AN ANALYSIS OF THE RELATIONSHIP BETWEEN CELLULAR AND FUNCTIONAL RECOVERY IN CLINICAL HEART TRANSPLANTATION

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Objective: Cardiac transplantation is faced with a decreasing number of donors and a higher proportion of borderline donor hearts. The main cause of early death is donor organ failure, which is more commonly right ventricular (RV) failure for incompletely understood reasons. We aimed to describe the high energy phosphate (HEP) metabolism and endothelial cell activation (ECA) after brain death and subsequently throughout transplantation and to make correlations with functional performance.

Patients and methods: Sixty-nine donor hearts (including 6 domino) were studied. Transmural biopsies were taken from both ventricles at 2 time points during the donor operation and repeated 3 times during implantation. In addition, heart transplant patients had postoperative biopsies taken at 1 week, 1 month and 3 months postoperatively during rejection surveillance. HEP were measured by bioluminescence and ECA markers were assessed in situ by immunohistology. The following markers were studied: P-sel, E-sel, VCAM-1, thrombomodulin, iNOS, hsp70 and the apoptotic markers Bcl-2 and Bax. 17 donors and 5 recipients also had intraoperative measurements with a conductance catheter inserted in the RV.

Results: Brain death does not affect HEP metabolism quantitatively but is associated with upregulation of adhesion molecules and thrombomodulin depletion, a phenomenon which occurs in domino hearts too. The 2 ventricles are not affected differently, however important time-dependent changes are seen. HEP levels fall significantly during warm ischaemia and recover partially after 10 minutes of reperfusion. HEP levels before reperfusion are poorly predictive of donor organ failure. Dysfunctional organs sustain the biggest injury at reperfusion and overall fail to replenish their energy stores. The expression of adhesion molecules is progressively upregulated during the procedure and remains high postoperatively in the absence of histological rejection. Bcl-2 and hsp70 are not expressed in the acute phase. Conversely, Bax and iNOSs are uniformly present in all vessels and the intensity of staining in the surrounding myocytes increases in time-dependent fashion. None of the tissue markers studied was predictive of donor organ failure. A dissociation was observed between HEP metabolism and contractile performance by pressure-volume loops.

Conclusion: Clinical transplantation is associated with cumulative injury to the myocardium and the endothelium. Despite this, most hearts perform well postoperatively. The pathophysiology of donor organ failure remains unclear, but the biggest insult seems to be sustained at reperfusion. This characterisation should assist in preservation and organ protection efforts for cardiac transplantation.
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### COMMON ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
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<td>AMP</td>
<td>adenosine 5'-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell leukaemia/lymphoma 2-like proteins</td>
</tr>
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<td>BSD</td>
<td>brain stem death</td>
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<tr>
<td>CAM</td>
<td>cell adhesion molecule</td>
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<td>CAV</td>
<td>cardiac allograft vasculopathy</td>
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<tr>
<td>CP</td>
<td>creatine phosphate</td>
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<tr>
<td>EC</td>
<td>energy charge</td>
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<tr>
<td>ECA</td>
<td>endothelial cell activation</td>
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<td>EDPVR</td>
<td>end-diastolic pressure-volume relationship</td>
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<td>E-selectin</td>
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<td>ESPVR</td>
<td>end-systolic pressure-volume relationship</td>
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<td>Hsp70</td>
<td>heat shock protein 70</td>
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<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IR</td>
<td>ischaemia-reperfusion</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>P/V</td>
<td>pressure-volume</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>PAR</td>
<td>protease-activatable receptor</td>
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<tr>
<td>PRSW</td>
<td>preload-recruitable stroke work</td>
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<tr>
<td>P-selectin</td>
<td>P-selectin</td>
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<tr>
<td>TM</td>
<td>thrombomodulin</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
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<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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<td>vWF</td>
<td>von Willebrand factor</td>
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CHAPTER 1
HEART FAILURE AND THE ROLE OF TRANSPLANTATION

1.1 THE DIMENSION OF CARDIAC FAILURE

Heart failure is increasingly a public health burden in the developed world. How is this possible in the presence of better prevention, new medical and surgical therapies? This section analyses the public health dimension and defines the subgroup of patients with the worst prognosis, requiring intensive hospital therapy.

Epidemiology

Heart failure has a prevalence of close to 2% in the adult population of the western world. The overall annual incidence of clinically overt cardiac failure in middle-aged subjects is 0.1-0.2% (McMurray, 2000). Defining heart failure in the general population is however difficult. The problems arise from the type of study, cross-sectional or longitudinal, the age group and geographic location of the subjects and the variable definition of heart failure based on clinical, echocardiographic or therapeutic criteria. The difficulties are compounded by the following disturbing notion: a number of patients have clinical heart failure in the presence of preserved left ventricular function indices, whereas another group with left ventricular dysfunction remain asymptomatic for variable periods. One thing, however, is clear from all the studies. The prevalence, incidence and severity of cardiac failure all increase with age. This further reflects directly on the frequency and length of repeated hospital admissions. It is estimated that in the UK 1.2% of the health care expenditure is consumed to treat this condition, approximately 70% of the total cost being represented by hospital admissions (McMurray, 1993).

