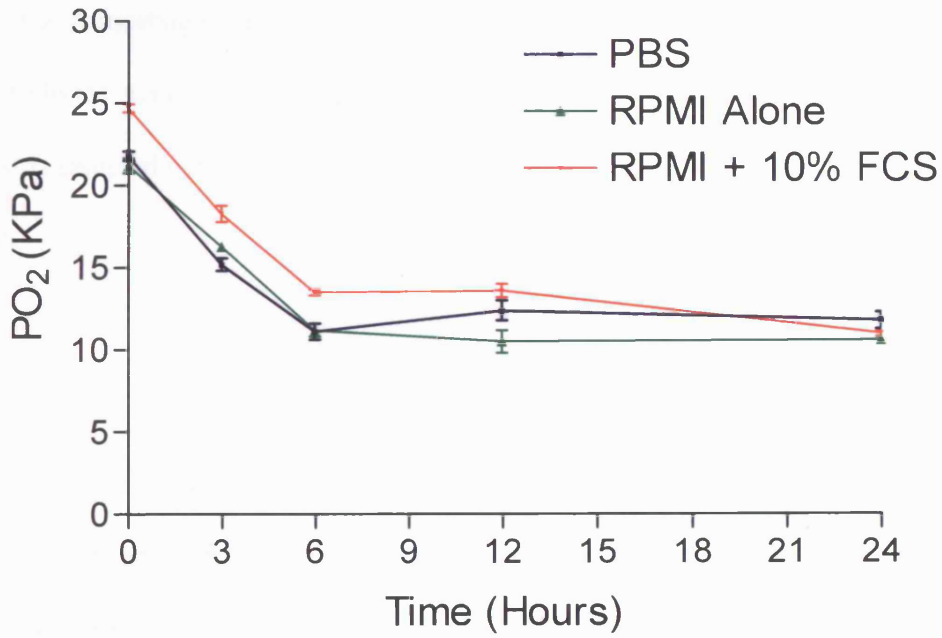
Figure 4.2: Photograph Showing the ATMOSBAG filled with 95% Nitrogen/5% Carbon Dioxide for Handling of Deoxygenated Culture Medium in a Deoxygenated Environment.

The ATMOSBAG is evacuated using the bench vacuum and refilled using the 95% nitrogen / 5% carbon dioxide gas mixture from the pink gas cylinder.

Results

Each of the three fluids tested had a mean PO_2 in room air of greater than 20KPa and this could not be reduced to below 10KPa by simple gassing as indicated in Figure 4.3 below.

Figure 4.3: Partial Pressure of Oxygen in Tissue Culture Fluids After Gassing with 95% Nitrogen/ 5% Carbon Dioxide (Mean/SD).



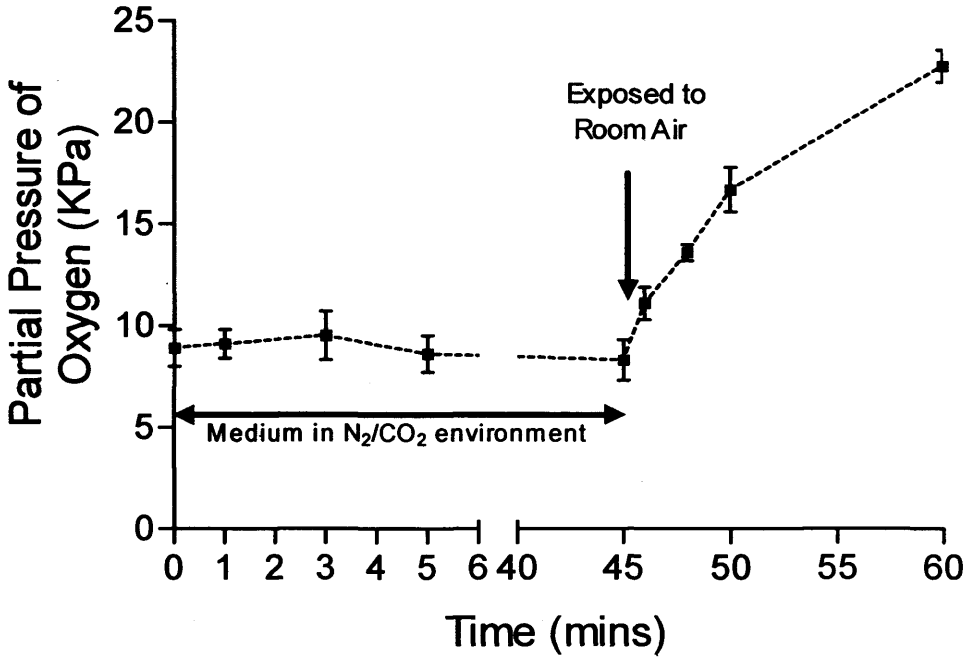
2) Reoxygenation Experiment.

The aim of the second experiment was to investigate the rate at which hypoxic medium reoxygenates in air. Aliquots of 2ml of RPMI with 10%FCS (n=6) were pipetted into a petri dish, placed in the airtight chamber and gassed with 95% nitrogen / 5% carbon dioxide. A sachet of anaerogen (Unipath), a commercially available product used to generate anaerobic conditions in closed spaces for bacteriology, was added to the chamber which was then sealed immediately. The chamber remained sealed on the bench for 24 hours and was then placed in the Atmosbag, which was evacuated and flushed as described. At time zero the chamber was opened to the Atmosbag environment and the medium sampled over time; samples remained capped within the Atmosbag. At 45 minutes the Atmosbag was opened to the room air and the medium was sampled again over time. Analysis of the medium was by blood gas analyser as described.

Results

The results are illustrated graphically in Figure 4.4. Maintaining the medium in a deoxygenated gas environment for 24 hours did not result in a further decrease in the partial pressure of oxygen; but reoxygenation did not occur. On exposure to room air, reoxygenation is rapid.

Figure 4.4: Graph Showing the Partial Pressure of Oxygen in Culture Medium Whilst in a Deoxygenated Gas Environment and on Exposure to Room Air (Mean/SD).



Conclusion

Oxygen in solution in tissue culture medium rapidly equilibrates with the oxygen in room air. These results suggest that simple gassing of the medium with an oxygen-free gas is not sufficient to ensure anoxic culture. Handling of deoxygenated medium for experimentation cannot be done in room air because the rate of reoxygenation is so rapid. However, the use of the Atmosbag and the gas-tight chamber do prevent reoxygenation and facilitate experiments with deoxygenated medium.

3) Assessment of Cell Viability After Warm Culture in Deoxygenated Medium.

Introduction

The previous experiment established a technique whereby tissue culture medium could be

(i) deoxygenated down to a PO_2 of around 10KPa (hypoxic medium)

(ii) maintained in a deoxygenated state and prevented from reoxygenating by storing in a sealed container gassed with oxygen free gas.

(iii) manipulated in a deoxygenated gaseous environment such that it can be pipetted into tissue culture plates.

To assess the validity of this model in the study of warm ischaemia, cells growing in culture were exposed to the deoxygenated medium. The aim of the experiment was to investigate the effect of culture in hypoxic medium on the viability of the cultured cells. The cells studied were the immortalised pig renal proximal tubule endothelial cell line LLC-PK1. A proximal tubular cell line was selected because this region of the nephron is considered the most metabolically active and the use of an immortalised cell line guarantees that the cells going into the model are the same for each experiment. Protocols relating to tissue culture are given in Appendix 1 and the details of regents and suppliers are given in Appendices 7 and 8 respectively.

Method

Cells were cultured in RPMI with 10% FCS to confluence in 7 of the 8 wells in each column of a 96 well plate under normally oxygenated conditions. Tissue culture medium, also RPMI/10%FCS was deoxygenated according to the protocol in the previous experiment, transferred within the deoxygenated Atmosbag to a sealed universal container. The tissue culture plate was then removed from the incubator and placed in the Atmosbag, which was again deoxygenated by repeated evacuation and flushing with 95% nitrogen/5% carbon dioxide. Each well was aspirated of oygenated medium, washed with 200µl of deoxygenated medium, which was then discarded and replaced by a further 200µl of deoxygenated medium. The plate was placed in the gas tight chamber, which was sealed, removed from the Atmosbag and placed in an incubator at 37°C. At set time intervals the plate was returned to the Atmosbag and viability assay commenced on two columns of wells (n=12) for each time interval. The MTT assay was used according to the protocol in Appendix 2, page 138. MTT was added to each well containing cells and medium in the unused wells was aspirated into a gas tight syringe for analysis of oxygen content on the blood gas analyser as described. The plate was then returned to the chamber and placed in the incubator. Incubation with MTT was for 4 hours after which time the medium was aspirated and discarded and the cells and MTT crystals disrupted with dimethyl sulfoxide in ethanol (all within the Atmosbag environment). Medium containing the dissolved formazan crystals was then transferred to a separate plate for reading of the assay on an automated 96 well plate reader. The absorbance recorded by the plate reader reflects the concentration of the coloured product and is proportional to the number of viable cells.

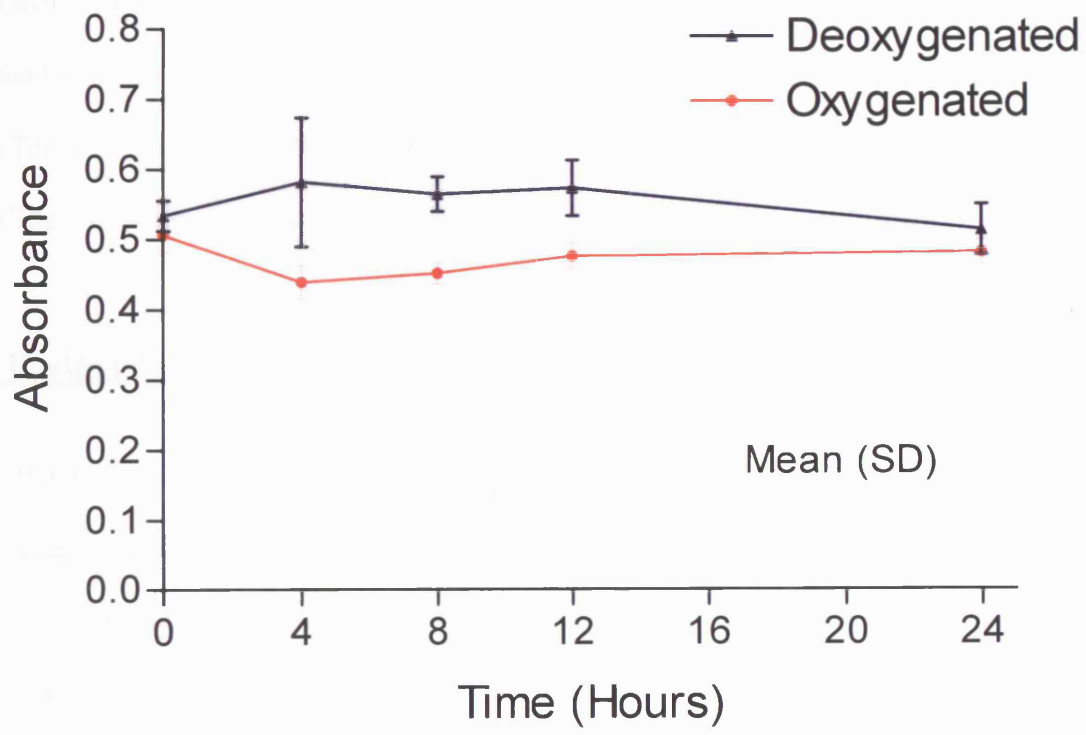
The viability of the cells in the two experimental conditions (as measured by the MTT assay) was compared using Student's t test with $p<0.05$ considered significant.

Results

The medium from only a single well was assayed for oxygen content for each time period and this is considered to reflect the conditions in the other wells for that time period. The PO_2 in the deoxygenated medium was between 10 and 11KPa for all wells assayed at all time intervals.

The results for the MTT assay are shown graphically in Figure 4.5. The probability of there being no difference between the viability of the cells at baseline, 4, 8, 12 and 24 hours was $p=0.72$, $p<0.001$, $p<0.001$, $p<0.001$ and $p=0.0525$ respectively. However, it is the deoxygenated culture which shows the greatest viability as measured by the MTT assay. This result is difficult to explain but certainly no decrement in viability as a consequence of culture in deoxygenated medium has been identified.

Figure 4.5: Graph Showing the Viability, Measured by the MTT Assay, of LLC PK1 Cells Cultured in Oxygenated and Deoxygenated Medium.



Conclusion

These cells tolerate prolonged culture in deoxygenated medium without significant effect on viability. There are three possible explanations:

(1) It is possible that the degree of deoxygenation of the medium i.e. to PO_2 of 10-11 KPa was not sufficient to induce necrosis: in vivo arterial blood has a PO_2 of 12-14Kpa and venous blood has a PO_2 of 5KPa.

(2) The MTT assay is unable to detect cell death in this system.

(3) These cells do not have the same response to hypoxia as renal tubular cells in vivo.

4) Further In Vitro Modelling of Warm Ischaemia

Having been unsuccessful in inducing necrosis in LLC PK1 cells by culture at PO_2 of 10-11 KPa, it was necessary to address two key issues:

(i) Can the medium be deoxygenated further?

(ii) Is the MTT assay suitable for use in this model? Is there a positive control?

i) Decreasing the PO_2 in the Medium

It has already been shown that more prolonged exposure of the medium to a deoxygenated gaseous environment did not results in greater degrees of hypoxia. Consequently it was decided to add an oxygen scavenger to the medium. Sodium dithionite is a powerful reducing agent that removes dissolved oxygen from solutions. Adding dithionite to the medium in the deoxygenated gaseous environment of the Atmosbag caused the PO_2 to fall to

less than 0.2KPa (i.e. below the level detectable by the blood gas analyser). A 100mM solution of sodium dithionite was made up by dissolving 0.087g of dithionite in 5mls of balanced salt solution. By a process of serial dilutions it was established that the lowest concentration of dithionite which could achieve this effect in culture medium was 5mM i.e. addition of 1ml of the 100mM dithionite solution to 19mls of medium. At this concentration the deoxygenating effect of dithionite was rapidly ablated by exposure to room air, necessitating continued handling of 'anoxic' medium in the Atmosbag. In addition the use of dithionite caused the medium to become more acidic- this could not be easily counteracted using RPMI medium and so subsequent experiments use Dulbeccos Modified Eagle Medium (DMEM) and 20mM Hepes buffer which generated a medium with PO_2 at or below 0.2kPa with a pH 7.1

ii) Developing a Positive Control

If the MTT assay is to be considered sensitive to changes in viable cell number it was necessary to establish a positive control model. To establish the positive control the effects of two metabolic poisons on cell viability, as assessed by the MTT assay, were studied. Cyanide and azide are both potent inhibitors of oxidative phosphorylation. They bind to Cytochrome oxidase and prevent electrons being passed along the electron transport chain. Potassium cyanide is a donor of the cyanide ion. Models of 'chemical hypoxia' using hepatocyte cultures show potassium cyanide to cause rapid cell death at concentrations of 1mM. Concentrations of potassium cyanide of 2.5mM, in conjunction with inhibitors of glycolysis, cause a 95% decrease in cellular adenosine triphosphate within five minutes (Gores et al, 1988). Under such conditions, viability is reduced by more than 50% within one hour

(Gores et al, 1989). In isolated proximal tubular cells, incubation with 1mM potassium cyanide inhibits oxygen consumption by 85% with immediate effect (Lash et al, 1989). The establishment of positive control conditions was assessed by adding these compounds to the medium of cultures of LLC-PK1 cells at concentrations of 0.5mM, 1mM and 2mM for potassium cyanide and 0.1%, 1% and 2% sodium azide (weight for volume i.e. 1g of azide in a litre of culture medium gives a 1% solution). The plates were incubated at 37°C under normal oxygenated conditions and assayed using the MTT assay (with 4 hour incubation of MTT as in the previous experiment) at baseline, 1 hour, 2 hours and 4 hours with n=8 wells for each time interval.

Results

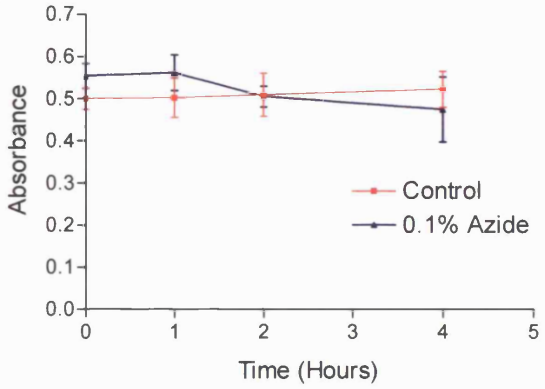
The results are shown graphically in figures 4.6 and 4.7. Culture with cyanide did not result in any decrease in cell viability as measured by the MTT assay. However, co culture with azide produced a decrease in formation of the tetrazolium product and this was accompanied by visible detachment of cells from the base of the well. The use of 2% azide caused the measured viability to decrease most rapidly and in a time period that is not dissimilar to the onset of necrosis seen in organs in vivo.

Conclusion

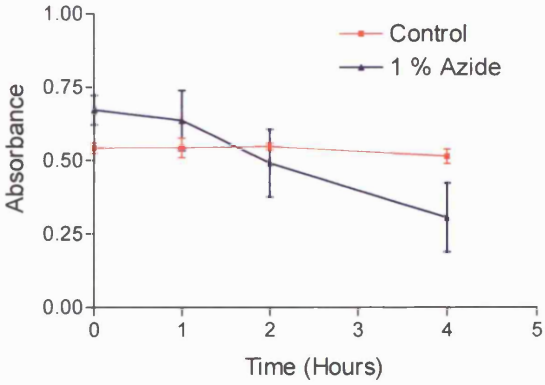
Addition of 2% azide to the culture medium produces a decrease in the viable cell number in the culture well which can be measured objectively with the MTT assay: it may therefore be considered an appropriate positive control.

Figure 4.6: Graph Showing the Effect on Viability of Culture of LLC PK1 Cells in the Presence of Azide (mean/SD)

i) 0.1% Azide



ii) 1% Azide



iii) 2% Azide

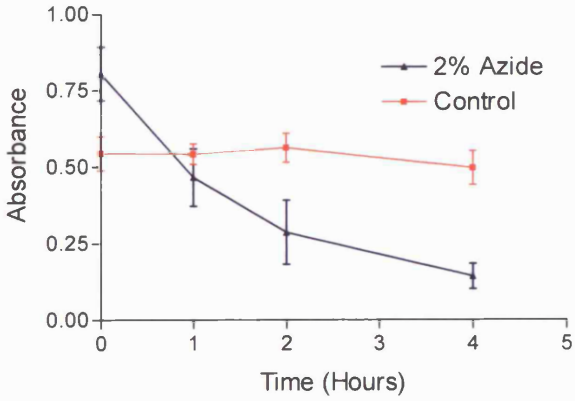
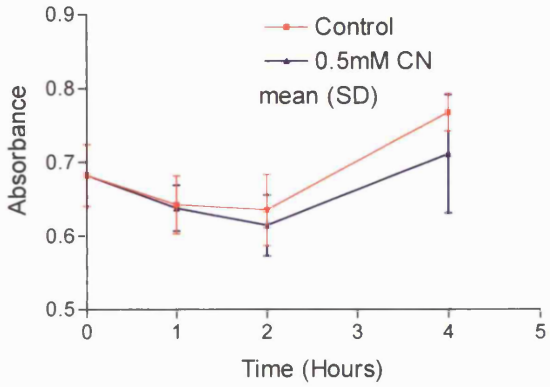
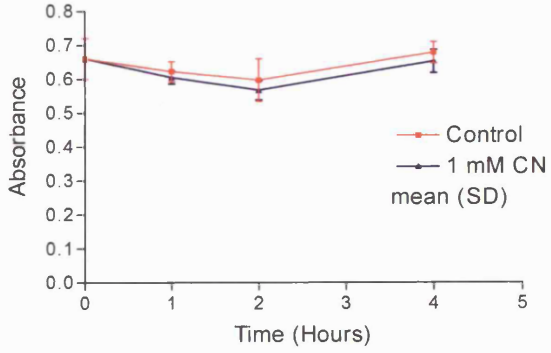


Figure 4.7: Graph Showing the Effect on Viability of Culture of LLC PK1 Cells in the Presence of Cyanide (mean/SD)

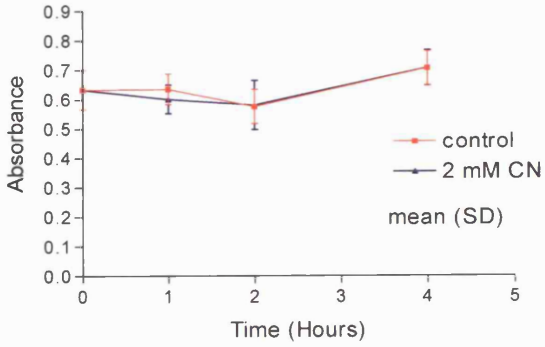
i) 0.5mM Cyanide



ii) 1mM Cyanide



iii) 2mM Cyanide



5) Assessment of the Viability of Cells after Culture in Anoxic Medium: Testing the Model

With the model of warm ischaemia now established as described above, the viability of cultured cells under conditions of anoxia (with a degree of acidosis) was tested.