Aetiology

Long-term surveillance of the cohorts in the Framingham heart study showed that important changes took places in their aetiological profile (Kannel, 1994). Between 1950 and the late 1980s the prevalence of valvular disease and hypertension diminished dramatically whereas that of coronary artery disease and diabetes increased. This is explained by parallel therapeutic developments.
CHAPTER 1

Prognosis

The natural history of cardiac failure is clearly unfavourable. The Framingham Heart Study found that patients with a diagnosis of cardiac failure had a median survival of 3.2 years for males and 5.4 years for females. This was after excluding from the analysis the cases with the worst prognosis, i.e. those dying within 90 days of the diagnosis (Ho, 1993). In the last two decades a fourfold increase in unadjusted mortality rates from heart failure was observed. The overall five-year mortality of 50% is identical to colorectal cancer, and worse than for breast or prostatic cancer for an age-matched population (Hobbs, 1999). The explanation for the increasing prevalence and mortality is an ageing population and increased survivorship after myocardial infarction with introduction of novel therapies. This trend is thought to continue (Mcmurray, 2000). Coronary artery disease is clearly the most powerful risk factor for cardiac failure and its most frequent precursor. The common practice of coding the cause of death as the underlying aetiology masks the true level of the association. It is in fact very probable that the contribution of heart failure to overall mortality and coronary artery disease related mortality is underestimated.

Management of refractory cardiac failure

Management strategies require prognostic information. Prognostic markers are broadly classified as clinical, haemodynamic, biochemical and electrophysiological (Cohn, 1988). Of the clinical factors the New York Heart Association (NYHA) class is the most important, its predictive value being further increased in combination with maximal exercise capacity. The most prominent haemodynamic indices have been the left ventricular ejection fraction and the maximal oxygen uptake during exercise (VO_{2\text{max}}). VO_{2\text{max}} less than 12 ml/kg/min is associated with 1-year mortality of approximately 50% and also with functional benefit from cardiac transplantation (Mancini, 1991). Biochemical indices are usually soluble factors and they reflect the major activation that takes place along the neurohormonal axis. In the search for prognostic markers, the biochemistry of the heart pump was investigated too (Neubauer, 1997). Using ^{31}\text{P} magnetic resonance spectroscopy it was shown that in cases of dilated cardiomyopathy the phosphocreatine/ATP ratio holds prognostic information which extends beyond that of NYHA class and ejection fraction. Finally, ventricular arrhythmias are the principal adverse electrophysiological indicator. Analyses regarding the value of these predictors as independent or combined factors are abundant in the literature. The volume of this work reflects the underlying
clinical problem: how to predict outcome and how to best allocate individual patients to the therapeutic resources.

In the following sections the discussion is focused on refractory heart failure, defined as persistence of symptoms despite maximal medical therapy. A typical exponent of this category would be the middle-aged adult with rapid and relentless progression of underlying cardiomyopathy. The mortality in symptomatic NYHA class IV cases managed as outpatients can be as high as 50% at 6 months (Aaronson, 1999). For this group emerging therapeutic modalities, reviewed next, are compared against the gold standard which remains cardiac replacement by orthotopic transplantation.

**Cardiac transplantation - recipient selection**

In the early days of transplantation the recipients were hospitalised patients with advanced heart failure. The subsequent success of the procedure meant that it could be extended to those ambulatory outpatients with a significantly reduced life expectancy. With the arrival of waiting lists and increasingly sophisticated therapies the potential recipients have become a fluid population. Their status may change repeatedly and the following scenarios are possible: worsening condition requiring treatment in the intensive care setting, improvement with ongoing medical therapy and removal from the transplant list or, sadly, death on the waiting list. The waiting list in a transplant centre is a dynamic entity which undergoes regular reappraisal. In addition, patients deferred are periodically re-evaluated. It is for these reasons that selection of patients for transplantation is best discussed in the context of other maximal therapies.

In a new case of refractory cardiac failure the following questions are generally asked:

- What is the aetiology, the speed of progression and the prognosis?
- Are all associated risk factors known and controlled?
- Is the patient on maximal medical therapy?
- Is the compliance optimal?
- Is this single organ failure?

Tailoring of intravenous medical therapy against haemodynamic targets is the next step before deciding that all pharmacological means are exhausted. Haemodynamic monitoring permits safe and simultaneous adjustment of systemic vascular resistance and filling pressure. Sixty to seventy per cent of patients discharged remain stable at one month (Fonarow, 1995) and, in the case of a
first presentation, this interval allows for the functional reserve to be formally tested. Also, any other investigations pertinent to alternative surgical therapies may be undertaken as appropriate. To describe the more general scene for modern transplantation, these new surgical pathways are very briefly discussed below.

**Alternative surgical therapies**

*High-risk revascularisation*

Haussman and colleagues summarised the profile of a suitable candidate for revascularisation (Haussman, 1997). Favourable indicators are: short duration of cardiac failure, absence of right ventricular failure, low dose of diuretics, cardiac index of >2.0 l/min/m², and end-diastolic left ventricular pressure <24 mm Hg. In addition, the patient should represent a first revascularisation of hibernating myocardium with good target vessels. Liberal use of intra-aortic balloon contrapulsation is recommended, often prophylactically. In the University of Yale series the operative mortality was below 5% and the 1-year survival was 87% (Elefteriades, 1997).

*Surgery for mitral regurgitation*

As the failing left ventricle dilates the coaptation of the mitral valve leaflets is decreased and the ensuing regurgitation aggravates ventricular dysfunction. In the early 1980s mitral valve replacement was associated with prohibitive mortality, and it was suggested that this was a consequence of removing the 'blow off' mechanism into the left atrium. More recent results, however, show that in patients with an ejection fraction of less than 25% and without ischaemia mitral valve repair is associated with small operative risk and good medium-term survival of 72% at 2 years (Bolling, 1998).

*Left ventricular restoration*

Large postinfarct ventricular aneurysms can by themselves lead to heart failure. The two types of surgical solutions are linear closure and endoaneurysmorrhaphy, also called intracavitary repair (Cooley, 1958; Cooley 1989). The aim of the latter is to restore the contour and volume of the left ventricle while preserving surface anatomy. Others expanded the concept of intracavitary repair. Jatene emphasises the notion of reconstruction and used purse string-sutures to restore the
ventricular ellipse (Jatene, 1985), whereas Dor and associates added a subtotal, unguided endocardectomy in order to reduce the risk of ventricular arrhythmias (Dor, 1989).

Partial left ventriculectomy

Batista and colleagues proposed a new procedure to restore ventricular anatomy and function in patients with dilated cardiomyopathy. A wedge resection of posterolateral left ventricular wall is performed, either between or including the papillary muscles (Batista, 1997). Although adopted as an alternative to transplantation, the procedure has not lived up to initial expectations. The high operative mortality and unproved long-term benefits only make it applicable to a reduced number of cases.

Cardiomyoplasty

Basic physiology research led to the finding that skeletal muscle could be trained and transformed sufficiently to assist the left ventricle. Carpentier reported in 1985 the first successful clinical application using the latissimus dorsi muscle in a patient with a left ventricular tumour (Carpentier, 1985). A review of the world-wide experience over the subsequent 15 years concluded that carefully randomised studies are needed before this operation is more widely adopted (Acker, 1999).

Ventricular assist devices (VADs)

VADs have become an indispensable adjunct in the management of patients with terminal or fulminant heart failure. The two accepted indications have been postcardiotomy cardiogenic shock and bridge to transplant. In the second use improved cardiac output corrects end-organ failure while the myocardium rests. With portable devices patients generally regain mobilisation and may progress to relative independence and leisure activities. Support can take place for months or even years. The main complications are related to driveline infection and thromboembolism. Patient selection is of utmost importance and cost makes VADs a scarcely used resource. An important lesson learned was that timely implantation of the device, before end organ dysfunction, can further improve results. Recent figures from large-volume centres are encouraging: 80% of bridged cases reach transplantation and 87% of those transplanted are discharged home, with an overall success for bridging of 70% (Kormos, 1998). One of the
revelations of using VADs for chronic support was that the left ventricle may improve markedly in terms of anatomy and physiology, a novel process described as reverse remodelling (Frazier, 1999). Bridge to recovery thus became a realistic proposition, the only problem being selection of candidates. The Berlin group provided a large experience in patients weaned from assist devices (Hetzer, 1999). In the absence of a prospective study, it is difficult to apply strict criteria and selection of patient for weaning is based mostly on clinical judgement. It appears that young patients with acute onset of cardiomyopathy (very likely non-ischaemic in origin) and a shorter duration of LVAD support have the highest chance of sustained recovery after weaning (Muller, 1997). Finally, high hopes are built around the new axial flow impeller pumps, totally implantable, regarded as the next generation of artificial hearts (Westaby, 1998).

Of the surgical methods described, high-risk revascularisation, mitral valve reconstruction and bridge to transplant have a definite role in the repertoire of operations for cardiac failure. The others retain an experimental title and their evolution is unpredictable at this stage. Rigid algorithms of management are no longer tenable, firstly because of the dynamic nature of the patient population and, secondly, because of overlapping possibilities. For example, LVAD support has been described as a bridge to partial left ventriculectomy (Frazier, 1999). Transplantation is the gold standard against which these therapies are judged and it is unlikely that any of them will replace it in the immediate future (Koerner, 2000). Heart failure surgery and transplantation should in fact be seen as complimentary and not mutually exclusive. Contrary to the general public's perception, heart replacement does not restore normal life expectancy. As a result, there is a wide scope in the individual cases for deferring transplantation in a controlled manner until the time when all other avenues have been explored and excluded.