Method

Experiments were repeated under these conditions using three cell types: immortalised LLC-PK1 cells, a non-immortalised human renal tubular epithelial cells and endothelial cells derived from human umbilical veins (HUVECs). Cells were grown to confluence in 12 columns of 6 on 96 well plates. Three plates of cells were used for each cell type: a control plate with normally oxygenated medium, a positive control plate with the medium containing 2% azide and an experimental plate in which the medium was changed for anoxic medium and cultured in an atmosphere of 95% nitrogen and 5% carbon dioxide within the model system described above. Two columns of each plate were used as baseline controls and the medium of the remaining wells changed accordingly. Viability was assayed at 2, 4, 6, 12 and 24 hours using the MTT assay as described and Student's t test was used to compare the groups.

Results

The results are shown graphically in Figures 4.8, 4.9 and 4.10 and the result of statistical analysis by t-test is given in the accompanying Tables 4.1, 4.2 and 4.3.

Figure 4.8: Graph Showing the Effect of Culture in Anoxic Medium on Viability of LLC PK 1 Cells as Measured by the MTT Assay

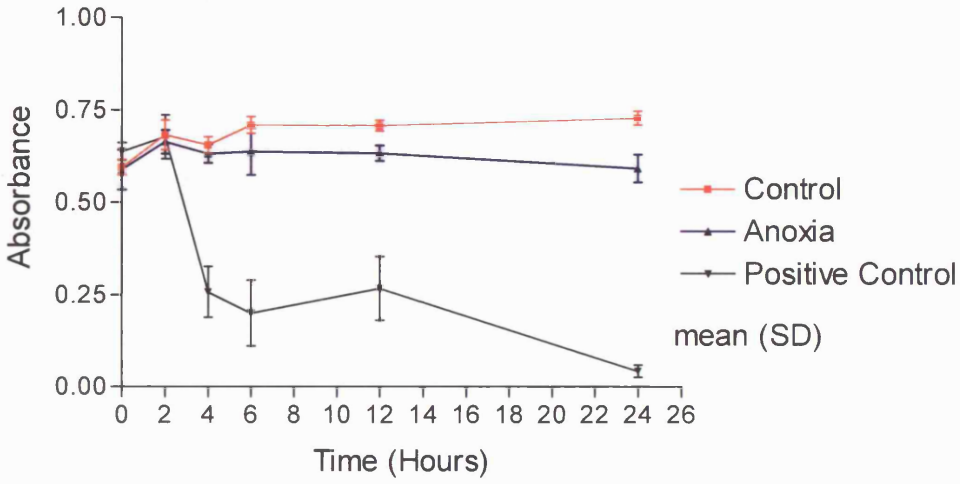


Table 4.1: Comparison Between the Viability of LLC PK1 cells in Control and Anoxic Conditions

Time	Baseline	2 Hours	4 Hours	6 Hours	12 Hours	24 Hours
t test	p=0.7685	p=0.22	p=0.02	p=0.0015	p<0.0001	p<0.0001

Figure 4.9: Graph Showing the Effect of Culture in Anoxic Medium on Viability of Human Tubular Epithelial Cells as Measured by the MTT Assay.

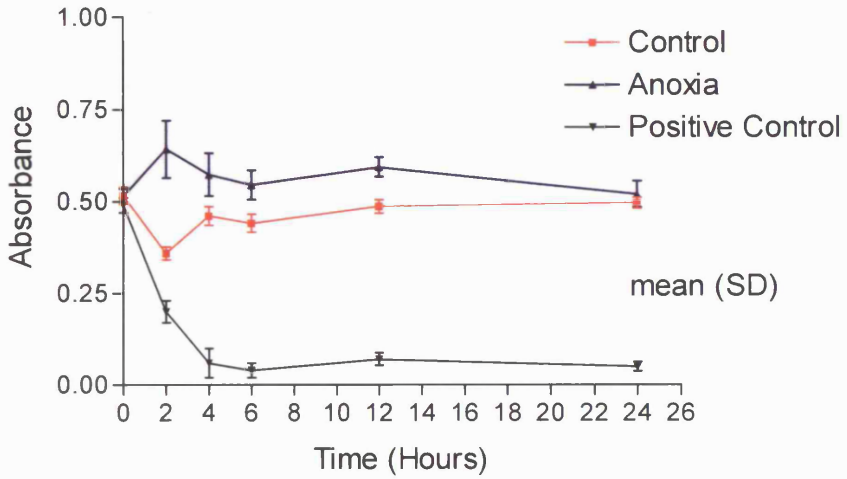


Table 4.2: Comparison Between the Viability of Human Tubular Epithelial Cells in Control and Anoxic Conditions

Time	Baseline	2 Hours	4 Hours	6 Hours	12 Hours	24 Hours
t test	P=0.827	P<0.0001	P<0.001	P<0.0001	P<0.0001	P=0.0525

Figure 4.10: Graph Showing the Effect of Culture in Anoxic Medium on Viability of Human Umbilical Vein Endothelial Cells Measured by the MTT Assay

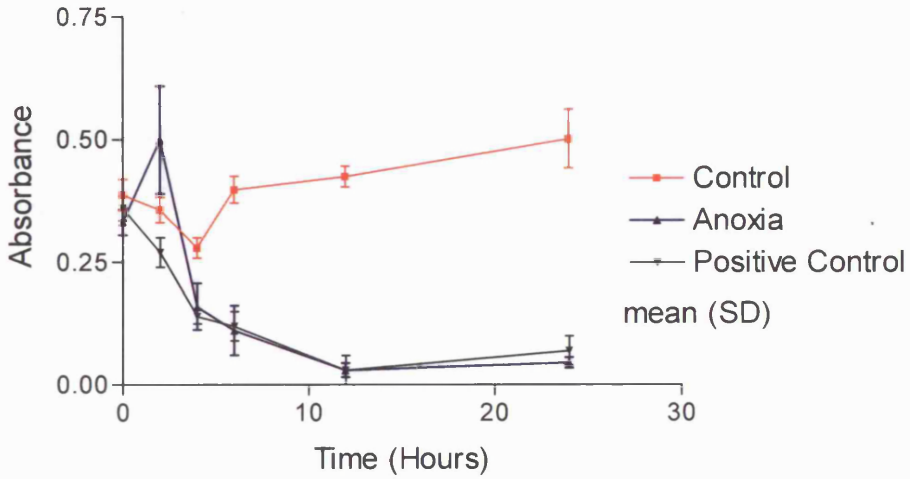


Table 4.3: Comparison Between the Viability of Human Umbilical Vein Endothelial Cells in Control and Anoxic Conditions

Time	Baseline	2 Hours	4 Hours	6 Hours	12 Hours	24 Hours
t test	p=0.008	p=0.0002	p<0.001	p<0.001	p<0.001	p<0.001

Discussion

Culture of these cells in an anoxic and acidic environment results in a statistically significant decrease in viability as measured by the MTT assay. However, the magnitude of this effect only appears biologically significant for the human umbilical vein endothelial cells. Indeed the human tubular epithelial cells and LLC PK1 cells seem to tolerate these conditions remarkably well. There is no doubt that such conditions would not be tolerated by the kidney in vivo and this raises the question whether in vitro modelling is an appropriate technique to study whole organ viability tests, or perhaps the wrong cells are being studied.

The endothelial cells are central to the effects of hypoxia and reperfusion on the whole organ and so the sensitivity of the endothelial cells to the anoxic and acidic environment in the in vitro model may be relevant to the whole organ situation. Firstly, vasospasm in the kidney is counteracted by the potent vasodilator nitric oxide, which is released by endothelial cells at the basolateral cell membrane. If these cells are highly sensitive to warm ischaemia then perhaps it is no surprise that there is such marked vasospasm in non heart-beating donor kidneys. Secondly, hypoxia has been shown to increase the expression of certain adhesion molecules on the luminal aspect of endothelial cells and key among these is Intercellular Adhesion Molecule-1 (ICAM-1). ICAM-1 mediates the adherence of polymorphonuclear lymphocytes to the endothelial cells and hence the migration of these cells into the tissues. Polymorphonuclear lymphocytic infiltration occurs in response to renal artery cross clamping with distribution primarily to the cortex and inner and outer medulla (Willinger et al, 1992). Hypoxia also induces the expression of ICAM-1 on tubular epithelial cells (Combe et al, 1997). The release of proteases and free radicals from activated polymorphonuclear lymphocytes may then cause

direct tubular injury over and above the direct effects of hypoxia, which may be sublethal. The breakdown of cell-cell and cell-extracellular matrix adhesion complexes may result in tubular epithelial cells being shed into the lumen of the nephron without undergoing necrosis. This perhaps explains the fact that large numbers of the epithelial cells found in the urine of patients with acute renal failure are actually viable. The administration of a monoclonal antibody to ICAM-1 protects against ischaemic acute renal failure in an animal model (Kelly et al, 1994).

Hypoxia may cause the in vivo picture of 'acute tubular necrosis', without necrosis being a direct result of the hypoxia. However, the remarkable tolerance of the cultured cells to oxygen deprivation needs to be explained: their demand for oxygen must be low. This may be the case either because of a low metabolic rate, which is not the case with tubular epithelial cells in vivo, or high-energy phosphates must be generated by an alternative metabolic pathway, perhaps by glycolysis. In the following chapter the glycolytic activity of the cells in normally oxygenated and anoxic culture will be investigated.

CHAPTER 5

ASSESSMENT OF ANAEROBIC METABOLISM BY CULTURED CELLS.

ASSESSMENT OF ANAEROBIC METABOLISM BY CULTURED CELLS.

Introduction

The finding that tubular epithelial cells tolerate prolonged warm ischaemia suggests that modelling of this type may not be suitable to use in the search for a whole organ viability test. A donor kidney would certainly not be viable if it were subjected to storage at 37°C for 24 hours with a PO_2 of 0.2KPa in the perfusion fluid. This suggests that the metabolism of the cultured cells is different to that of the whole organ in vivo. Metabolic poisons, such as azide and cyanide, which arrest oxidative phosphorylation would be expected to cause rapid depletion of cellular ATP and thus necrosis unless the cells have an alternative energy generating metabolic pathway. It is possible that anaerobic metabolism i.e. glycolysis might be this alternative pathway. The pathway of glycolysis, which is much less efficient than oxidative phosphorylation, converts one glucose molecule into two molecules of lactate, generating only two high energy phosphate bonds (two ATP molecules) in the process: oxygen is not required. The aim of this experiment was to study the anaerobic metabolism of cells cultured in oxygenated and anoxic conditions by measuring the release of lactate into the culture medium.

Method

Each of the three cell types, LLC-PK1, human tubular epithelial cells and HUVECs were studied. For each cell type, cells were grown to confluence in six well culture plates. Two

sets of four plates (of six wells each) were used for each cell type. One set of four plates served as a control and was incubated in a 5% CO_2 incubator at 37°C. The other set of four plates was subjected to anoxic culture by having the medium exchanged for medium deoxygenated by 5mM dithionite and culture within the nitrogen/carbon dioxide environment of the Atmosbag and then inside the gas tight chamber at 37°C. Foetal bovine serum, a constituent of the culture medium, contains large amounts of lactate and so for this experiment previously dialysed foetal bovine serum was added to the base culture medium (DMEM): dialysis removes most of the lactate. Three samples of the medium used were kept to represent the baseline.

At time zero the medium in the culture wells was changed for either fresh oxygenated medium or fresh deoxygenated medium in the appropriate gaseous environment. One plate was taken out of both the control and anoxic groups at 2 hours, 4 hours, 6 hours and 12 hours after change of the medium. The medium from each well was aspirated and stored at –20°C; a 200µl aliquot was taken from one sample for analysis on the blood gas analyser. The cells were washed with two changes of 500µl of phosphate buffered saline and then allowed to air dry whilst remaining adherent to the culture plate. The culture plates were then covered and stored at –20°C.

When all the experiments were completed the samples were assayed. Medium was assayed for lactate content by colourimetric assay: the concentration of lactate in the sample is calculated from a regression line derived by the analysis of known standard concentrations of lactate (protocol in Appendix 3, page 140). To control for the possibility of differing cell numbers in the wells at the start of the experiment, the plates were assayed for the DNA mass

in each well again using a colourimetric assay and reference to a regression line of known standards (protocol in Appendix 4, page 142).

For each time interval, the mass of lactate in the medium of each well was calculated (Mass=Concentration x Volume). The mass of lactate in the medium at time zero was taken to be the mean of the three baseline samples. The mass of lactate added to the medium was considered to be the mass at time of analysis minus the mass present at time zero. The results are expressed in terms of mass of lactate added per mass of DNA (nmols/μg). The results of culturing a single cell type in the two different environments are compared by t-test at each time interval using the Bonferroni correction for multiple analyses. Statistical significance was therefore assumed at $p<0.0125$ (0.05/4) for any single comparison.

Results

The control medium had a PO_2 of greater than 18KPa at all time points for all cultures. The anoxic medium had a PO_2 of less than 0.2KPa at all time points for all cultures, but was acidic (pH 7.19 to pH 7.23) as a consequence of using dithionite. All three cell types produced lactate in both oxygenated and anoxic conditions. There was no statistically significant difference between the amount of lactate produced by the LLC PK1 cells in control oxygenated or in anoxic culture (Figure 6.1 and Table 6.1). For both the human tubular epithelial cells and the human umbilical vein endothelial cells, the amount of lactate produced in the anoxic conditions was less than that produced in oxygenated conditions and this difference is statistically significant (Figure and Table 6.2; Figure and Table 6.3).

Figure 6.1: Graph Showing the Amount of Lactate Released into the Culture Medium by LLC PK1 Cells During Control (Oxygenated) and Anoxic Culture.

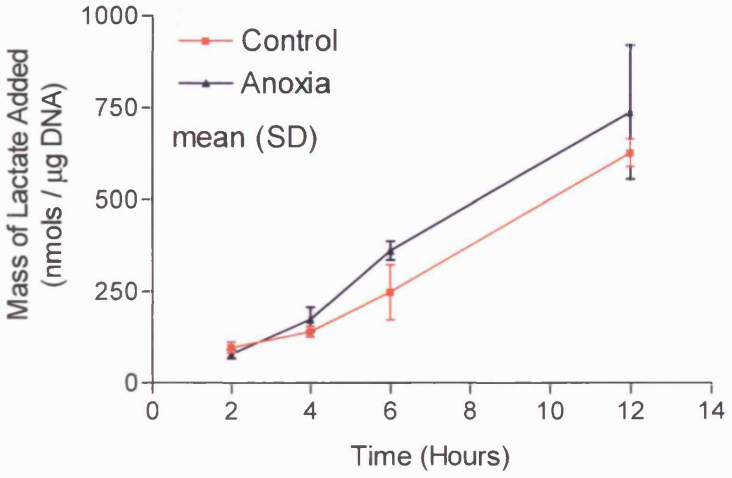


Table 6.1: Mass of Lactate (nmols/µgDNA) Released into the Culture Medium by LLC PK1 Cells During Control (Oxygenated) and Anoxic Culture Expressed as Mean (SD)

	2 Hours	4 Hours	6 Hours	12 Hours
Control	97.56 (14.6)	140.4 (14.6)	248.6 (75.6)	628.8 (37.61)
Anoxic	78.66 (11.5)	174.2 (33.3)	362.6 (25.75)	739.5 (182.8)
t- test	p=0.088	p=0.11	p=0.029	p=0.281

Figure 6.2: Graph Showing the Amount of Lactate Released into the Culture Medium by Human Tubular Epithelial Cells During Control (Oxygenated) and Anoxic Culture.

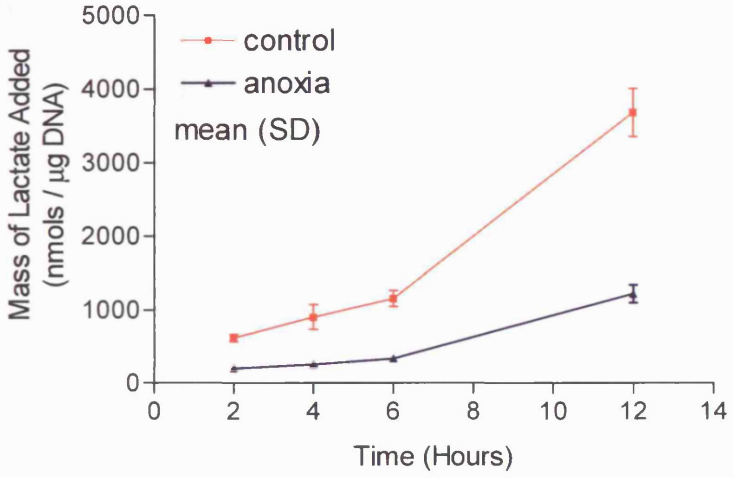


Table 6.2: Mass of Lactate (nmols/µgDNA) Released into the Culture Medium by Human Tubular Epithelial Cells During Control (Oxygenated) and Anoxic Culture Expressed as Mean (SD)

	2 Hours	4 Hours	6 Hours	12 Hours
Control	614.3 (50.7)	904.3 (168)	1159 (109.5)	3691 (328.1)
Anoxic	202.5 (12.9)	255.9 (19.5)	341.0 (44.75)	1223 (122.5)
t- test	p< 0.0001	p<0.0001	p<0.0001	p<0.0001

Figure 6.3: Graph Showing the Amount of Lactate Released into the Culture Medium by Human Umbilical Vein Endothelial Cells During Control (Oxygenated) and Anoxic Culture.