1.2 THE PRACTICE OF CARDIAC TRANSPLANTATION

Human heart replacement is a success story of modern medicine. The problems facing the pioneers were multiple and it is thanks to their unabated efforts and visionary attitude that what was once a 'fantastic speculation' is now part of the clinical reality (Lansman, 1989). A few memorable landmarks are presented here. The discussion leads on to the problems of transplantation as it is practised today.
The early days

Carrel and Guthrie performed the first heterotopic transplant in a dog in 1905. Almost three decades later, in 1933, Mann developed a technique for cervical cardiac implantation and improved coronary perfusion. Starting in 1946, Demikhov in Russia described technical solutions for implanting the heart in the chest. A dog transplanted by him survived for 15 hours sustained by its new heart. In 1957 Webb and Howard demonstrated that successful canine cardiac transplantation is possible after storage at low temperatures, thus anticipating long-distance procurement two decades later (for references see Lansman, 1989). The development of cardiopulmonary bypass in the late 1950s started the era of unprecedented innovations in cardiac surgery. Lower and Shumway published a seminal paper in 1960 in which an integrated approach to canine heart transplantation was presented (Lower, 1960). Together with Cass and Brock in London (Cass, 1959), they are credited with the now standard technique of preservation of a posterior atrial cuff. It then became apparent that technical problems were largely overcome. Donor organ procurement and graft rejection represented the only remaining barriers to clinical adoption of this operation. Ethical and logistic issues were far from negligible, death being defined as cardiorespiratory arrest, rather than cessation of brain activity. The requirement for the donor and recipient to 'die' at almost the same time was a major obstacle. In this context Hardy performed a primate to human transplant in 1964 and Lower described a mirror image human to primate operation in 1966 (Cripp, 1984). These pioneer xenografts failed to sustain the circulation for more than a few hours.

In 1967 Barnard carried out the first human cardiac transplant in an orthotopic position. The patient succumbed 18 days later to pseudomonas pneumonia. A wave of popularity for the procedure followed, but the enthusiasm of most groups waned in front of the combined problems of rejection and infection. A handful of dedicated centers continued their efforts and in the 1970s better means to diagnose and treat rejection became available (Griep, 1972, Caves, 1974, Calne, 1978). When endocardial sampling and cyclosporin were introduced in routine clinical practice it meant that the biggest threat to the early success of the operation, acute rejection, could now be monitored and treated promptly. When series of successful cases with long-distance procurement of the organs were reported in the late 1970s (Thomas, 1978) it was becoming clear that cardiac transplantation was here to stay. The introduction of cyclosporine-based triple immune suppression in early 1980s dramatically improved freedom from rejection and marked the
beginning of a new era. Many centers started clinical programmes so that by 1989 the number of cases world-wide exceeded 3000.

Transplantation today

A summary of indications, techniques and results is presented here. Donor selection is one of the main subjects in this research proposal and is discussed separately in the next section.

Indications and contraindications

The most common indications for cardiac transplantation are ischaemic cardiomyopathy and idiopathic cardiomyopathy, representing together just over 90% of all heart transplant cases. The remaining cases include: congenital heart disease, valvular disease, retransplantation and miscellaneous (Fig. 1.2.1) (Hosenpud, 1999). Results of surgery for congenital heart disease are continually improving, and so it is likely that an increasing proportion of these patients will be referred for transplantation in early adulthood. Absolute contraindications for cardiac transplantation are HIV positive serology, high fixed pulmonary hypertension (>5 Wood units), severe chronic pulmonary obstructive disease, cirrhosis of the liver, active gastrointestinal bleeding and active drug abuse (Koerner, 2000). Relative contraindications are patient-specific and vary between centres.

Operative technique

The donor cardiectomy is part of the multi-organ procurement operation. When all surgical teams are ready heparin is administered, the superior vena cava is ligated and the inferior vena cava is clamped and divided, resulting in exsanguination. The aortic cross-clamp is then applied and cardioplegia is infused in the aortic root. Crystalloid cardioplegic solutions are the norm, with up to eight types being used in the early 1990s (Wheeldon, 1992). A randomised trial recently showed that arrestsing the heart with donor blood cardioplegic solution might be beneficial to allograft function (Luciani, 1999). In the majority of cases the thoracic organs are nowadays explanted as a block, which entails additional measures for lung preservation. The block is split, when required, on a side table and the anastomotic sites are usually prepared at the recipient hospital before implantation.
The quest for the optimal preservation method has been long and not particularly rewarding (Stoica, 2001). The principles of organ preservation for cardiac transplantation and the rationale for intracellular and extracellular preparations are described elsewhere (Mendler, 1992; Jahania, 1999). A survey from 1992 showed that a single flush of cardioplegic solution followed by static hypothermic storage is the preservation method employed by more than 90% of transplant centres (Wheeldon, 1992). Continuous perfusion techniques lend themselves to interesting experimental variations, but the lack of clear clinical advantage and the cumbersome addition of equipment during transport makes them virtually absent from clinical practice (Wheeldon 1992). More disturbingly, another survey of 147 transplant centres from the United States showed that up to 167 solutions were in use, with 2 states using up to 17 different solutions (Demmy, 1997). This immense variability testifies to the lack of consensus in the field.