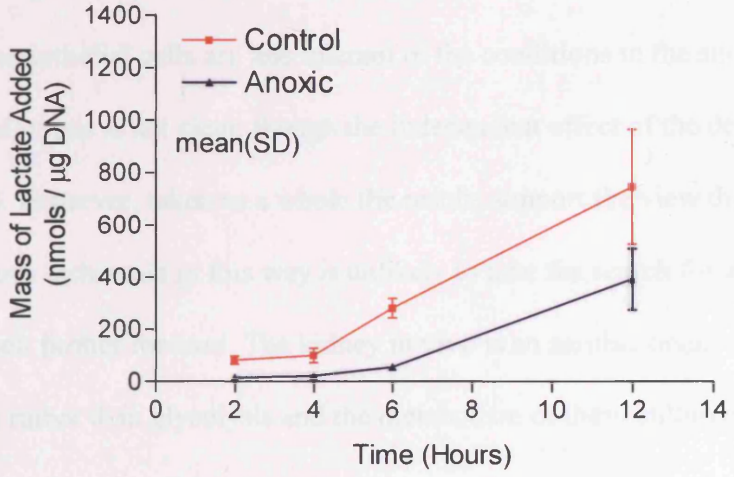


Table 6.3: Mass of Lactate (nmols/µgDNA) Released into the Culture Medium by Human Umbilical Vein Endothelial Cells During Control (Oxygenated) and Anoxic Culture Expressed as Mean (SD)

	2 Hours	4 Hours	6 Hours	12 Hours
Control	82.73 (16.03)	100.6 (27.79)	282.0 (37.93)	747.7 (219.4)
Anoxic	15.55 (2.84)	22.81 (6.83)	56.51 (9.99)	392.6 (117.7)
t- test	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$

Discussion

All three cell types release lactate into the medium in conditions of oxygenated culture suggesting that despite the presence of oxygen, glycolysis is a major metabolic pathway. The reason why the endothelial cells are less tolerant of the conditions in the anoxic culture model than the other cell types is not clear, though the independent effect of the decrease in pH has not been studied. However, taken as a whole the results support the view that in vitro modelling of warm ischaemia in this way is unlikely to take the search for a whole organ viability test much further forward. The kidney in vivo is an aerobic organ utilising oxidative phosphorylation rather than glycolysis and the metabolism of these cultured cells is markedly different despite optimal conditions.

An increase in the glycolytic capacity and decrease in the oxidative metabolism of whole tubules in culture has been reported (Dickman et al, 1989). This differentiation was prevented by agitation but on plating out the cultures into formal tissue culture, this effect was accelerated. With increasing time in culture one group has found this differentiation away from oxidative phosphorylation to become more pronounced (Aleo et al, 1992). High glycolytic capacity has also been reported in coronary endothelial cells in culture (Mertens et al, 1990). The results presented here are consistent with these published results. Quite why this switch to glycolysis occurs is not known. It may represent an adaptive response to the various stresses of repeated trypsinisation and handling or it is possible that the amount of oxygen actually available to the mitochondria in culture is much less than the partial pressure in the medium would suggest.

It is of note that lactate production in the immortalized cells was unchanged by anoxic culture whereas in the human tubular epithelial cells and endothelial cells which are not immortalized lactate production was markedly reduced. This may not be due to oxygen deprivation but rather to the decrease in pH which occurs in the anoxic system. In vivo, the enzymes of glycolysis are inhibited by increasing acidity and it is possible that this response is retained by the non-immortalized cells.

Conclusion

In vitro modelling of warm ischaemia by oxygen deprivation does not mimic the in vivo situation because the cultured cells utilise the glycolytic pathway even in the presence of oxygen. Models of this type are of no value in the search for a whole organ viability test in renal transplantation.

CHAPTER 6

DISTRIBUTION AND PREDICTIVE VALUE OF APOPTOSIS IN PREPERFUSION BIOPSIES FROM DONOR KIDNEYS.

DISTRIBUTION AND PREDICTIVE VALUE OF APOPTOSIS IN PREPERFUSION BIOPSIES FROM DONOR KIDNEYS

Introduction

Current tests of renal viability prior to transplantation are designed to detect features of cell death typical of necrosis. This is a logical approach since prolonged severe hypoxia certainly results in necrotic cell death manifested initially as a component of the phenomenon of 'acute tubular necrosis' and subsequently as cortical necrosis. However, a growing body of literature suggests that necrosis is not the only process whereby cells are injured by periods of ischaemia or ischaemia followed by reperfusion. Apoptosis, or programmed cell death, is another mechanism by which cells may die. The importance of this process is increasingly being recognised in a wide range of tissues and circumstances including a number of in vivo situations in which hypoxia or hypoxia followed by reperfusion occurs. For example, after myocardial infarction reperfusion of cardiac myocytes induces cell death by apoptosis thus leading to a worsening the original injury (Gottlieb et al, 1994) despite the fact that restoration of blood flow is the most effective method of limiting the overall injury; a situation that is analogous to transplantation of solid organs. In transplantation, apoptotic cell death has attracted increasing attention particularly with respect to heart and liver (Kohli et al, 1999) transplants where the organs tolerate ischaemia poorly.

That apoptosis plays a role in the renal response to both partial and complete ischaemia is well established. Inducing stenosis in the rat renal artery by partial ligation

results in tubular apoptosis at two days and this process subsequently contributes significantly to the renal atrophy that may occur following renal artery stenosis (Gobe et al, 1990). In another rat model, 30 minutes of warm ischaemia followed by 12 hours of reperfusion led to the production of large numbers of apoptotic bodies in the renal tubules; more severe insults produce more dramatic evidence of apoptosis (Schumer et al, 1992). Apoptosis in cortical tubular cells has been identified after renal transplantation, which inevitably involves a period of ischaemia and reperfusion (Matsuno et al,1996; Burns et al, 1998). NHBD kidneys are subject to a period of ischaemia prior to procurement and this may be accompanied by an element of in situ reperfusion due to external cardiac massage. It is therefore possible that the full insult received by a NHBD kidney is a consequence of cell death by both necrosis and apoptosis. It is possible that the number and distribution of apoptotic cells may reflect the severity of the ischaemic injury suffered. In the same way that markers of necrosis have been used to study the renal injury prior to transplantation, detection of apoptotic cell death in biopsies taken before reperfusion might reveal information about the severity of the injury and thus about the potential for function after transplantation.

Apoptosis: an Overview

In order to understand the potential importance of this form of cell death and the methods by which it may be studied and perhaps even modified, it is necessary to have some understanding of the process of apoptosis.

Morphology of Apoptosis

The word 'apoptosis' is derived from the Greek word of the same spelling which was used to describe the 'falling off' or 'dropping off' of leaves from a tree and was proposed and explained in an elegant footnote to the definitive apoptosis paper (Kerr et al, 1972). Apoptosis, in contrast to necrosis, involves shrinkage and fragmentation of cells with intact membranes and their rapid removal by phagocytosis such that an inflammatory reaction is prevented. The morphological changes occur in three phases (Wyllie et al, 1980). In the first phase, the earliest changes are detectable in the nucleus where the chromatin condenses into well circumscribed masses which become marginalised to the periphery of the nucleus and the nucleolus dissociates. Indeed, one of the pathognomonic features of apoptosis is the cleavage of DNA at internucleosomal linker regions into short lengths which are integer multiples of 50-200kilobase pairs. Concomitantly the cell volume decreases by loss of water and organelles become compacted. Specialized surface structures such as microvilli and contact regions are lost and the cell becomes detached from its neighbours. In the second phase there is convolution of the nuclear and plasma membranes. This results in blebbing or budding and finally pinching-off of cell fragments as discrete membrane bound fragments known as 'apoptotic bodies', some or all of which will contain nuclear fragments. In vivo these bodies may be shed from epithelial surfaces or engulfed by adjacent cells or macrophages. There is no release of intracellular contents into

the extracellular space and no inflammatory reaction. Phase three comprises progressive deterioration of the residual nuclear and cytoplasmic structures. In vivo this usually occurs within the phagosome of the engulfing cell and it is in this state that most of the apoptotic bodies are seen in tissues studied by light microscopy. In vitro, apoptotic bodies are seldom phagocytosed but eventually undergo degenerative changes known as 'secondary necrosis' one of the features of which is membrane rupture and permeabilty to vital dyes.

The time scale of these events is dramatic. Time lapse photographic studies (Evan et al, 1992) reveal that after exposure to the lethal stimulus there is an initial pause the duration of which is tissue and stimulus specific (Wyllie et al, 1980). The first two phases of the apoptotic process, which result in the production of apoptotic bodies, are then completed within a few minutes. The apoptotic bodies themselves are detectable for up to 6 hours reflecting the time taken for breakdown within the phagosome.

Apoptosis differs from necrosis in a number of ways. Necrotic cell death is a passive process characterised by a failure to regulate the internal milieu of the cell. Membrane pumps fail due to depletion of high energy phosphates. Failure to exclude sodium results in a massive influx of water with subsequent swelling of organelles and the whole cell. Finally the plasma membrane ruptures and intracellular contents including lysosomal enzymes and other inflammatory mediators are released into the extracellular matrix. By contrast, apoptosis is an energy dependent process, usually affecting single cells rather than sheets of adjacent cells. Cells are deleted by a well ordered non-random process in which membrane integrity is maintained and there is no release of inflammatory mediators into the interstitium.

Enzymology of Apoptosis

The last four years have seen great advances in our understanding of the molecular events that constitute apoptosis. Research has largely been performed in a small number of experimental models, such as glucocorticoid treated thymocytes and some caution is required when extrapolating these findings into other fields of research. However, apoptosis as a process seems to be extremely well conserved throughout phylogeny so such extrapolation is likely to be valid. Much of the early work was performed in the study of the nematode *Caenorhabditis elegans* in which 131of the 1090 somatic cells undergo apoptosis during development. The finding that this apoptosis did not occur in the absence of certain cell death (ced) genes started the search of the human genome for ced homologues. This approach has led to the discovery of a large number of enzymes vital to the control of cell death in man.

Apoptosis is the result of a genetically encoded suicide program which may be divided into a number of phases:

1. Induction - by a signal or signals known as Triggers. These include such diverse stimuli as cytotoxic lymphocytes, cytokines, growth factors, removal of growth factors, ionising radiation, and chemotherapeutic pharmaceuticals. The pathway by which these signals are transduced and apoptosis subsequently stimulated is established for cytotoxic lymphocytes (Fas/Fas Ligand binding) but remains unclear in most cases and will not be further discussed.

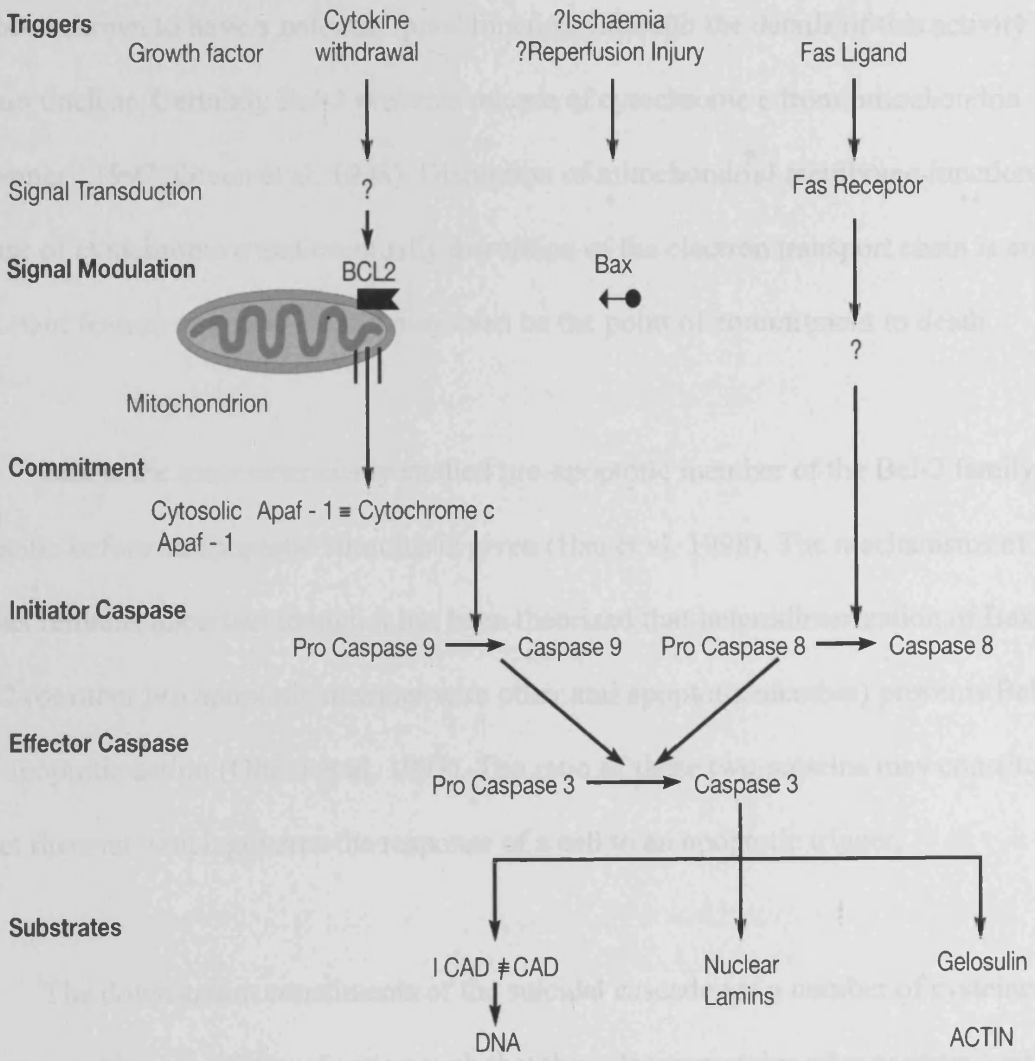
2. Determination - a number of intracellular proteins have important actions in determining whether or not an apoptotic signal is of sufficient strength to cause the cell to become committed to die. The genes which encode these modulator proteins are among the most important oncogenes.

3. Execution - once the cell is committed to apoptosis the effectors of the suicide program are activated and the cell destroys itself in a specific and non-random manner.

The Apoptotic Machinery

For most triggers, though perhaps not for cytotoxic lymphocytes, the activation of a cytosolic protease called the Apoptosis protease activating factor (Apaf-1) (Zou et al, 1997) constitutes the important step of commitment to cell death by apoptosis (See Figure 6.1). Control over the activation of Apaf-1 is a central event in the determination stage of apoptosis and is influenced by a number of modulator proteins as already described. Apaf-1 will only become active in the prescence of the cofactor cytochrome c (Li et al, 1997) which is contained within the mitochondria of non-apoptotic cells. The release of cytochrome c from mitochondria may be in large part a function of the Bcl-2 family of proteins. This 'family' contains both pro and anti-apoptotic members (Adams et al, 1998). By some mechanism altered expression of these proteins, or changes in the ratio in which they are found in the cell, is able to gauge whether a survival or apoptotic trigger has been received and convert the competing signals into a life or death outcome.

Figure 6.1: Schematic Showing the Phases of Cell Death by Apoptosis and the Enzymes and Substrates Involved.



Bcl-2 itself is an antiapoptotic protein and has been shown to protect cells from a wide variety of cytotoxic insults including irradiation, cytokine withdrawal and cytotoxic drugs. It is found on the cytoplasmic surface of the outer layer of the mitochondrial membrane in a patchy distribution associated with areas of contact between the inner and outer layers. It has been shown to have a potential 'pore' function although the details of this activity remain unclear. Certainly Bcl-2 prevents release of cytochrome c from mitochondria (Kroemer , 1997; Green et al, 1998). Disruption of mitochondrial membrane function, with release of cytochrome c and eventually disruption of the electron transport chain is an important feature of apoptosis and may even be the point of commitment to death.

Bax is the most extensively studied pro-apoptotic member of the Bcl-2 family. It is cytosolic before an apoptotic stimulus is given (Hsu et al, 1998). The mechanisms of action of Bax remains uncertain though it has been theorized that heterodimerization of Bax with Bcl-2 (or other pro apoptotic member with other anti apoptotic member) prevents Bcl-2 anti-apoptotic action (Oltavi et al, 1993). The ratio of these two proteins may constitute the preset rheostat which governs the response of a cell to an apoptotic trigger.

The downstream constituents of the suicidal cascade are a number of cysteine proteases with a specificity of action such that they cleave proteins after aspartic acid residues- as a result of their structure and specificity they have been called Caspases (C-aspase's). Caspases exist in the cytosol as precursors (Procaspases) which are only activated by proteolytic cleavage. The family of caspases is broadly divided into two groups:

i) Initiator caspases- of which the best known is caspase-9.

Initiator caspases cause activation, or inactivation, of other enzymes, including other caspases. Different triggers cause activation of different initiator caspases. The Bcl-2 inhibitable, cytochrome c/Apaf-1 pathway which activates procaspase-9 to the active enzyme is common to many cytotoxic stimuli but other stimuli may induce apoptosis by different pathways. For example, cytotoxic lymphocyte killing, by binding of Fas with its ligand is Bcl-2 independent (Strasser et al, 1995) and is initiated by activation of caspase-8 rather than caspase 9.

ii) Effector caspases- of which the best known is caspase-3

Activated by their initiator cousins eg Caspase 9 and by auto catalysis of their own precursors, effector caspases are responsible for the familiar morphological changes which comprise apoptosis. Knowledge of their substrates is incomplete but these are likely to be specific because the amino acid sequences for recognition of substrate are specific. Thus effector caspase activity is part of a specific self destruct mechanism in which a select set of proteins is cleaved in a coordinated and predetermined manner; random autodigestion is not seen.

The activation of effector caspases has direct effects on cell structures and also indirect effects via activation or inactivation of critical regulatory enzymes.

Direct effects include the direct disassembly of cell structures. For example the nuclear lamina is seen to fragment as part of the process which facilitates the dissolution of the chromatin in apoptotic bodies (Takahashi et al, 1996).

Indirect effects are of two main types: (i) cleavage of enzymes involved in regulation and repair. For example gelosulin is a protein which severs actin filaments in a controllable manner- caspase cleavage generates a fragment which is constitutively active (Kothakota et al, 1997) and this may be responsible for the morphological changes seen in the cytoskeleton (ii) inactivation of proteins that protect cells from the intrinsic apoptosis activity. The endonuclease responsible for the pathognomonic DNA cleavage (CAD-caspase activated deoxribonuclease) exists constitutively bound to its inhibitor I^{CAD} in nonapoptotic cells. Caspase activity cleaves ICAD from CAD and inactivates it, leaving the deoxyribonuclease active (Liu et al, 1997; Enari et al, 1998).

The control of the caspase cascade is incompletely understood. In common with the complement and coagulation cascades it is likely that there are a number of intrinsic inhibitors which can influence the caspase cascade but these remain to be isolated. However, a number of experimental caspase inhibitors have been identified and these may prove to be effective in preventing cell death by apoptosis in circumstances where it is undesirable.