**Figure 1.2.1** Indications for cardiac transplantation (adapted from Hosenpud, 1999).

CAD, coronary artery disease.

In terms of implantation, the standard biatrial cuff technique has been modified by other groups. In the bicaval (Wythenshawe) method the caval veins are anastomosed end-to-end to their counterparts (El Gamel, 1995). Total orthotopic transplantation involves in addition complete excision of the recipient heart and separate pulmonary venous anastomoses (Dreyfus, 1991). In
return for increased technical complexity it was claimed that ventriculo-atrial regurgitation is reduced, atrial transport function is improved and this is reflected in decreased postoperative morbidity. Direct comparisons with the standard technique have been undertaken and the superiority of any one approach still remains to be determined (El Gamel, 1995; Bainbridge, 1999). The prevalence of different implantation practices is revealed by a recent survey (Aziz, 1999). Topical cold irrigation is still the preferred method of protecting the heart at implantation. Secondary cardioplegia is used by up to 50% of the responding centres. Furthermore, results of a small clinical trial of retrograde warm blood reperfusion during implantation were encouraging (Carrier, 1996). A note on the heterotopic technique, introduced in clinical practice in 1974 by the Cape Town group. It now represents 2.5% or less of all transplants performed but is of relevance to the following circumstances, especially in patients with poor outlook without expeditious transplantation: significant donor-recipient size mismatch, long ischaemic time, elevated pulmonary vascular resistance and potentially reversible conditions, when it is envisaged that the heterotopic organ could be later explanted. With the advent of ventricular mechanical assist the last two indications tend to be very infrequent.

Results - complications and quality of life

Thirty-day mortality ranges between 5% and 10%, depending on the case mix and the centre expertise (Sarris 1994). The most important cause of early death is non-specific graft failure, accounting for 30% of cases, followed by acute rejection and sepsis (Hosenpud, 1998). Between 30 days and 1 year there is an equal representation of infection and rejection. The overall 1-year survival rate is 79%. New immunosuppressive regimens made an important contribution to the continued trend in improved survival. In the 1990s the patient 1/2 life (time to 50% survival) was 9.4 years. In those surviving the first year the patient 1/2 life is 11.4 years, from year 1 onwards the mortality rate being 4% per year (Hosenpud, 1998). Late after transplantation the most common causes of death are cardiac allograft vasculopathy (CAV) and malignancy. Data from the annual reports of the Registry of the International Society for Heart and Lung Transplantation show that an excellent improvement is observed in the quality of life of transplanted patients. At 4 years over 90% of patients consistently report no limitations in their activities. The employment status is also encouraging. Between year 1 and year 4 postoperatively approximately 40% of patients are in employment.

21
DR. TRENDS AND THE DOMINO PROCEDURE

Heart transplantation is a successful procedure in terms of both survival and quality of life. Unfortunately in recent years the number of transplants per year has been declining. After reaching a peak of 4068 cases worldwide in 1995, in 1998 the number of cases was only 2961 (Fig. 1.3.1) (Hosenpud, 2000). A similar trend has occurred in the UK (UKTSSSA, 1996). The reason for this phenomenon is the scarcity of suitable donor organs. Safer road traffic conditions in the UK meant that road traffic accidents have fallen from the leading to the second commonest cause of donor death, after intracranial pathology, the latter retaining a constant incidence. This leads to a situation where an increasingly successful intervention is needed by an increasing number of patients but the resource is less and less available. In this context the British Transplantation Society demanded standards to be established for solid donor organs and their use (BTS, 1998). Only a few countries have adopted legislation of presumed consent for organ donation. Understandably, it will take time for the public attitude to change for the current rates of donation to increase significantly. Until that time the only way of meeting the supply to demand imbalance is by maximal use of currently available donors.

Figure 1.3.1  The trend in number of donors world-wide (adapted from Hosenpud, 2000).

With one notable exception discussed below, hearts for transplantation are procured from individuals with cessation of cerebral function. The circulation and respiration of these patients is,
of necessity, maintained artificially. The concept of cerebral death was described in 1959 but was accepted by the medical establishment and the general public only a decade later (Guerit, 1999). In many countries clinical and legal guidelines are available. In the UK it is sufficient for the brain stem death (BSD) criteria to be fulfilled, as tested by two independent clinical practitioners at two separate assessments, for the individual patient to be declared dead (DHSS, 1983). If there are no contraindications for organ donation, trained donor coordinators approach the family with this request. Valuable insight into the situation of organ donation in England was presented by Gore and colleagues in 1989. It was estimated that 98% of notified solid organ donors in England come from intensive care units. The audit carried out for 3 months at the beginning of 1989 showed that of 234 patients (8% of those dying in intensive care units) 148 patients (5% of the audited deaths) became solid organ donors. Consent by the family was given in 70% of cases. More detailed data are available from the United Kingdom Transplant Support Service Authority (UKTSSA) which published the cardiothoracic transplant audit for the interval 1985-1995. During this period the number of solid organ donors in the UK varied between 728 and 1005 (mean 901). After 1987 the percentage of donors whose hearts were offered was between 35% and 72% (mean 53%) but the percentage of donors whose hearts were used varied within a narrower interval of 34% to 38% (mean 36%). In 1993 zonal retrieval arrangements were made between the designated transplant centres and this strategy was effective for better allocation of organs. In 1995 392 hearts were accepted in total for adult transplantation and of these 82 (21%) were not used, the most common reason being 'non-viable organ'. Data however are not available for these organs to account for rejection on the basis of visible/palpable pathology, inability to sustain the circulation in the donor or a combination of factors.