Detection of Apoptosis in Tissue Specimens

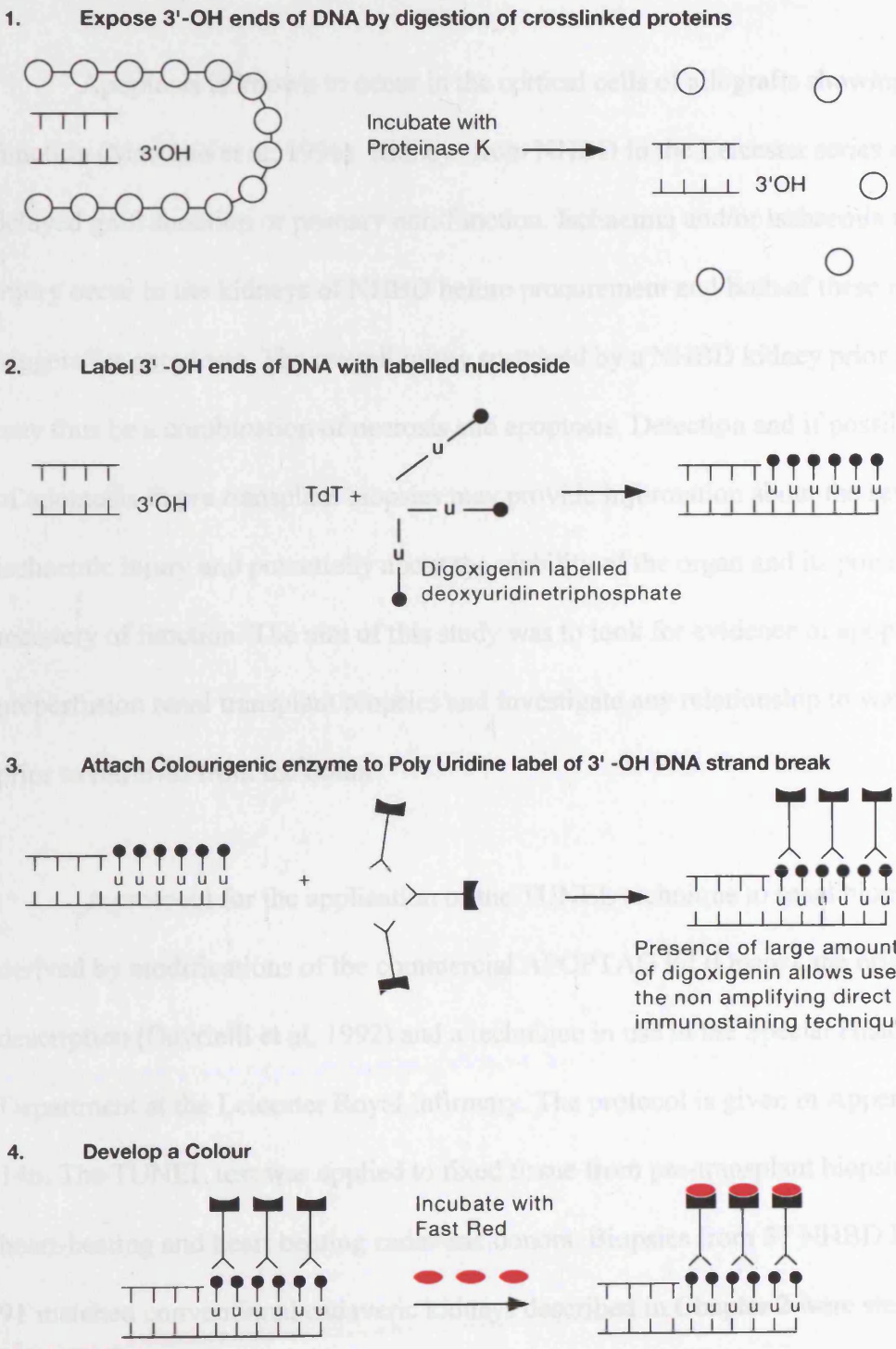
The process of apoptosis from trigger to disappearance of the cell may take as little as 1 to 6 hours depending on cell type. Relatively low rates of apoptosis i.e. proportion of apoptotic to normal cells, can thus cause large changes in cell populations in a relatively short time. Cellular and nuclear morphology remain viable techniques to detect the presence of an apoptotic process but histological snapshots of cellular morphology tend to underestimate apoptotic activity because of the delay between trigger induced enzymatic commitment to death and the morphological changes. Assessment of morphology by light microscopy is a poor technique for quantifying the rate of apoptosis in a tissue specimen.

A number of detection techniques utilise the DNA changes which are pathognomonic of apoptosis to highlight apoptotic cells before morphological changes become apparent. The internucleosomal cleavage of DNA into integer multiples of 50 to 200kb produces a ladder effect when the DNA is run out by agarose gel electrophoresis. This is in stark contrast to the smear generated by the random DNA cleavage seen in necrosis but only provides evidence of the process and is only semi-quantitative at best.

In cell suspensions flow cytometry may also be used by gating for cell size and DNA content by using fluorescent nuclear dyes. This technique is quantitative but is only suitable for application to cell culture suspensions. The greatest advance in the detection of apoptosis in clinical specimens was the development of the TUNEL test. TUNEL is an acronym for TdtT-mediated dUTP Nick End Labeling. The large number of double stranded DNA breaks produced in apoptosis by activated deoxyribonuclease may be utilised as the target for the technique of insitu labelling (Gavrieli et al, 1992). The TUNEL test is an immunohistochemical technique which labels the 3'-OH ends of DNA strand

breaks. The TUNEL test utilises the specific binding of the enzyme terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of DNA. By incubating with a solution of digoxigenin labelled deoxynucleotide (uridine) base a polydeoxynucleotide polymer is added to each 3'-OH DNA strand break. An antidigoxigenin antibody is then used to carry a reporter enzyme to the labelled strand and this reporter enzyme is subsequently exposed to a chromogenic substrate. Thus, the 3'-OH ends of fragmented DNA are labelled with a coloured precipitate, which is detectable by light microscopy (See Figure 6.2). The specific staining of free 3'-OH ends found in high concentration in apoptotic nuclei produce an intense signal. False positive staining of normal nuclei is minimal. Whilst the TUNEL test is sensitive as a test for apoptosis and preferentially labels apoptotic cells, it may not be 100% specific (Kressel et al, 1994). It is theoretically possible that some cells undergoing a necrotic process may also be labelled but the random nature of DNA cleavage in necrosis produces a much lower concentration of 3'-OH ends. The number of sites available to be labelled per nucleus is consequently much smaller in necrosis than in apoptosis. Nevertheless, it has been suggested that positive TUNEL findings should be confirmed by other means, such as electron microscopy or DNA extraction for agarose gel electrophoresis (Grasl-Kaupp et al, 1995).

Figure 6.2: Schematic Showing the Steps of the TUNEL Immunohistochemical Technique for Labeling Nuclei Undergoing Apoptosis



Is Apoptosis in Pretransplant Biopsies Predictive of Ischaemic Injury Prior to Retrieval or Outcome after Transplantation?

Apoptosis is known to occur in the cortical cells of allografts showing delayed graft function (Matsuno et al, 1996). Kidneys from NHBD in the Leicester series all show delayed graft function or primary non-function. Ischaemia and/or ischaemia reperfusion injury occur in the kidneys of NHBD before procurement and both of these insults may be triggers for apoptosis. The overall injury sustained by a NHBD kidney prior to procurement may thus be a combination of necrosis and apoptosis. Detection and if possible quantitation of apoptosis in pre transplant biopsies may provide information about the severity of the ischaemic injury and potentially about the viability of the organ and its potential for recovery of function. The aim of this study was to look for evidence of apoptosis in preperfusion renal transplant biopsies and investigate any relationship to warm ischaemia prior to retrieval from the donor.

A protocol for the application of the TUNEL technique to renal biopsy tissue was derived by modifications of the commercial APOPTAG kit (Oncor), the original description (Gavrielli et al, 1992) and a technique in use in the Special Histochemistry Department at the Leicester Royal Infirmary. The protocol is given in Appendix 5, page 146. The TUNEL test was applied to fixed tissue from pre-transplant biopsies from non heart-beating and heart beating cadaveric donors. Biopsies from 37 NHBD kidneys and the 91 matched conventional cadaveric kidneys described in Chapter 2 were studied. Sections of human tonsil, supplied by the Department of Histopathology at the Leicester Royal Infirmary, were used as control tissue. In normal germinal centres of tonsil, and lymph

node, a high proportion of the cells, primarily B lymphocytes, undergo apoptosis. This makes tonsillectomy tissue ideal as a positive control tissue for apoptosis related experimentation. The TUNEL technique is delicate, expensive and time consuming. For this reason, sections were stained in batches. Each batch contained two control slides. The positive control slide comprised a section of tonsil stained in the manner described such that if the technique worked successfully, follicular nuclei would be labelled. The negative control slide also comprised a section of tonsil but during the staining technique the TdT enzyme aliquot was replaced by an aliquot of distilled water. Thus, if any staining occurred on these slides it demonstrated that staining did not necessarily indicate apoptotic cells.

Results

All positive control specimens showed TUNEL positive stained cell nuclei in the follicles (Figure 6.3) and all negative control specimens showed no stained nuclei. This confirms that the technique was reliable. No TUNEL positive cortical tubular cells were seen in the pre-transplant biopsies from the heart beating cadaveric donors or from the NHBD. Six biopsies from NHBD also contained some renal medulla. Four of these showed TUNEL positive cells in the tubules of the medulla (Figure 6.4). In one of these four the cytoplasm of the stained cells appears swollen, suggesting that the cells may be undergoing a necrotic process rather than an apoptotic one (Figure 6.5). Ten biopsies from the heart beating cadaveric controls contained some renal medulla: no TUNEL positive cells were seen. These results are not suitable for any statistical analysis.

Figure 6.3: Photomicrograph of a Section of Human Tonsil Stained Using the TUNEL Technique for Apoptosis (Positive Control)

Nuclei undergoing apoptosis are stained red- this is seen to occur in cells within the germinal centre of the tonsillar tissue.

x100

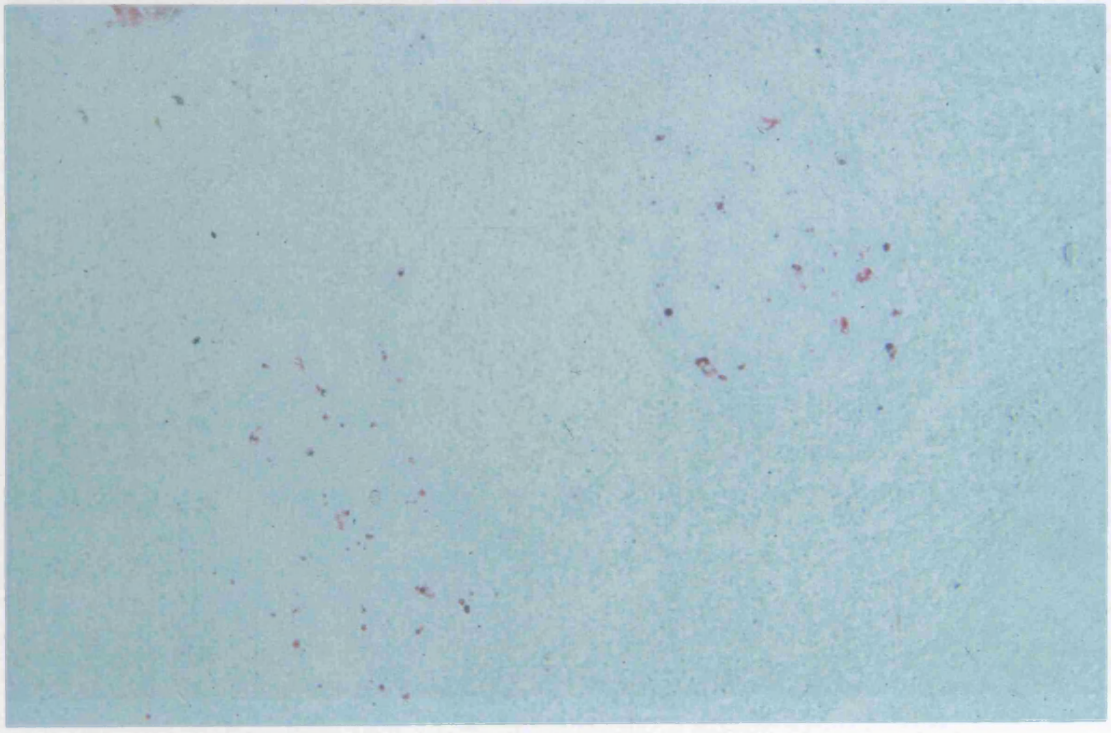


Figure 6.4: Photomicrograph Showing A Section of a Pre-Perfusion Biopsy from a Non Heart-Beating Donor Kidney Stained Using the TUNEL Technique

Positive (red) staining is seen in the nuclei of tubular cells in the medulla indicating that these cells are undergoing apoptosis.

x200

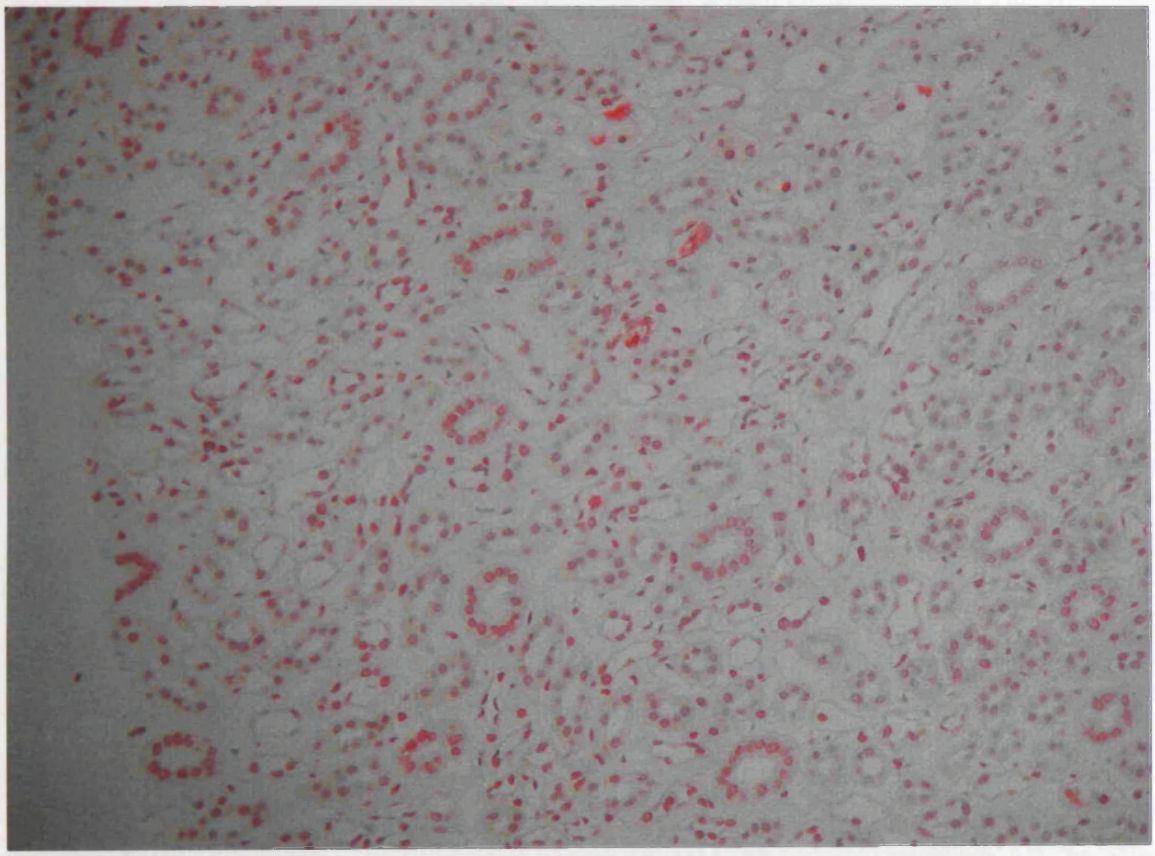
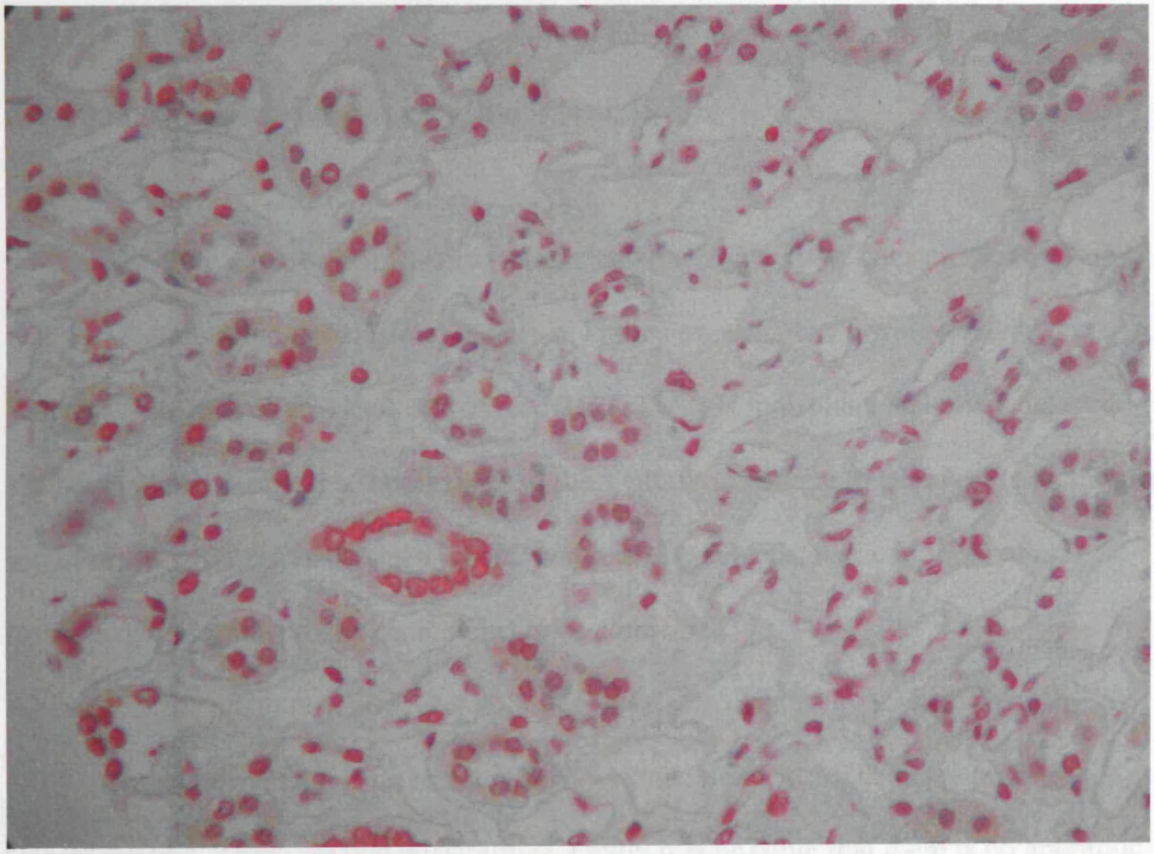


Figure 6.5: Photomicrograph of a Section of a Pre-Perfusion Biopsy from a Non Heart-Beating Donor Kidney Stained with the TUNEL Technique

Positive (red) staining of the nuclei of tubular cells is seen in association with some swelling of the cell cytoplasm. TUNEL staining is considered specific for apoptosis but in this section the swelling of the cytoplasm and interstitial oedema may indicate a necrotic process.

x200



Discussion

Application of the TUNEL test to pre-perfusion cortical biopsies from NHBD kidneys shows no evidence of tubular apoptosis: this test has no use as an indicator of viability or as an indicator of the warm ischaemic injury which the kidney has suffered. The number of biopsies studied is small but the finding is consistent and the positive controls show that the experimental technique is sound. No other studies investigating apoptotic activity in pre-perfusion biopsies from NHBD kidneys are available for comparison. In conventional cadaveric donor kidneys, cortical tubular apoptosis was found to be a very rare event in pre-perfusion biopsies (Burns et al, 1998).

However, apoptosis in cortical tubular cells, which may be detected by the TUNEL test, is a feature of post perfusion biopsies in kidney transplants showing delayed graft function (Matsuno et al, 1996). All of the NHBD kidneys transplanted showed delayed graft function at best and so application of the TUNEL technique to post perfusion biopsies might reveal differences between NHBD and heart beating cadaveric kidneys after transplantation. This prediction remains to be confirmed.