The domino operation

To address the issue of maximal use of transplantable organs, the so-called domino procedure was developed (Yacoub, 1990). This is based on the observation that about a half of the heart-lung recipients have near normal hearts that could be used further for transplantation. The moderate degree of right ventricular hypertrophy in the face of elevated pulmonary pressures is thought to be possibly beneficial for the new recipient. If such an organ is implanted into a recipient with a high transpulmonary gradient, the likelihood of postoperative right ventricular failure is less. Its other advantages are the absence of brain death injury, short ischaemic times and the theoretical possibility of performing HLA matching. In the Papworth experience the
results of the domino procedure have been good (Oaks, 1994; Smith, 1996). The debate continues over the best use of the heart-lung block on utilitarian vs. deontological grounds.

**Brain death - old and new ideas**

Donor management and selection are best discussed in the context of BSD changes. Nowadays in the Western World cerebral death represents death of the individual. The diagnosis of BSD has become an accepted clinical exercise. Its first part is an exclusion process which involves three steps:

- What is the cause of the neurological deterioration?
- Can the presentation be simulated by other conditions?
- Can it be reversed?

Three major groups of lesions causing BSD can be differentiated: supratentorial, infratentorial and metabolic. Whatever the cause, the final pathway of injury is the same. Progressive brain oedema increases intracranial pressure to an extent which impairs and then stops cerebral perfusion. Death of neuronal tissue ensues shortly thereafter. The natural history of this condition is short: cessation of respiration leads to hypoxic cardiac arrest. If the cardiorespiratory system is artificially supported, the natural disintegration can be delayed for hours or rarely days. Indefinite support is clearly futile (Jennett, 1981). When the clinician is satisfied that all reversible causes have been excluded, clinical testing can be carried out. This has to essentially confirm cessation of respiration by apnoea testing and the absence of brain stem reflexes. Hypothalamic-pituitary dysfunction can be documented but is not necessary to establish the diagnosis of BSD. Nor are other ancillary tests such as the electroencephalogram in the UK (DHSS, 1993). In the absence of contraindications for solid organ donation, and provided the donor's family are in agreement, the transplant coordinator steps in at this stage and the search starts for a suitable recipient.

The state of the donor circulation is largely governed by the changes ensuing after brain death. A few decades back the effects of BSD were considered an extension of the Cushing response observed with raised intracranial pressure. Novitzky and Cooper first provided a detailed description of the acute changes that follow BSD in animals and humans and established the rationale of hormonal treatment (Novitzky, 1990; Cooper, 1989). In the agonal period all the sympathetic reserves are mounted in the attempt to perfuse the brain. The resultant catecholamine or sympathetic 'storm' produces profound pathophysiological derangements (Wilhelm, 2000). Within minutes there is an acute increase in cardiac afterload, accompanied by tachycardia. This
combination may create an important mismatch between myocardial oxygen supply and demand. Depending on the severity of this response and on pre-existing pathology, such as ventricular hypertrophy or coronary artery disease, the effects range unpredictably from subclinical dysfunction to acute heart failure, with or without myocardial infarction. The subendocardium of the left ventricle appears particularly vulnerable. Over the next 4-6 hours there is a gradual decline in vascular tone and cardiac output. The haemodynamic, electrocardiographic and histopathological effects are all well documented. In the clinical setting it is however difficult to establish with certainty the exact moment of BSD and to quantify the magnitude of the sympathetic response. The mode of BSD also plays a role. An explosive increase in intracranial pressure is associated with the highest degree of myocardial damage (Shivalkar, 1993). Polytrauma and intensive therapy have general effects that should not be overlooked. The lungs for example can be compromised by aspiration, pulmonary oedema, sputum retention, atelectasis, fat embolism or a combination of all these.