The important question remains as to why apoptosis is not detected prior to transplantation. There are two possibilities. Firstly it is possible that there is no apoptotic trigger prior to reperfusion at transplantation. The point of induction of apoptosis in the process of ischaemia/reperfusion injury is not known. A number of workers have failed to detect evidence of morphological apoptosis or raised molecular markers of apoptosis in models lacking a reperfusion element (Gottlieb et al, 1994; Eschwege et al, 1998). Evidence of apoptosis is absent from the post mortem kidneys of individuals suffering

acute renal hypoperfusion by virtue of sudden death without resuscitative efforts (Jaffe et al, 1997). In human renal allografts from conventional cadaveric donors, which subsequently show delayed graft function and have apoptosis demonstrable in post reperfusion biopsies, apoptosis is a rare finding in the preperfusion cortical biopsies (Burns et al, 1998). The in vitro, animal and human evidence suggests that apoptosis is a consequence of reperfusion. Whilst external cardiac massage and artificial ventilation is performed on the NHBD after a period of cardiac standstill, the evidence suggests that renal reperfusion by this technique may not occur as previously discussed: there may be no in situ reperfusion injury. If ischaemia alone is not an apoptotic trigger then apoptosis would not be found in pre-perfusion biopsies.

The second possibility is that commitment to apoptosis is made in the donor, following whatever trigger, but because of cold storage, the enzymatic machinery of apoptosis proceeds so slowly that insufficient numbers of free 3'-OH ends of DNA are created for the TUNEL test to be positive in pre-transplant biopsies. Distinguishing between these two possibilities will be important. If cells are already commited to die by apoptosis prior to procurement, then there are currently no techniques that will detect this injury or predict its severity. This means that predicting viability in kidneys which subsequently show delayed graft function or worse, will remain difficult. However, if commitment to apoptosis only occurs on reperfusion then there are possible therapeutic interventions that may be of use. That component of reperfusion which induces apoptosis is not known. However, apoptosis may be prevented by interventions 'downstream' of the unknown trigger. It is possible to increase cellular levels of Bcl-2 such that commitment to apoptosis may be prevented by maintaining the distributon of cytochrome-c (Bilbao et al, 1999). In addition a number of caspsase inhibitors are available and experimental work in

animals suggests that adding these agents to the perfusion fluid of ischaemic livers protects against ischaemia/reperfusion injury (Cursio et al, 1999). Whether or not it is advisable to prevent the apoptotic death of damaged cells by pharmacological means is unknown. It is uncertain whether this activity will produce improved long-term graft outcomes in renal transplantation. Furthermore, since the Bcl-2/Bax ratio is important not only in apoptosis due to pathological stimuli but also in oncogenesis, the long term effects of attempting to manipulate apoptosis must be considered with respect to the development of malignancy. This is particularly relevant in the immunocompromised patient.

Quite why TUNEL positive cells are seen in the outer medulla of some biopsies is also unclear. Traditionally it is the renal cortex which has been studied in viability tests based on the premise that segments of the proximal convoluted tubule are the most sensitive to hypoxia (Endre et al, 1989). However it is known that the medulla has a PO_2 which is below that of the cortex and often below that of venous effluent blood (Aukland et al, 1960). This is probably a result of direct diffusion of oxygen from arterial to venous vessels (shunt diffusion). These vessels are particularly closely apposed in the long vascular loops in the renal medulla. The tubular cells of the thick ascending limb of the loop of Henle are highly metabolically active and selective injury to these cells has been demonstrated in animal models that resemble the clinical situation of hypoperfusion (Heyman et al, 1988; Heyman et al, 1991). Furthermore, vascular obstruction by aggregated erythrocytes occurs in the medullary circulation after relief of total renal ischaemia (Mason, 1986). This prolongs ischaemia in the medulla and inner cortex, exacerbating post-ischaemic renal failure. Clearly then the renal medulla is at risk in the kidneys of NHBD and the finding of TUNEL positive cells is perhaps not so surprising. The renal medulla is certainly worthy of greater interest in the assessment of renal viability prior to transplantation.

CHAPTER 7

SEVERITY OF INTERSTITIAL FIBROSIS IN THE CORTEX OF DONOR KIDNEYS AS AN INDICATOR OF ORGAN QUALITY.

SEVERITY OF INTERSTITIAL FIBROSIS IN THE CORTEX OF DONOR KIDNEYS AS AN INDICATOR OF ORGAN QUALITY.

Introduction

The majority of potential non heart-beating donors present in Accident and Emergency departments having suffered cardiac arrest within the department or prior to their arrival. Efforts are correctly directed towards resuscitation and only after declaration of death by the attending physicians is the possibility of organ donation considered. Eligibility for donation is based largely on experience of organs procured from conventional cadaveric donors, the selection criteria being based on donor age and the absence of known or suspected renal disease as outlined in Chapter 2. Kidneys are procured from non heart-beating donors with normal serum creatinines who are not known to renal services on the assumption that the renal function is sufficient for the needs of the donor and therefore will be sufficient for the needs of a recipient. It is presumed that there is no progressive renal disease in the donor but, in fact, this may not be the case. Individuals who die from cardiovascular disease can be expected to have widespread atherosclerosis. Occult renovascular lesions occur in post mortem (Holley et al, 1964) and aortography (Eyler et al, 1962) series of patients with atherosclerosis at other sites and in patients undergoing coronary angiography (Harding et al, 1992).

Serum creatinine measurements are usually made by the attending medical team but serum creatinine alone is not a reliable indicator of the GFR: GFR declines with age but so

does lean body mass and consequently the serum creatinine tends to remain within the quoted normal range (Davies et al, 1950). Mathematical formulae exist for the estimation of creatinine clearance (Cockroft et al, 1976; Kampmann et al, 1974) but the mass of the individual may be required to apply these formulae and presentation in cardiac arrest does not easily allow the potential non heart-beating donor to be weighed. No rapid test of organ quality i.e. glomerular filtration rate (GFR) exists for application to this situation.

Whilst no rapid biochemical test exists for in situ measurement of renal function in the non heart-beating donor situation, attempts have long been made to correlate structure in the kidney with its function. It is standard practice for a renal cortical biopsy to be taken prior to transplantation so that any significant renal disease in the donor may be assessed. Indeed it has been suggested in some American series that significant glomerulosclerosis (>10%) in a wedge cortical biopsy from the donor means that the kidney will give a poor functional result after transplantation. Double transplantation of en bloc kidneys has been advocated in this situation (Johnson et al, 1996a; Johnson et al, 1996b). However, there is little evidence to support this contention. The ground breaking studies of Risdon (Risdon et al ,1968) and Schainuk (Schainuck et al, 1970) clearly demonstrated that the histological variable most closely correlated with the glomerular filtration rate was not glomerular damage but rather the severity of interstitial fibrosis. Interstitial fibrosis is the final common pathway of all chronic, progressive renal disease, including renovascular disease, hypertensive nephropathy (Mai et al, 1993) and diabetic nephropathy and leads ultimately to the clinically familiar picture of the small fibrotic kidneys of end stage renal failure.

Quite how interstitial fibrosis causes a decrement of glomerular filtration rate is unclear. It is theorized that worsening fibrosis causes compression of post glomerular capillaries, which are ultimately obliterated (Bohle et al, 1987). Certainly a paucity of capillary loops is a histological feature of the fibrotic kidney and a reduction in the interstitial capillary area correlates with the decline in GFR (Seron et al, 1990). Compression of post glomerular capillaries would decrease blood flow through the glomeruli and thus reduce GFR. However, a rise in the flow resistance would increase the filtration pressure and result in the opposite effect. Quite why the flow effect should predominate is an unexplained weakness of this theory. Similarly obliteration of the renal tubules has some merit as a potential mechanism since there was a significant correlation between tubular atrophy and creatinine clearance in the original work by Risdon. Ischaemic atrophy of the tubules consequent upon ischaemia caused by obliteration of capillaries is a speculative mechanism. It is also possible that there is a contribution from the tubuloglomerular feedback mechanism. If tubular atrophy, or separation of the tubules from the interstitial capillaries by fibrosis, causes impaired reabsorption of sodium chloride proximal to the macula densa, then the increased sodium chloride concentration at the macula densa may produce a decrease in glomerular filtration via the physiological tubuloglomerular feedback mechanism (Mackensen-Haen et al, 1981; Bohle et al, 1981).

Mechanisms Underlying the Formation of Interstitial Fibrosis

Clearly, since it represents a final common pathway, interstitial fibrosis occurs in response to a wide range of stimuli many of which may be present in non heart- beating donors. However, despite recognition of its importance our knowledge of the processes underlying the formation of interstitial fibrosis is incomplete.

The fibrotic interstitium contains a large number of different proteins in the extracellular matrix. Many of these are constituents of the normal interstitium present in greater than normal amounts: fibronectin, collagen I, III, V and VII but others are matrix proteins restricted to tubular basement membranes in health (Eddy, 1996). The relative contribution of each of these constituents to the interstitial fibrosis is variable and may even be disease specific.

The cellular source of the increased amounts of extracellular matrix protein is not clear. In concert with fibrogenic processes elsewhere the fibroblast lineage seems most likely but in vivo studies of interstitial fibroblasts are problematic because of a lack of cell markers. However, it has been shown that fibroblasts from fibrotic human kidneys synthesize more total collagen than those derived from normal kidneys (Rodemann et al, 1991a) and fibroblasts derived from scarred rabbit kidneys show an increased rate of spontaneous proliferation in vitro than fibroblasts derived from normal kidneys (Rodemann et al, 1991b). It is not clear if these fibroblasts are resident or recruited from the circulating fibroblast population or whether there is significant transformation of other types of cell, especially perivascular cells, locally.

Other cell types are also capable of laying down extracellular matrix substance. Macrophages have been shown to synthesize collagen I and fibronectin in vitro (Vaage et al, 1990) and an interstitial infiltrate of macrophages is commonly seen in kidneys with progressive renal disease. Tubular epithelial cells respond to profibrotic cytokines in vitro by increased production of collagen I whereas the production of collagen IV remains unchanged (Creely et al, 1992). Cells that stain positively for epithelial markers, presumably tubular epithelial cells, have been identified in the interstitium of human end stage kidneys. This is considered to be a consequence of remodelling of the fibrous architecture (Nadasdy et al, 1994). The contribution made by these cells to the extracellular matrix is unclear.

The control of deposition of extracellular matrix compounds is a function of a number of cytokines acting in a paracrine fashion. This has been neatly demonstrated by co-culture of tubular epithelial cells and interstitial fibroblasts (Knecht et al, 1991). Deposition is stimulated by the fibrogenic cytokines Transforming Growth Factor β (TGFβ) and Platelet Derived Growth Factor (PDGF) (Nath, 1996). TGFβ has been most extensively studied. In rats it is released by both interstitial and tubular cells perhaps under the influence of Angiotensin II, and is chemoattractant and mitogenic for fibroblasts and can induce the activation of fibroblasts to myofibroblasts. However, incontrovertible evidence for the effect of TGFβ is difficult to obtain because prolonged systemic depletion is fatal. Treatment of rats with recombinant PDGF results in the accumulation of interstitial myofibroblasts and interstitial fibrosis whilst TGFβ mRNA levels are not raised (Tang et al, 1996)

There is also good evidence that increasing interstitial fibrosis is not only a function of increasing deposition, but also a consequence of reduced collagen breakdown. In models of renal fibrosis due to renal vein or ureteric ligation there are considerable reductions in collagenolytic activity (Gonzalez-Avila et al, 1988). Breakdown of collagen is a function of the Matrix Metalloproteinase family of enzymes and their respective inhibitors- Tissue Inhibitors of Metalloproteinase 1, 2 and 3 (TIMP1 ,TIMP2 and TIMP3). TIMP 1, the most extensively studied of the family, is not detected by immunohistochemical techniques in the interstitium of normal rat kidneys but is detectable in fibrotic kidneys. TIMP1 mRNA levels are found to be several times control levels in a number of experimental models of interstitial fibrosis including diabetic nephropathy (Wu et al, 1995). These findings are entirely compatible with the theory of decreased matrix degradation at a time of increased matrix deposition.

Interstitial Fibrosis: Balance Between Formation and Degradation

Accumulation of excess extracellular matrix is not haphazard but may follow a sequential pattern dependent upon the site studied. Indeed, Kuncio (Kuncio et al, 1991) has proposed three arbitrary phases of the fibrogenic process: Induction, Matrix Deposition and Resolution, though these may proceed in parallel rather than sequentially. This is supported by the fact that the nature of the fibrosis changes as it ages. Fibronectin, which is chemoattractant for fibroblasts (Gharaee-Kermani et al, 1996), appears first in fibrotic interstitium, perhaps to act as a scaffold for the subsequent deposition of collagen I and collagen III. In advanced interstitial fibrosis fibronectin is not a major contributor to the increase in extracellular matrix. Indeed, in a rat model of acute tubular necrosis secondary to ischaemia-reperfusion injury, the

deposition of interstitial fibronectin is found to be only transient (Walker, 1994) and subsequent deposition of collagens does not occur. Deposition of extracellular matrix proteins is a physiological response to injury that is necessary for the formation of scars and the healing process in general. However, progressive fibrosis in response to a long vanished insult is pathological and represents an imbalance between the processes of deposition and degradation, the controls of which are as yet unknown.

Predicting Donor Organ Quality by Assessment of Interstitial Fibrosis

Despite our incomplete knowledge of the signalling, control and source of interstitial fibrosis, correlation between the severity of fibrosis and the GFR has been confirmed in a large number of renal diseases. Assessment of the severity of interstitial fibrosis thus has the potential to provide information about the function of a kidney prior to transplantation i.e. to act as a surrogate marker of GFR. It has been assumed that kidneys from NHBD have the same functional potential as those from cadaveric donors. However, the mode of presentation of many NHBD i.e. with cardiovascular deaths suggests that they may have occult renovascular lesions and so may have greater levels of interstitial fibrosis and worse glomerular filtration rates than cadaveric donors. The aim of this study was to investigate the potential that quantitative analysis of interstitial fibrosis in pretransplant biopsies might have in predicting the results of renal transplantation. Immunohistochemical identification of collagen III was used as the marker of interstitial fibrosis.

Material and Methods

Cortical biopsies, obtained prior to reperfusion, from the 39 NHBD kidneys and 91 cadaveric kidneys identified as a matched group in Chapter 2 were studied. These kidneys are matched for the key parameters known to influence the outcome of renal transplantation. Wax embedded renal cortical tissue obtained from needle core biopsies was cut into 4μm sections and mounted on standard microscope slides. Collagen III was stained by an immunohistochemical technique using a primary goat anti human collagen III antibody and a peroxidase conjugated rabbit anti-goat secondary antibody with diaminobenzidene as the chromagen (see Appendix 6, page 150 for the protocol). A counterstain was omitted in order to facilitate subsequent image analysis (Figure 7.1). The slides were then analysed by a computerised image analysis technique. The sections were viewed on a photomicroscope (Zeiss) with an attached video camera. Images were grabbed onto an Apple Macintosh 7100/80 AV microcomputer and analysed using the freeware program 'NIH image'. Sequential microscope fields (x120 magnification) of cortex along each section were captured, without overlapping, up to a maximum of ten fields. The proportion of stained pixels in the interstitium was determined by the image analysis software: the threshold to distinguish between the stained and unstained pixels was set manually at commencement of the analysis and remained constant. The interstitial volume fraction of interstitial fibrosis (collagen III) for each field was calculated and a mean cumulative interstitial volume fraction calculated for the section. The reproducibility of this technique has been established in a previous study (Nicholson et al, 1999). Data concerning post transplant function were retrieved from an existing prospective transplant database. The mean interstitial volume fraction of interstitial fibrosis was compared for the different donor types using Student's t-test and the relationship

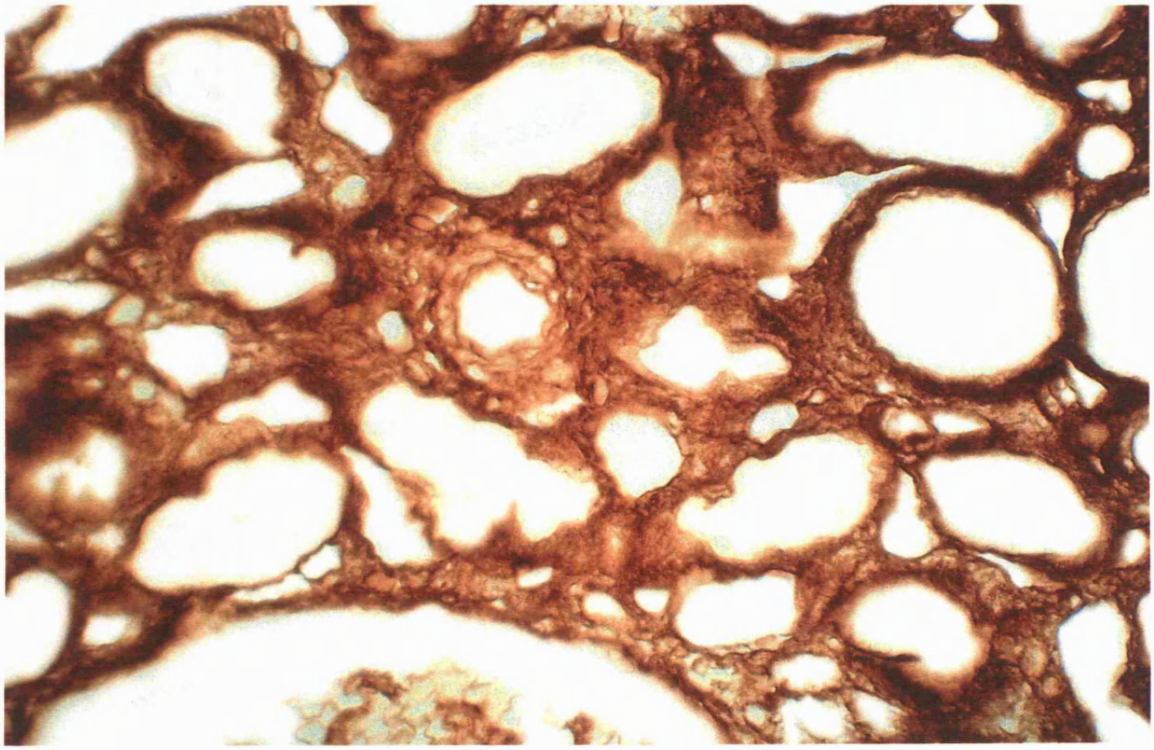
between the severity of interstitial fibrosis and (i) donor age and (ii) post transplant function represented by serum creatinine at 3 months, 6 months and 1 year, was investigated by Pearson correlation coefficients and calculation of the linear regression line.

Results

The interstitial volume fraction of collagen III in pre-perfusion biopsies is shown in Figure 7.2 and Table 7.1 for non heart-beating donors and in Figure 7.3 and Table 7.2 for cadaveric heart beating donors (HBD). Mean interstitial volume fraction of interstitial fibrosis was higher in the NHBD kidneys than the HBD kidneys (31% vs 28%, P=0.02; Figure7.4) but the ages of the donors were not different (mean 45 yr vs 47 yr, P=0.34; Figure 7.5). Correlation between donor age and severity of interstitial fibrosis was poor considering all biopsies (r^2=0.02, p= 0.132), HBD kidneys alone (r^2=0.015, p=0.375) or NHBD kidneys alone (r^2=0.12, p=0.054) (Figure 7.6). Severe interstitial fibrosis was seen in the biopsies of some kidneys from younger donors and mild fibrosis was seen in some biopsies from older donors. There was no significant correlation between 1/creatinine and the IVF of collagen III at 3 months (Figure 7.7), 6 months (Figure 7.8) or 1 year (Figure 7.9) in the HBD kidneys (r^2=0.014, 0.0003, 0.02) or the NHBD kidneys (r^2=0.04, 0.01, 0.02) respectively.

Figure 7.1: Photomicrograph of a Section of Renal Cortex Stained by Immunohistochemistry for Collagen III

x120



Diaminobenzidine gives an intense brown stain over the labelled collagen III.

Figure 7.2: Frequency Histogram Showing the Distribution of the Severity of Interstitial Fibrosis in Non Heart-Beating Donor Biopsies.

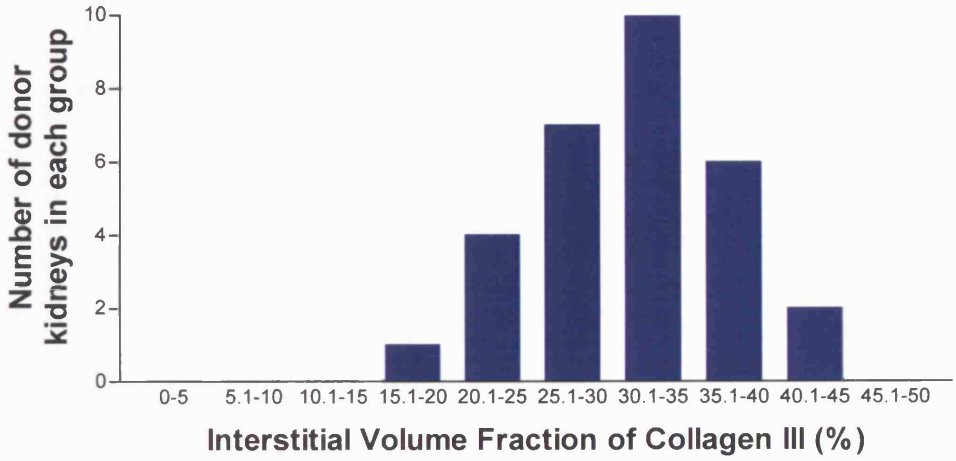


Table 7.1: Descriptive Data for the Interstitial Volume Fraction (%) in Non Heart-Beating Donor Biopsies

Number of Biopsies Studied	31
Mean (%)	30.98
Standard Deviation	6.176
Standard Error	1.109
95% Confidence Interval	28.71 – 33.24
Median (%)	30.59
Range (%)	16.86 – 43.97

Figure 7.3: Frequency Histogram Showing the Distribution of the Severity of Interstitial Fibrosis in Heart-Beating Donor Biopsies

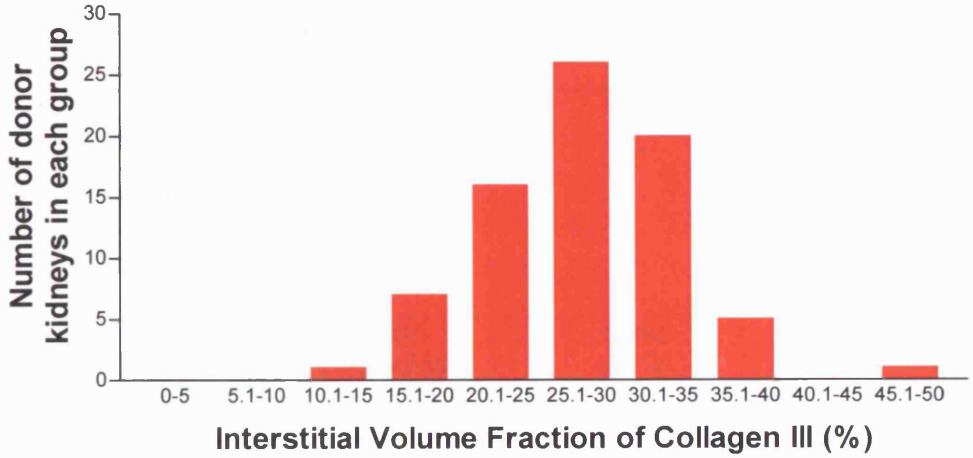


Table 7.2: Descriptive Data for the Interstitial Volume Fraction (%) in Heart-Beating Cadaver Donor Biopsies

Number of Biopsies Studied	75
Mean (%)	28.03
Standard Deviation	5.82
Standard Error	0.6675
95% Confidence Interval	26.7 – 29.36
Median (%)	28.08
Range (%)	14.49 – 49.57

Figure 7.4: Scattergram Showing the Interstitial Volume Fraction of Interstitial Fibrosis in Pre-perfusion Renal Biopsies by Donor Type

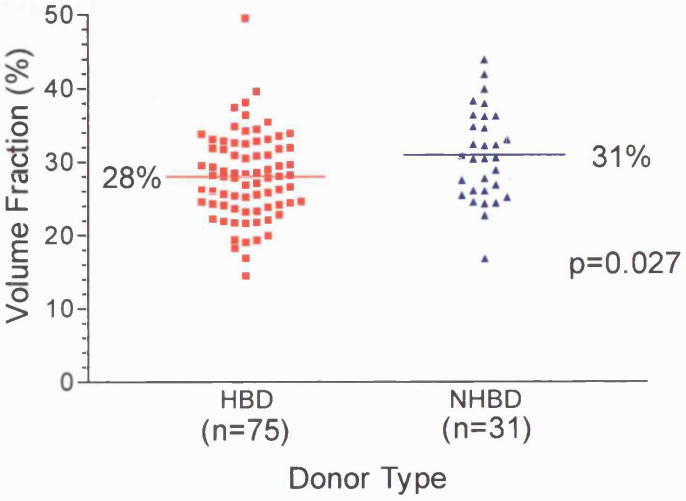


Figure 7.5: Scattergram Showing Donor Age by Donor Type for the Study Groups

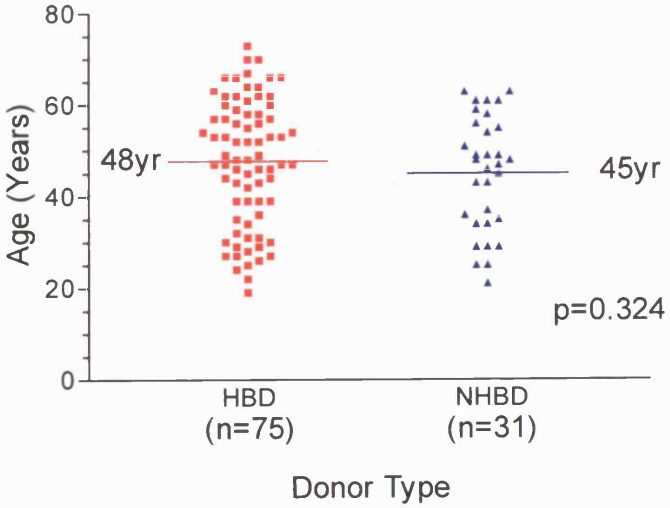
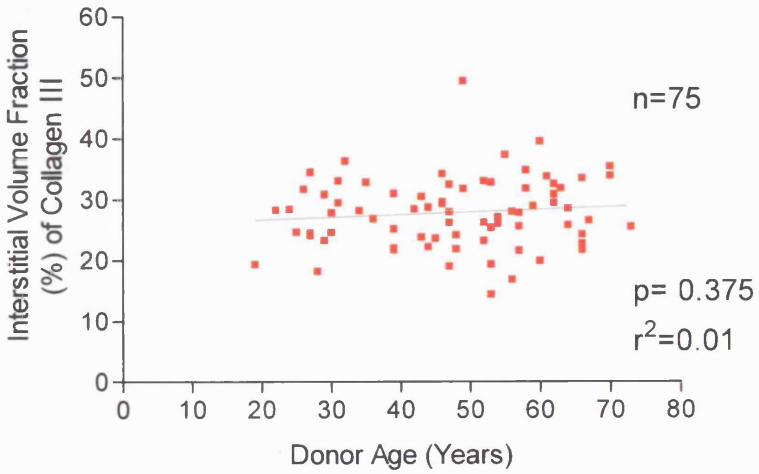


Figure 7.6: Graphs to Show the Relationship Between Renal Transplant Cortical Fibrosis and Donor Age in Heart Beating Cadaveric Donors and Non Heart-Beating Donors

i)Heart Beating Cadaver Donors



ii)Non Heart-Beating Donors

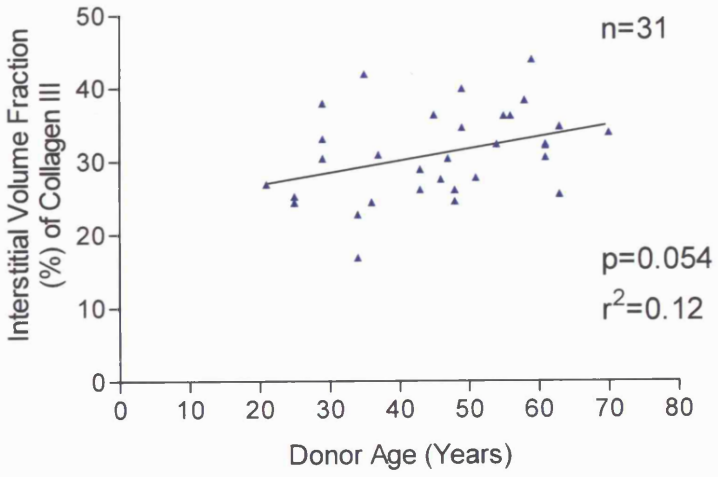
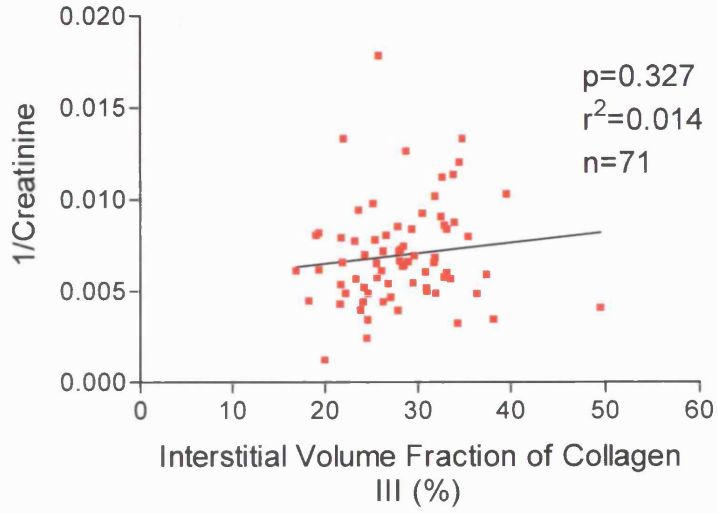


Figure 7.7:Graphs Showing the Relationship Between the Severity of Interstitial Fibrosis in Pre-Perfusion Donor Kidney Biopsies and 1/Creatinine at 3 Months Post Transplantation.

i) Heart Beating Donors



ii) Non Heart-Beating Donors

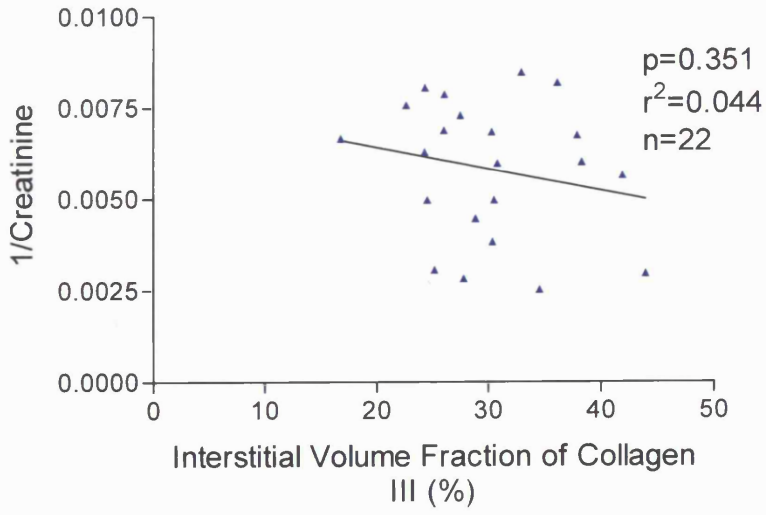
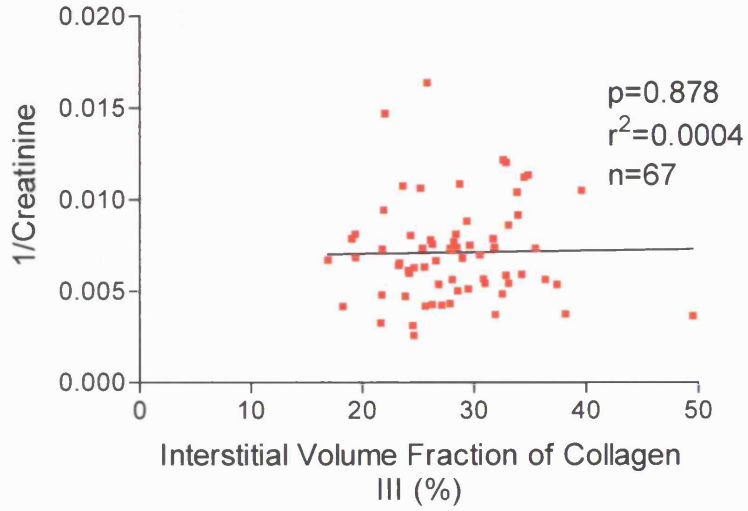


Figure 7.8: Graphs Showing the Relationship Between the Severity of Interstitial Fibrosis in Pre-Perfusion Donor Kidney Biopsies and 1/Creatinine at 6 Months Post Transplantation.

i) Heart Beating Donors



ii) Non Heart-Beating Donors

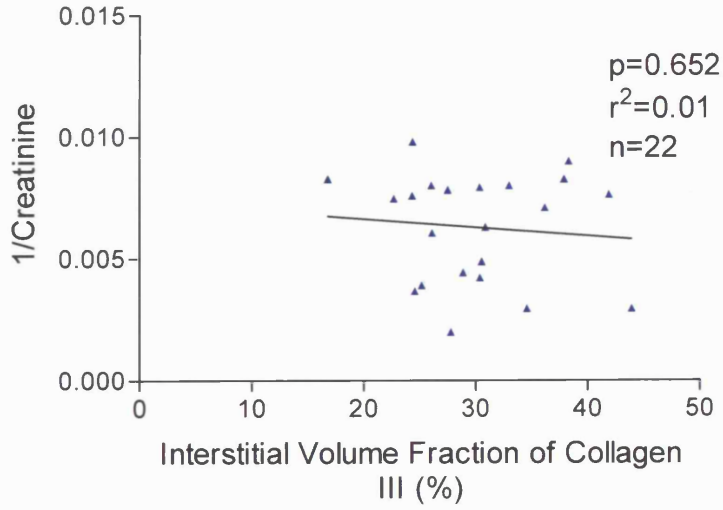
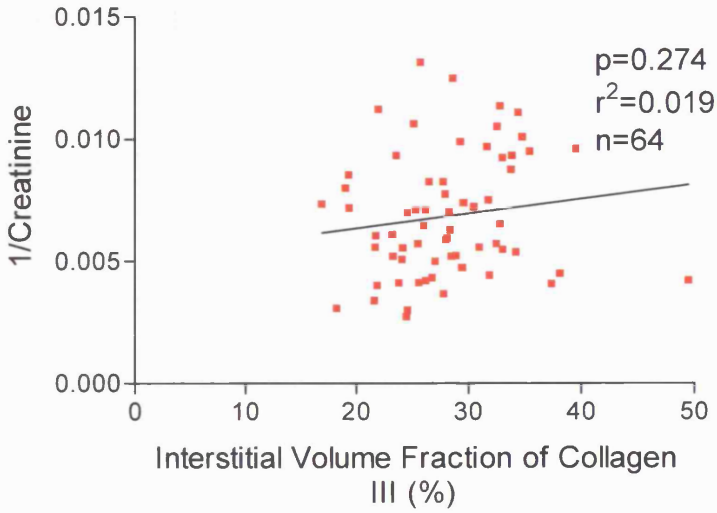
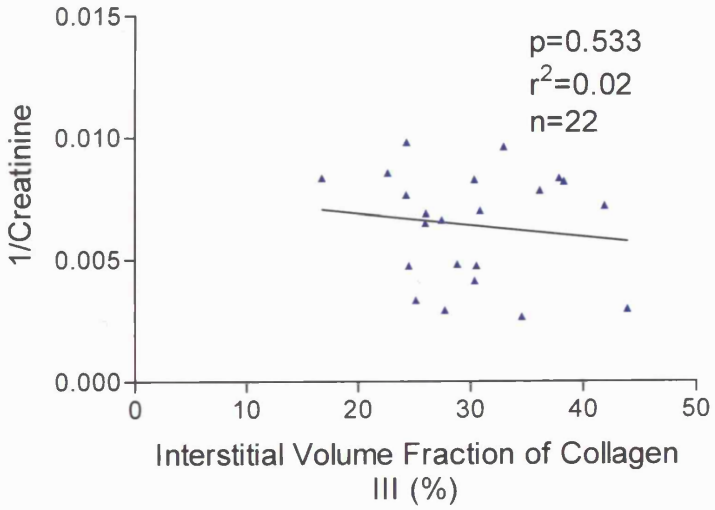


Figure 7.9: Graphs Showing the Relationship Between the Severity of Interstitial Fibrosis in Pre-Perfusion Donor Kidney Biopsies and 1/Creatinine at 1 Year Post Transplantation.

i) Heart Beating Donors



ii) Non Heart-Beating Donors



Discussion

Estimates of the interstitial volume fraction of interstitial fibrosis in normal kidneys range from 11-33%. (Kappel et al, 1980; Dunnill et al, 1973). When compared with the measurements of renal cortical interstitial fibrosis in other series the results presented here are somewhat higher. This could be a consequence of methodological differences in assessment of the interstitium. The image analysis system requires the threshold for discriminating stained i.e. counted pixels from unstained pixels to be set manually and this is certainly a source of potential discrepancy. Other image analysis systems, including those based on confocal microscopy (Ueda et al, 2002), are available, but the requirement for manual threshold discrimination remains. In addition this system includes in its assessment of the interstitium collagen incorporated in the walls of arteries. The original work comparing interstitial changes with GFR was done using a point counting rather than immunohistochemical technique (Risdon et al, 1968; Schainuck et al, 1970). This allows the walls of vessels to be excluded from the assessment but does not indicate what particular component of the extracellular matrix is most important. A number of proteins are deposited in the extracellular matrix of the renal cortex with advancing age and in disease states. These include Collagen III, Collagen IV, laminin and fibronectin. Collagen III was selected for study because it is the most abundant and is found in increased amounts in animal models of interstitial fibrosis (Downer et al, 1988). It is not clear if assessment of the other proteins, in particular collagen IV or fibronectin, would provide more information. It is also possible that using a non immunohistochemical technique such as Sirius Red which stains both Collagen I and Collagen III, would produce different results (Moreso et al, 1994). Furthermore, it has been assumed

that all of the biopsies have been taken of a similar level of cortex, or at least that differences in the level are evenly distributed between the two groups. The area fraction occupied by the interstitium is considerably greater in the renal medulla and may be greater in the juxta medullary cortex than in the superficial cortex.