Hypothalamic-pituitary dysfunction is another hallmark of BSD. Loss of thermoregulation produces hypothermia while plasma levels of free thyroxine, arginine vasopressin, insulin and cortisol all decrease. Depletion of thyroid hormone directly affects the aerobic mitochondrial respiration of the cardiomyocytes. Thyroid hormone replacement has produced equivocal results in animal and human studies and is not universally embraced in practice (Wilhelm, 2000). At Papworth Hospital the view is favoured that, in an environment of intense catecholamine activity, haemodynamic correction is best undertaken with naturally occurring pressor and inotropic agents, such as vasopressin and thyroxine. Whether donor heart dysfunction is primarily mediated by neurological or hormonal pathways is not completely settled (Galinanes, 1994; Szabo, 2000). In recent years however two major contributions to BSD-related pathophysiology have been made. Firstly, with the knowledge that acute RV dysfunction and elevated pulmonary vascular resistance are prominent risk factors for early death, the role of the RV was investigated more closely (Kirklin, 1988). Bittner and colleagues performed a series of controlled animal experiments using load-independent indices of function (Bittner, 1999). They showed that RV dysfunction is primarily a consequence of BSD, since hearts from live donors were able to adapt and cope with elevated pulmonary vascular resistance in the recipient. An earlier analysis of adrenergic receptor function showed that this pathway is not underlying the observed dysfunction. Increased β-adrenergic receptor density together with increased adenylate cyclase activity were actually found after brain death (Van Trigt, 1995). It seems logical that perioperative RV injury accounts for the RV dysfunction seen in clinical practice, but no data are
yet available to confirm this hypothesis. Secondly, Tilney’s group elegantly demonstrated that BD induces a profound inflammatory response in peripheral solid organs, in which the heart is clearly not spared (Takada, 1999; Wilhelm 2000). These changes establish an unfavourable baseline, further to be modified in cumulative and deleterious fashion by ischaemia and reperfusion with non-autologous blood (Kupatt, 1999; Kevelaitis, 1999; Stoica, 2001). The implications extend beyond immediate surgical outcomes and appear to directly influence the long-term success of the procedure (Day, 1995). The concept of endothelial activation in transplantation is further discussed in chapter 2.

Donor selection

BSD leads to a wide spectrum of clinical pictures. In practical terms, hypothermia, hyper- or hypotension, cardiac arrhythmias, ECG abnormalities, hypoxia, polyuria, and electrolyte changes are often seen but not always corrected. The procurement operation has in fact all the features of a major procedure performed on a labile patient arriving from ITU. The surgical procedure adds to the irritability of the heart by hypothermia, blood loss, and direct manipulation. In an increasingly older donor population coronary artery disease and left ventricular hypertrophy are also more common. All of these factors may have a contribution to the fluctuating and often marginal donor circulation. In actual fact the donor patient is often the subject of two periods of intense activity separated by a relative period of neglect. Between demise in intensive care and organ procurement in theatre the circulation is characterised by a variable decline, at times very severe. The simple principle that hypotension can result from pump failure or vascular bed failure is sometimes overlooked. The first task of the organ procurement team is to reverse the trend of homeostatic disruption. Only when this is accomplished, in parallel with assessment of organ function, the team is able to decide as to the suitability of the organs for transplantation.

The practice and the results of donor resuscitation have been discussed elsewhere (Wheeldon, 1995; Stoica, 2001) and are described briefly in chapter 3. Donor heart acceptance criteria are loosely based on the entry criteria for mechanical assist of the circulation and are used as an approximate guide. The haemodynamic parameters generally employed are mean arterial pressure (MAP), central venous pressure (CVP), pulmonary capillary wedge pressure (PCWP), left ventricular stroke work index (LVSWI) and doses of inotropes (Table 1.3.1). Some of these are measured directly whereas others are calculated based on thermodilution measurements. The practical advantages of the Swan-Ganz catheter are: firstly, it gives data about the pump function
and about the vascular bed resistances and, secondly, the measurements can be repeated so as to provide a semi-continuous assessment of function. The Swan-Ganz catheter is however not always sufficient to help in the donor heart selection dichotomy 'to take or not to take'. The statistics of the UK central transplant authority show that in recent years up to a half of donor hearts offered to any unit will be deemed unsuitable for transplantation, with up to a quarter of all donor hearts rejected on grounds of poor ventricular function (UKTSSA, 1999). It therefore becomes imperative to formulate better descriptors of donor organ function. They could be generically classified as haemodynamic, already discussed, imagistic, and biochemical.

Table 1.3.1: Haemodynamic and respiratory resuscitation targets.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP</td>
<td>&gt;60 mmHg</td>
</tr>
<tr>
<td>CVP</td>
<td>&lt;12 mmHg</td>
</tr>
<tr>
<td>PCWP</td>
<td>&lt;12 mmHg</td>
</tr>
<tr>
<td>CI</td>
<td>&gt;2 L/min/m²</td>
</tr>
<tr>
<td>LVSWI</td>
<td>&gt;15 mg-M/beat/m²</td>
</tr>
<tr>
<td>Inotropes</td>
<td>one inotrope only, equivalent to dopamine in a dose of 5 µg/kg/min or less</td>
</tr>
<tr>
<td>PO₂</td>
<td>&gt; 50 kPa on FiO₂=100%</td>
</tr>
</tbody>
</table>

Abbreviations: MAP, mean arterial blood pressure; CVP, central venous pressure; PCWP, pulmonary capillary wedge pressure; LVSWI, left ventricular stroke work index.