Close scrutiny of the data from Kappel and Olsen (Kappel et al, 1980) reveals that interstitial fibrosis was more severe in the autopsy series than in the 'healthy' cadaveric organ donor series for any given age. The NHBD kidneys could well have belonged to Kappel and Olsen's autopsy series which, for a forty year old man, had a mean interstitial volume fraction of 28%. This value is not greatly different to the mean interstitial volume fraction in the NHBD which have a similar mean age. Biopsies from NHBD kidneys show statistically greater degrees of severity of interstitial fibrosis than biopsies from conventional cadaveric kidneys and this is not due to differences in age between the groups. However, the difference is small and the data must be considered with regard to three important caveats: (i) the reproducibility of the technique may not be sufficiently good for the difference to be confidently considered real (ii) the biopsy studied may not reflect the level of interstitial fibrosis globally within the cortex i.e. the results are explained by a sampling error (iii) the difference may be real but is small and may not be biologically significant. Nevertheless the possibility remains that NHBD kidneys may not have the same functional potential at procurement as cadaveric kidneys at a given age. It is therefore vital to consider all the available information when considering the merits of a potential non heart-beating organ donor and not to rely too heavily on age alone. Donor age as a variable is certainly predictive of transplant outcome but probably only as a surrogate marker of donor renal function and

perhaps only as a surrogate marker of the severity of interstitial fibrosis. If donor age and interstitial fibrosis show no relationship in NHBD this may be because of the presence of an underlying disease process in the kidney, such as renovascular disease from atherosclerosis and in this situation age may be less predictive of post transplant outcome than in cadaveric kidneys.

There are a number of potential pitfalls in using assessment of interstitial changes to predict organ quality. No data exists concerning any possible correlation between the GFR and the severity of interstitial fibrosis in humans with a normal serum creatinine who are not being investigated for renal disease i.e. potential NHBD, rather a relationship is inferred from data collected from patients with known renal disease. Biopsies from live kidney donors represent the only source of material whereby this relationship may be confirmed. How the severity of interstitial fibrosis, the transplanted nephron dose and the functional requirements of the recipient would combine as determinants of transplant outcome is not known. Serum creatinine is a readily available outcome measure but does not always correlate well with the glomerular filtration rate in the post transplant setting and methods derived to calculate GFR from known variables are also unreliable (Butterworth et al 1997; Perrone et al, 1992). The lack of measured GFR data is unfortunate but it is perhaps not surprising that there is no correlation between the interstitial volume fraction of collagen III and the post transplant serum creatinine in a small study using univariate analysis. The end result of placing a fibrotic kidney into a fibrogenic environment is unknown but data on graft survival will be interesting.

CHAPTER 8

CONCLUSIONS, THE FUTURE OF VIABILITY TESTING AND SUGGESTIONS FOR FURTHER WORK

CONCLUSIONS, THE FUTURE OF VIABILITY TESTING AND SUGGESTIONS FOR FURTHER WORK

Non Heart Beating Donors are a practical source of organs by which we may address the organ shortage. The short-term results of renal transplantation from NHBD are not dissimilar to the results for conventional cadaveric heart beating donors. The major problems remain the incidence of primary non-function, potentially a high discard rate for poorly perfused kidneys and the incidence of delayed graft function. No satisfactory test of viability exists which would prevent the transplantation of kidneys that will never function and prevent the discard of kidneys, which, though they may appear substandard to the naked eye, would function well after transplantation.

In this thesis the potential for an in vitro model of warm ischaemia, which might lead to the development of a viability test, has been explored: differences in the metabolism of cells in vitro compared with in vivo mean that such a model is unlikely to be helpful. The detection of apoptosis in pre-perfusion biopsies suggests that processes other than necrosis have a role in post transplantation renal dysfunction. The role of apoptosis after transplantation is not determined. Reperfusion may result in a greater role for apoptosis than can be seen in the pre-perfusion biopsies. The major problem in studying apoptosis in vivo remains the fact that it is a rapid process that is not detectable until well established and leaves no trace. Consequently a 'snapshot' view of the tissue may be difficult to interpret. However, this work suggests that closer study of apoptosis especially in the medulla is required. Serial biopsies in the first few hours following transplantation are likely to be most informative, but this approach would

require an animal model. The role of apoptosis over a slightly longer period might be revealed by studying a post perfusion biopsy and then weekly protocol biopsies during the period of delayed graft function in human NHBD transplants.

Interstitial fibrosis in pre-perfusion biopsies has been investigated as a marker of quality of the donor kidney: whilst there are statistically significant differences between the severity of interstitial fibrosis in NHBD kidneys compared with conventional cadaveric kidneys, the magnitude of the difference is unlikely to be biologically significant and the severity of interstitial fibrosis cannot be used on its own as a viability test. The quality of kidneys used in transplantation has not been well studied. In cadaveric transplantation clinical experience has shown that this is not necessary, since even kidneys from older donors may give excellent functional results. However, non heart-beating organ donors may have different comorbidities to their heart beating counterparts especially if elderly. Kidneys from such donors may be less tolerant of warm ischaemia or of prolonged cold ischaemia after warm ischaemia although the rarity of renal failure after cardiac arrest is low. Further study of the quality of NHBD kidneys is required to look for functional correlates.

The way forward in viability testing may lie down the previously well-trodden path of assessment by pulsatile machine preservation. Machine perfusion preservation is probably better for the preservation of ischaemically damaged kidneys than is static cold storage. Whether or not this will be seen in the long-term graft survival data is not clear- the significance of delayed graft function in NHBD series is not known.

Continuous perfusion also facilitates the measurement of substances released from the kidney over time- though these have not been as predictive as had been hoped. However, there is good data that the vasculature of ischaemically damaged kidneys may be improved by continuous perfusion and this may explain the good early function of non heart-beating donor kidneys which have been preserved in this way. In recent times great emphasis has been placed on the perfusion characteristics of individual kidneys on the machine. However using this data to support a decision to discard a kidney rather assumes that the best technique for perfusion has been well established and close perusal of the literature shows that this is not the case.

Prolonged pulsatile machine perfusion for renal preservation (for 24 hours or more) was first reported to give immediate graft function in a canine model with immediate contralateral nephrectomy on retransplantation in 1966 (Ackermann et al, 1966), though the publication by Belzer in the Lancet received greater attention (Belzer et al, 1967). However, the benefits of pulsation over constant pressure have not been clearly established but are assumed because this was considered more 'physiological'. The early reports described the selection of perfusion pressure based on achieving an immediate flow of 60-120ml per minute: the perfusion pressure tended to be in the range 50/20 to 80/40mmHg. No clear indication is given as to why these flow rates were selected. Canine kidneys preserved at mean arterial pressure of 20mmHg have been shown to give more immediate function on transplantation than the Belzer protocol (Grundmann et al, 1975). Despite this functional data, theoretical concerns about renal damage at higher pressures and the paucity of supporting experimental data, Belzer's perfusion protocol has been avidly taken up worldwide for pulsatile machine perfusion.

The importance of constant pressure perfusion over constant flow was established in rabbits (Pegg et al, 1973) but quite what the optimal initial perfusion pressure is has not been established by experiment in human kidneys. Certainly in human cadaveric kidneys pulsatile perfusion at 55mmHg was not inferior to perfusion at 60mmHg (Murata et al, 1978). The optimal perfusion protocol has yet to be determined.

The benefits of machine perfusion may well be the result of clearance of the vascular tree i.e. improved washout rather than by any particular improvement in cellular preservation per se. Obstruction of the intrarenal vasculature, particularly the medullary capillaries, is caused largely by erythrocyte aggregation rather than by activation of any part of the coagulation cascade (Mason et al, 1984). Whilst not influencing filtration directly it has been postulated that depression of tubular reabsorption in the thick ascending loop of Henle increases macula densa sodium chloride concentration, which activates the tubuloglomerular feedback mechanism. This component of acute renal failure / acute tubular necrosis thus may decrease filtration (Mason et al, 1987) though experimental attempts to confirm this theory by blocking tubuloglomerular feedback have not been confirmatory.

In a pig model, warm ischaemia prior to nephrectomy has been shown to result in marked vasospasm, which is associated with poor washout on initial perfusion and poor flows during machine pulsatile perfusion at 60mmHg systolic. In general flow rates improved with time of pulsatile perfusion though this was variable. Despite the absence of functional data after retransplant, these results were extrapolated to the human and it was suggested that

cadaver kidneys with low flow rates during preservation be discarded and not transplanted (Belzer et al, 1970).

There is no data in human kidneys which correlates flow characteristics with post transplant function in all kidneys rather than in a selected subset. Flow characteristics have not been shown to be better than subjective clinical assessment (Metcalfe et al, 2001). Whilst perfusion characteristics may reassure the transplant surgeon of what he already suspects from his clinical assessment they cannot, yet, be regarded as true viability tests. However, the determinants of renal blood flow, especially intrarenal vascular resistance do merit further study. The role of the endothelial cell is not well investigated as the focus has rather been on the proximal tubular cell. The data presented in this thesis suggests that, in vitro, non immortalised human endothelial cells may tolerate warm ischaemia less well than both non immortalised human tubular epithelial cells and immortalised pig proximal tubular epithelial cells. Since nitric oxide released by endothelial cells is the most important vasodilator, endothelial cell function is an important determinant of intrarenal vascular resistance and so the endothelial cells should certainly be targets for future research.

The body of evidence available suggests that a single assay of viability is unlikely to be predictive of post transplant function. A combination approach is more likely to be successful and I suggest that this would involve:

1) Some measure of tissue adenine nucleotide energetics - perhaps whole organ MRS- can the tissues be recharged?

2) Perfusion characteristics

3) Some measure of the quality of the kidney prior to retrieval

4) Attention to the component of medullary injury rather than focusing purely on the cortex.

In animal models it is essential that all kidneys be transplanted so that the effects of reperfusion can be factored into the equation. Only in this way can clinical correlation genuinely be made with the putative viability test or index. Retransplant experiments are the only way in which a test can be developed which confidently allows the discard of such a valuable resource.

APPENDICES

APPENDIX 1

CELL CULTURE

It should be noted that cell culture was performed by staff members of the Leicester General Hospital Transplantation Laboratory and not by the author. Prepared tissue culture plates were supplied as the cells grew to confluence. The protocols below are included for completeness.

1.(i) Cell Lines

The LLC-PK1 renal proximal tubular cell line is an immortalised cell line derived from the Hampshire Pig. It was selected for use in these experiments because it is considered to be a relatively differentiated cell line.

Non-immortalised human tubular epithelial cells were obtained by culture of biopsies of renal cortex: the protocol is outlined below in (iii).

Endothelial cells were obtained initially from fresh human umbilical vein and then by subculture: the protocol is outlined below in (iv).

1.(ii) General Cell Culture Techniques

All manipulation of fresh tissue and cultured cells was performed in a Class II laminar flow cabinet using sterile equipment. Cultured cells were fed at intervals of 48-72 hours and incubated in 5% CO_2 incubators at 37°C. Subculture was performed at 70-90% confluence. Subculture was achieved by suspending the cells by the addition of a 1:10 dilution of Trypsin/EDTA followed by incubation at 37°C for one minute. Full

mobilisation of cells was confirmed under invert microscopy before the trypsin was quenched by the addition of 10mls 10% fetal calf serum in calcium and magnesium free HBSS. The supernatant was transferred to a 25ml universal container and the cells washed twice in calcium and magnesium free HBSS by centrifugation at 167g for 5 minutes and resuspension. The pelleted cells were finally resuspended in 10mls of the appropriate media: 5mls was pipetted into a further 500ml flask for subculture and the remainder pipetted into plates for experimentation (see Appendix 7 for reagents).

1.(iii) Preparation of Human Non-Immortalised Tubular Epithelial Cells

Needle core biopsies of renal cortex were taken from kidneys immediately prior to transplantation. The biopsies were transported immediately to the laboratory in a 25ml universal containing RPMI medium. The biopsies were disrupted by pressing the tissue through a sterile sieve with a syringe plunger, collecting the disrupted tissue in a petri dish containing 12ml of kidney epithelial cell culture medium. The disrupted biopsy tissue and culture medium were transferred to an $80cm^3$ tissue culture flask, which was incubated in a humidified 5% CO_2 incubator for 14 days at 37°C. The epithelial cells are adherent and may be trypsinised and subcultured as above.

Kidney Epithelial Cell Culture Medium Comprises (200ml):

RPMI 1640	174ml
Foetal Calf Serum	20ml
Insulin, Transferin, Selenite solution 5mg/ml	2ml
Hydrocortisone 50ng/ml	1ml
Epidermal Growth Factor25ng/ml	1ml
Antibiotic/antimycotic solution	2ml

1.(iv) Preparation of Human Umbilical Vein Endothelial Cells (HUVECs)

Human umbilical cords were collected by staff in the maternity unit of the Leicester General Hospital. The cords were stored in 150ml sputum pots and transported to the laboratory within 24 hours. Endothelial cells were harvested from the umbilical vein by collagenase digestion using the following protocol. All manipulation was performed in a Class II laminar flow hood and the cells subsequently cultured in endothelial cell culture medium in 250ml flasks placed in a 5% CO2 incubator.

Endothelial Cell Culture Medium (ECM) comprises:

RPMI 1640 (10mM Hepes)	153ml
Foetal Calf Serum	40ml
Penicillin/Streptomycin	2ml
Heparin (9mg/ml in RPMI 1640)	2ml
L-Glutamine (200mM)	2ml
Endothelial Cell Growth Supplement (1:3 in RPMI)	1ml
Sodium Pyruvate (100mM)	0.4ml

1.(v) Protocol for HUVEC Primary Culture

1) Place the following equipment inside the hood:

1 kidney dish

70% propan-2-ol in a squeezy bottle

1 pair of disposable forceps

1 pair of stainless steel (non-disposable) forceps

2 stainless steel cannulae

2 stainless steel clamps

suture or string

1 sterile scalpel

1 drawing up (blunt) needle

1 30ml syringe

3 10ml syringes

Calcium and magnesium free Hanks balanced salt solution (CMF-HBSS)

Hanks balanced salt solution containing calcium, magnesium, 20% fetal calf serum, penicillin and streptomycin (HBSS-FCS)

Endothelial cell culture medium (ECM)

1 150µl aliquot of 0.1M Calcium Chloride

4 sterile universal containers

1 plastic disposal bag

In a water bath heated to 37°C place:

1 universal containing 30ml HBSS

1 test tube containing 10ml of 1% collagenase solution

Note: Wear two pairs of gloves

Before using the forceps, cannulae and clamps immerse them in propan-2-ol to to sterilise and then into HBSS on each occasion.

Put rubbish into the plastic bag whilst it is still in the hood.

2) Place a layer of blue roll tissue on top of the inverted kidney dish.

3) Remove the cord from the pot using forceps and place it on the kidney dish. Swab the exterior of the cord with propan-2-ol. Gently remove any visible blood clots from within the cord by massaging towards one end.

4) Cut 2cm off each end of the cord using the sterile scalpel. Ensure that the areas crushed by the application of clamps during parturition are excised.

5) Identify the lumen of the umbilical vein at both ends of the cord. Gently distend the lumen of the vein using sterile forceps.

6) Into one end of the vein (end#1) insert a pre-sterilised cannula using the forceps. Gently push the cord over the cannula and secure it in place using a string.

7) Take a 30ml syringe and fill it with CMF-HBSS. Attach the syringe to the cannula and perfuse the vein such that the CMF-HBSS flows from the other end of the cord into the tub in which the cord was originally collected. Repeat as necessary until the effluent perfusate is free of blood and clots. Flush residual CMF-HBSS from the vein by injecting air down the cannula.

8) Introduce the second cannula into the other end (end#2) of the vein. Gently push the cord over the cannula and secure using a string as in (6).

9) Take the collagenase solution from the waterbath into the laminar flow cabinet. Draw the collagenase up into a 10ml syringe using the needle. Change the syringe at end#1 of the vein for this loaded syringe.

10) Insert an empty syringe into the empty cannula at end#2.

11) Introduce the collagenase at end#1- the syringe at end#2 should fill under hydrostatic pressure.

12) Wipe the cord with propan-2-ol and transfer it, with both syringes still in place, to the 50ml universal containing HBSS in the waterbath.

13) Incubate for 20 minutes.

14) Remove the cord from the universal container and return it back to the laminar flow cabinet.

15) Wipe the cord with propan-2-ol and gently massaging as this is done.

16) Holding the cord vertically so as to prevent loss of the contents introduce 10ml HBSS-FCS via the cannula at end#1. The effluent is collected in the syringe at end#2.

17) Transfer the contents of the syringe at end#2 to a 25ml universal container.

18) Replace the syringe and flush 10ml of air into the vein from end#1 to ensure that all the solutions have been removed from the vein. Transfer any additional vein effluent from the syringe at end#2 to the universal container.

19) Centrifuge the cord effluent at 1000rpm for 5 minutes.

20) Resuspend the cell pellet in 5ml ECM and centrifuge again at 1000rpm for 5 minutes.

21) Resuspend the cell pellet in 5ml ECM and transfer to a 50cm3 culture flask.

22) Place the flask in a 37°C, 5% CO_2 incubator with the top loosened. Allow 24 hours for adherence before changing the medium.

APPENDIX 2

Assessment of Cell Viability by the MTT Assay

2(i) Background Information

The MTT assay measures mitochondrial function. MTT (diMethylthiazol-2-yl-2-5 diphenyltetrasolium bromide) in solution enters the mitochondria by diffusion. The enzyme Succinate Dehydrogenase catalyses the ATP dependent reduction of MTT to an insoluble formazan salt which precipitates in the cell. This salt produces a bright purple colour when solubilised in ethanol- the salt needs initially to be liberated from the cell by the action of dimethyl sulfoxide. The purple colour of solubilised formazan provides the basis for a colourimetric assay. The quantity of formazan and thus the metabolic activity of the mitochondria is measured by the absorbance of light with a wavelength of 540nm as measured using a plate reader.

2(ii) Protocol for the MTT assay

See Appendix 7 for the reagents.

Note: MTT is carcinogenic and the powder should only be manipulated in a class 1 laminar flow cabinet to prevent accidental inhalation. Gloves must be worn at all times.

1) Weigh out 20mg of MTT into a 25ml universal container.

2) Add 4mls of HBSS to make up a 5mg/ml solution. Wrap the universal container in aluminium foil to protect the contents from the light and place it on an agitating plate in a 37°C warming oven. Leave for 15 minutes.

3) Filter the MTT solution through a 0.45μm filter to remove undissolved MTT.

4) Add 100µl of MTT solution to each experimental well- do not add to one column of the plate which will act as blanks. Incubate for 4 hours at 37°C..

5) Gently remove the medium using a pipette.

6) Wash the cells by gently adding 100µl of PBS and then aspirate it off.

7) Add 150µl of 1:1 DMSO:Ethanol to each well.

8) Agitate the plate on a plate agitator for 10 minutes until dissolution of the formazan is uniform.

9) Transfer the plate to a plate reader and read at 540nm and 690nm reference wavelength.

10) The true absorbance is calculated as the absorbance at 540nm minus the absorbance at 690nm.

APPENDIX 3

Measurement of Lactate in Tissue Culture Medium

3.(i) Background Information

Lactate (lactic acid) is the end product of glycolysis and is released by the cells into the culture medium. Lactate dehydrogenase catalyses the reaction

$$\text{Lactate} + NAD^+ \rightarrow \text{Pyruvate} + NADH$$

The NADH generates an optical density, which is detected by spectrophotometry at 340nm.

3.(ii) Protocol for the Lactate Assay

1) Equipment and Reagents (See Appendix 7)

12mm disposable tubes-one per sample

Deionised water

Glycine/hydrazine buffer pH9.2

Nicotinamide Adenine Dinucleotide

Lactate Dehydrogenase

Lactic Acid standard solutions made up to the following concentrations:

0 nmol/ml, 400nmol/ml, 800 nmol/ml, 1200nmol/ml

Quality control specimen of previously used medium

Spectrophotometer

2) Thaw and vortex each specimen.

3) Pipette 55µl of each of the standards and 3 aliquots of quality control into individual tubes.

4) Pipette 10 µl of each sample of medium into individual tubes. To each of these add a further 45 µl of deionised water to make a total volume of 55µl.

5) Add 350µl of the glycine/hydrazine/NAD^+ mixture to every test tube.

6) Vortex to mix.

7) Transfer 190µl from each tube to the cuvette of the spectrophotometer and read the optical density at 340nm.

8) To each tube add 4µl of lactate dehydrogenase and vortex to mix.

9) Incubate at room temperature on the bench for 40 minutes.

10) Transfer 190µl from each tube to the cuvette of the spectrophotometer and read the optical density at 340nm.

11) Calculate the regression equation for the optical density of the standards.

12) Use the increase in optical density between (7) and (10) in the equation to calculate the lactate concentration in the tube and then adjust for the starting dilution factor.

APPENDIX 4

Method for Extraction and Assay of DNA from Cultured Cells

4.(i) Assay Reagents (See also Appendix 7)

1. Acetaldehyde

Dissolve 50µl of acetaldehyde in 25ml of deionised water. Acetaldehyde boils in the pipette tip so it is necessary to chill the acetaldehyde bottle on ice and then draw the acetaldehyde in and out of the pipette tip four or five times to chill the tip. The transfer can then be made reliably into the water.

2. Perchloric Acid (70%w/w BDH)

For a 20% solution make up 290ml of the 70% stock solution to 1 litre with deionised water. For a nominally 10% solution make up 145ml of the 70% stock to 1 litre with deionised water.

3. Acetic Acid (Glacial - AnalaR)

4. Sodium Hydroxide Solution (5mM)

5. DNA (High MW) from calf thymus. Type 1. (For Calibration Standards)

6. Diphenylamine

1g dissolved in 25 ml of glacial acetic acid in a plastic pot.

NB. Diphenylamine is toxic. It is supplied in 25g bottles which should be divided into 1.00g portions in a fume cupboard (with the light switched off) whilst wearing gloves,

safety goggles and a respirator. Each 1g portion should be wrapped in foil and stored in the poisons cabinet.

Preparation of DNA Standards

Dissolve 20mg of calf thymus DNA in 50ml of 5mM Sodium Hydroxide, stirring in a magnetic stirrer for 30 minutes. Add 50ml of 20% Perchloric Acid: the DNA will precipitate. Cap off the container and transfer to a 70°C water bath. Incubate for 20 minutes after which time the precipitate should have dissolved giving a digest equivalent to 20mg/100ml or 20μg/ml. This digest may be stored in a capped universal container at -20°C.

To make up a series of DNA Standards, dilute the 20μg/ml DNA stock solution with 10% perchloric acid according to the table below:

Final [DNA] (μg/ml)	0	10	20	30	50	100	150	200
Volume(ml) of DNA hydrolysate (200μg/ml)	0	0.1	0.2	0.3	0.5	1.0	1.5	2.0
Volume (ml) of 10% Perchloric Acid	2	1.9	1.8	1.7	1.5	1.0	0.5	0

These standards can also be stored in capped tubes at -20°C.

Quality Control

A sample of rat muscle DNA digest in 10% perchloric acid was used for quality control.

4.(ii) Protocol for Performing the Assay

1. Thaw the stored culture plates, place the bottle of 10% perchloric acid on ice and chill the centrifuge to 1°C. Turn on the water bath to warm up to 70°C.

2. Into each well of the culture plate pipette 150µl of ice cold 10% perchloric acid.

3. Scrape each well with the rubber plunger of a 1ml syringe (or a rubber policeman) to remove the precipitated DNA.

4. Using a 3ml plastic Pasteur pipette transfer the 150µl of precipitate solution to a 1.5ml microcentrifuge tube.

5. Repeat steps 2-4 with a further 150µl of 10% perchloric acid.

6. Chill the precipitates in the microcentrifuge tubes for 30 minutes at 4°C to maximise precipitation.

7. Transfer the microcentrifuge tubes to the water bath and incubate at 70°C for 10 minutes. Then vortex each tube to coat any precipitate stuck high on the walls of the tube with hot acid. Return the tubes to the water bath for a further 12 minutes.

8. Chill the microcentrifuge tubes in a fridge at 4°C overnight to precipitate out protein.

9. Centrifuge the tubes for 10 minutes at 3500rpm at 1°C to spin down the protein precipitate and leave the soluble digest of DNA as supernatant.

10. Transfer 100µl of supernatant to labelled 1.5ml microcentrifuge tubes for assay of DNA. Also pipette out 100µl of DNA standard solutions and quality control solution.

11. Into each tube pipette 100µl of diphenylamine reagent and vortex immediately.

12. As soon as possible after adding the diphenylamine reagent, add 20µl of acetaldehyde solution and immediately vortex.

13. Cap the tubes and incubate overnight in a water bath at 25-35°C. (Keep the lid on the water bath to minimise evaporation and protect the samples from light.)

14. Transfer 190µl aliquots from each tube to a 96 well microplate.

15. Read the absorbance on a plate scanner using the 595nm filter and then the 710nm filter. (Remember to allow 10 minutes for each filter to warm up in the plate scanner beam before scanning.)

Calculation

1. The assay result is the absorbance at 595nm minus the absorbance at 710nm.

2. Plot and calculate the regression equation : absorbance against standard concentration of DNA.

3. Derive the concentration of DNA in each sample by inserting the absorbance result into the regression equation.

4. Convert the concentration of DNA in the 100µl aliquot to a mass of DNA in the original experimental well using Mass (µg) = Concentration (µg/ml) x 0.3ml

APPENDIX 5

Protocol for TUNEL Test Immunohistochemistry

5.(i) Protocol for TUNEL Immunohistochemistry

This protocol is a modification of the method described by Gavrielli et al, 1992

1) The following equipment is required (See Appendix 7 for details of reagents)

aliquot of TdT enzyme-stored in crushed ice

aliquot of reaction buffer

aliquot of equilibration buffer

100ml 10% neutral buffered formalin (4% paraformaldehyde)

100ml 0.05M glycine in Phosphate Buffered Saline (PBS) as post fix rinse- see 'preparation of reagents'(a)

PBS to excess

TBS (Tris Buffered Saline) at pH 7.6 in a squeezy bottle - see 'preparation of reagents'(b)

3 coplin jars

Plastic Pasteur pipettes

two plastic coverslips per slide

tissue paper

humidified chamber

50 ml stopwash buffer-prewarmed to 37°C in a water bath

aliquot of antidigoxigenin-alkaline phosphatase enzyme

substrate buffer - see 'preparation of reagents'(c)

solution of fast red in substrate buffer - see 'preparation of reagents'(d)

Mayers haematoxylin

Gelatin mounting medium

Glass coverslips

2) Dewax the tissue by immersing the slide in xylene for 10 minutes, with a change of xylene after 5 minutes.

3) Rehydrate the tissue by immersing the slide initially in 100% alcohol for 5 minutes, then 50% alcohol for 5 minutes, 30% alcohol for 5 minutes, 10% alcohol for 5 minutes. Immerse the slide in distilled water for 5 minutes and then repeat, with fresh distilled water for a further 5 minutes.

4) Remove the slide from the distilled water and tap off excess. Immerse the slide in 0.05M glycine in PBS for 2 minutes.

5) Remove the slide from the glycine and gently irrigate with PBS from the squeezy bottle. Repeat this irrigation twice.

6) Immerse the slide in Proteinase K (20μg/ml in PBS) in a Coplin jar for 15 minutes at room temperature.

7) Irrigate the slide with PBS from the squeezy bottle. Repeat the irrigation twice then remove excess PBS from the slide by blotting with the edge of a piece of tissue paper- take care not to touch the section on the slide.

8) Apply 15μl of equilibration buffer to one end of the tissue using a pipette.

9) Lower a plastic coverslip, cut such that it just covers the tissue, onto the slide starting at the end where the equilibration buffer has been placed and continuing such that the buffer is spread evenly over the tissue. Leave for 10 minutes.

10) Meanwhile add reaction buffer to TdT solution in ratio 4:1. Vortex to mix and replace in the bath of crushed ice.

11) Remove the coverslip from the slide and blot around the section with tissue paper.

12) Apply 15μl of TdT/Reaction buffer to the slide and apply a coverslip in the manner described in 9 above.

13) Place the slide in a humidified chamber and incubate for 2 hours at 37°C

14) Remove the coverslip and wash the slide in TBS by irrigating from the squeezy bottle.

15) Place the slide into the Coplin jar containing pre-warmed stopwash. Leave for 30 minutes.

16) Remove the slide from the Coplin jar and wash twice by irrigation with TBS.

17) Dry around the section with tissue paper.

18) Apply 15µl of Anti-Digoxigenin-Alkaline Phosphatase and coverslip as before (use a new coverslip). Incubate in a humidified chamber for 30 minutes at room temperature.

19) Remove the coverslip and wash the slide in TBS by irrigating from the squeezy bottle. Dry around the section using tissue paper.

20) Apply 15µl of substrate buffer and coverslip. Leave for 5 minutes and during this time make up the substrate buffer.

21) Remove the coverslip and wash the slide in TBS by irrigating from the squeezy bottle. Dry around the specimen using tissue paper.

22) Apply 15µl of fast red substrate buffer, coverslip and incubate in a humidified chamber for 1 hour at 37°C.

23) Remove the coverslip. Place the slide in a coplin jar containing TBS. Leave for 5 mins then agitate the slide and repeat with fresh TBS

24) Remove the slide from the coplin jar and dry around the section using tissue paper.

25) Drop sufficient haematoxylin onto the specimen to completely cover it. Wait for 75 seconds.

26) Place the slide in a coplin jar containing tap water previously passed through a 0.2µm filter. Wait for 2 minutes and repeat twice with fresh tap water.

27) Place the slide in a class 1 laminar flow cabinet to dry and then transfer to a warming oven at 37°C.

28) Mount the warm slides in warm gelatin mount using glass coverslips and allow to dry.

5.(ii) Preparation of Reagents for the TUNEL Test

a) Post Fix Rinse

Glycine dissolved in PBS to a concentration of 0.05M- stored at room temperature

b) TRIS Buffered Saline (TBS)

Sodium Chloride	8.1g
TRIZMA	0.6g
1M Hydrochloric Acid (dropwise to pH7.6) approximately	3.8ml
Distilled Water	1000ml

The sodium chloride and Trizma were dissolved in the water and 1M Hydrochloric Acid was added dropwise under continual pH monitoring until the required pH was achieved.

c) Substrate Buffer

TRIS (pH8.2)	10ml
5M NaCl	2ml
1M $MgCl_2$	5ml
Distilled Water	82ml
0.1M Levamisole	1ml

d) Fast Red Substrate

Naphthol-AS-MX phosphate	2mg
Substrate buffer	2mls
Fast Red TR salt	10mg

The Napthol and Fast Red Salt were only handled in a Class 1 laminar flow cabinet. Each was added to 1 ml of substrate buffer and agitated for five minutes at room temperature. These two solutions were then mixed together and then passed through a 0.45μm filter. This substrate was made immediately prior to use and the excess discarded.

Appendix 6

Protocol for Collagen III Immunohistochemistry

6.(i) Protocol for Immunohistochemistry

1. Dewax sections by immersing the slides in xylene for 10 minutes

2. Rehydrate sections in 99% alcohol: 3 changes of solution for 3 minutes each

3. Rinse with distilled water

4. Block endogenous peroxidase activity by immersing in 3%H_2O_2 /1MHCL for 20 minutes

5. Rinse with distilled water

6. Wash with TBS for 3 minutes

7. Digest protein by washing in pepsin solution at 37°C for 45 minutes

8. Wash with TBS for 3 minutes

9. Incubate in 1:5 normal rabbit serum/TBS for 20 minutes

10. Wash with TBS/BSA for 3 minutes

11. Incubate in primary antibody- goat anti type III collagen (1:25 diluted with TBS/BSA) overnight at 4°C

12. Wash with TBS/BSA for 3 minutes

13. Incubate in secondary antibody- peroxidase conjugated rabbit anti goat immunoglobulins (1:25 diluted with TBS/BSA) for 30 minutes

14. Wash with TBS/BSA for 3 minutes

15. Wash with Diaminobenzidene solution for 5 minutes

16. Wash with water

17. Enhance colour by washing in $CuSO_4$/NaCl for 5 minutes

18. Rinse with water

19 Mount

6.(ii) Preparation of Reagents (See also Appendix 7)

Tris Buffered Saline TBS

Tris (hydroxymethyl) methylamine	121.14g
NaCl	175.32g

Dissolve in 700ml of distilled water, adjust to pH 7.65 and make up to 1 litre with distilled water.

Pepsin Digestion

Distilled water	300ml
HCl	3ml
Pepsin	0.075g

Prepare fresh and pre warm to 37°C

TBS/BSA

TBS	1 litre
BSA	0.1%

Prepare fresh

Diaminobenzidene Solution

DAB tablet x1 dissolved in 1ml of distilled water: prepare and use in fume cupboard.

$CuSO_4$/NaCl

$CuSO_4$	4g
NaCl	7.2g

Dissolve in 1 litre of distilled water. Stable for six months but filter before re use

Appendix 7

List of Equipment and Reagents Used

ITEM	STORAGE	CATALOGUE NUMBER	SUPPLIER
General Tissue Culture			
10% Formalin Solution			LGH Pathology
Sodium Pyruvate	4°C	S8636	Sigma
L-Glutamine (200mM)	-20°C	G7513	Sigma
Heparin	4°C	H3149	Sigma
Endothelial Cell Growth Supplement	-20°C	E2759	Sigma
Collagenase (Blend H)	-20°C	C8051	Sigma
Propan-2-ol	RT	29694	Fisons
HEPES Buffer	4°C	H 0887	Sigma
Phosphate Buffered Saline	4°C	M34A	Microgen Bioproducts
Hanks Balanced Salt Solution (HBSS)			
with calcium and magnesium	4°C	H 1641	Sigma
without calcium and magnesium	4°C	H 9394	Sigma
RPMI 1640	4°C	R7006	Sigma
Dulbeccos Modified Eagles Medium	4°C	D 5546	Sigma
Foetal Calf Serum	-20°C	A5-302-50	Advanced Protein Products
Penicillin/Streptomycin	4°C	M971	Imperial Laboratories
Trypsin/EDTA	-20°C	T 4174	Sigma
Sodium Chloride	RT	10241	BDH
Magnesium Chloride (1M soln)	RT	M1028	Sigma
Calcium Chloride	RT	C7902	Sigma
Antibiotic/antimycotic solution	-20°C	A5955	Sigma
Epidermal Growth Factor 25ng/ml	-20°C	E1264	Sigma
Insulin, Transferin, Selenite solution 5mg/ml	-20°C	I1884	Sigma
Hydrocortisone 50ng/ml	-20°C	H6909	Sigma
Hypoxic Culture Model			
Sodium Hydrosulfite (dithionite)	RT	S-1256	Sigma
AnaeroGen	RT	AN 35	Unipath
Atmosbag-two hand (ethylene oxide treated)		Z11, 836-2	Aldrich
Sodium Azide	RT	S 2002	Sigma
Potassium Cyanide	RT	60178	Sigma

TUNEL Immunohistochemistry			
Glycine	RT	G-6761	Sigma
Proteinase K	RT	P6556	Sigma
ApopTag Plus Kit comprising		S7101-kit	Oncor Appligene
Equilibration Buffer	-20°C	S7101-1	
TdT enzyme	-20°C	S7101-3	
Reaction Buffer	-20°C	S7101-2	
Stopwash Buffer	-20°C	S7101-4	
Anti digoxigenin-Alkaline Phosphatase	4°C	1093 274	Boehringer Mannheim
Bovine Serum Albumin	RT	A 4161	Sigma
Levamisole	RT	L9756	Sigma
Trizma	RT	T 7149	Sigma
Fast Red TR Salt	-20°C	F 1500	Sigma
Naphthol AS MX Phosphate	-20°C	N 5000	Sigma
Glycerol Gelatin	4°C	GG 1	Sigma
Glass Coverslip	RT	KTH 507	Knittel GLASER
Silica Gel	RT	S 7625	Sigma
Magnesium Chloride (1M soln)	RT	M1028	Sigma
MTT Assay			
MTT	4C	M5655	Sigma
Ethanol	RT	SIN 1170	Hayman Limited
DiMethylSulfoxide (DMSO)	RT	D 05879	Sigma
Titertek Multiskan MC			Flow Laboratories
Lactate Assay			
Lactate Dehydrogenase	4°C	L 3379	Sigma
Glycine Buffer pH 9.2	4°C	G826-3	Sigma
Nicotinamide Adenine Dinucleotide (NAD^+)	-20°C	260-120	Sigma
Lactic Acid Standard Solution	-20°C	69780	Sigma
DNA Assay			
Acetaldehyde GPR	-20°C	27003	BDH
Perchloric Acid 70%	RT	10176	BDH
Acetic Acid (Glacial-AnalaR)	RT	622500	BDH
Sodium Hydroxide Pellets	RT	102527R	BDH
DNA (Calf thymus- type 1)	-20°C	D1501	Sigma
Diphenylamine	RT	D2385	Sigma

Collagen III
Immunohistochemistry

Pepsin 1:60000	-20°C	P 7012	Sigma
Goat anti Type III Collagen-unlabelled	-20°C	1330-01	Euro-Path
Peroxidase conjugated Rabbit anti-goat immunoglobulins	-20°C	P0449	Dako
Diaminobenzidene (as tablets)	-20°C	D 4168	Sigma
Copper Sulphate	RT	27848	BDH
Aquamount	RT	362262H	BDH
Hydrogen peroxide	RT	27657	BDH
Hydrochloric acid (1M)	RT	34865	BDH
Trizma	RT	T7149	Sigma

Appendix 8

Details of Suppliers

Aldrich
Subdivision of Sigma-Aldrich Company Ltd
The Old Brickyard
Gillingham
Dorset
SP8 4JL
United Kingdom
Telephone number for orders 0800 717181

Sigma Chemical Company
Subdivision of Sigma-Aldrich Company Ltd
Fancy Road
Poole
Dorset
BH12 4QH
United Kingdom
Telephone number for orders 0800 373731

Boehringer MannheimUK
(Diagnostics and Biochemicals) Ltd
Bell Lane
Lewes
East Sussex
BN7 1LG
Telephone number for orders 0800 521578

Flow Laboratories Ltd
Dolphin House
Rockingham Road
Uxbridge
Middlesex
UB8 2UE
United Kingdom

Hayman Limited
70 Eastways Industrial Park
Witham
Essex
CM8 3YE
United Kingdom

Imperial Laboratories (Europe) Ltd
West Portway
Andover
Hampshire
SP10 3LF
United Kingdom

Life Technologies Ltd
3 Fountain Drive
Inchinnan Business Park
Inchinnan
Renfrewshire
PA4 9RF
United Kingdom

Microgen Bioproducts Ltd
1 Admiralty Way
Camberley
Surrey
GU15 3DT
United Kingdom

Oncor Appligene
Pinetree Centre
Durham Road
Birtley
Chester-le-Street
County Durham
DH3 2TD
United Kingdom

BDH Laboratory Supplies Ltd
Poole
United Kingdom

Unipath Ltd
Wade Road
Basingstoke
Hampshire
United Kingdom

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