Angiography to assess ventricular ejection fraction is a scarce resource and is used anecdotally (Deibert, 2000). The main advantage of cardiac catheterisation would be to rule out coronary artery disease in older donors. Ex vivo contrast examination of the donor heart (bench angiography) has been proposed (Robicsek, 1992) but never gained wide use. It is hoped that epicardial Doppler probes will soon be able to perform that function. Transthoracic and transoesophageal ultrasound studies are faced with the following dilemma: since many wall motion abnormalities in the donor heart are transitory and generally improve after transplantation, which organs can then be safely selected (Stoddard, 1993; Vedrinne, 1996)? Testing the functional reserve of the heart, by dobutamine-stress for instance could add discriminating value (Kono, 1999). All the image-derived indices of function, as well as the SGC parameters, are load-dependent and therefore unable to determine intrinsic contractile impairment. The conductance catheter provides the only load-independent measure of ventricular function. As a result, it was
used as the principal tool used to assess the haemodynamic effects of BSD in animal experiments (Bittner, 1996; Szabo, 2000).

Biochemical markers have the attraction of being less invasive. Troponin T measured in 100 BSD patients inversely correlated with left ventricular ejection fraction (Riou, 1995). However, donor heart dysfunction may not be caused solely by myocyte disruption and loss of contractile elements. Yacoub's group showed that BSD is associated, particularly in poorly functioning hearts, with abnormalities in cytokine profile, transmembrane signalling and apoptotic pathways (Birks, 2000; Owen, 1999; Birks, 2000; Birks, 2000). Whatever the mechanism of donor heart dysfunction, one of the consequences may be a perturbed energy metabolism. Our group showed that recipients developing donor organ failure have a low baseline of adenine nucleotides at the time of donor heart assessment (Darracott-Cankovic, 1998).

Towards donor and recipient matching

The 'marginal donor' remains an ill-defined concept, generally denoting the presence of one or more risk factors. Along with suboptimal cardiac function, expansion of the donor pool has been advocated on criteria of age (Drinkwater, 1996; Loebe, 2000), long ischaemic time (Pflugfelder, 1991), left ventricular hypertrophy (Marelli, 1999), cardiotoxic cause of death (Tsui, 1999), systemic infection (Lamermeyer, 1990), weight mismatch (Jeevanandam, 1996), and after arrest and resuscitation (de Begona, 1993). These risk factors are complemented by multivariate analyses from the ISHLT registry (Hosenpud, 1999; Hosenpud, 2000). Donor management focused on haemodynamics is not inappropriate because, of all the risk factors, this is the only variable which can be actively and favourably modified at the time of organ procurement. In general, the cautious consensus is that the presence of up to two donor risk factors does not affect recipient outcome. A good donor heart implanted expeditiously in a low-risk recipient will generally produce a good outcome. At the other extreme, for example, a 60-year-old female donor whose heart is implanted with technical difficulty and after a long ischaemic time in an oversized male on a ventilator. Across the whole spectrum, donor to recipient 'quality matching' remains more of an intuitive rather than a rigorously scientific exercise. The standard has been raised by reports in which UK pooled national data are matched in the analysis according to the level of donor and recipient risk, (Fig. 1.3.2) (Anyanwu, 1999). In practical terms, Laks and associates have successfully pioneered the concept of the alternate list, allocating suboptimal donor organs to high-risk recipients who might otherwise be excluded from the regular list (Laks,
1999). In many of the outcome analyses undertaken to date a well-matched control group is difficult to form retrospectively. With the relatively small numbers described by an individual unit it means that too many variables have to be corrected for in the analysis, with unavoidable loss of power. Such studies would clearly benefit from the 'transformation model', very familiar in the business environment: input → process → output. Prospective, controlled investigation of the simplified sequence donor → ischaemic period → recipient would lead again to the question: what is a good donor heart?

Figure 1.3.2 The relationship between donor and recipient matching. A poor donor/recipient is defined as having in excess of 2 known risk factors (from Anyanwu, 1999).

1.4 OBJECTIVES OF THE CURRENT RESEARCH PROJECT

In the current environment of donor heart scarcity, this multidisciplinary study is part of a more general effort to maximise the donor pool and improve clinical outcomes. Our main purpose is to formulate more accurate descriptors of donor heart function. Since the interval between brain
death and completion of transplantation is characterised by complex pathophysiology, serial measurements were performed.

The first main objective of this project is therefore to describe donor heart function serially in terms of:

a) High-energy stores
b) Endothelial activation
and

c) Haemodynamic performance (courtesy of Drs D K Satchithananda and P A White)

Attention will then move towards correlating different descriptors of function between them and with the patient outcomes. The following main end points will be studied:

a) The effect of brain death
b) Allograft failure

Theoretical considerations for the methods employed are presented in chapter 2 and the methods are described in chapter 3. After presentation and discussion of results, the final chapter speculates on areas of future research.
CHAPTER 2
THEORETICAL CONSIDERATIONS FOR ASSESSMENT OF FUNCTION IN THE TRANSPLANTED HEART

This chapter describes the cardiac metabolism and endothelial activation in cardiac transplantation, along with the principles of studying these phenomena in the laboratory and clinically. The principles of load-independent measurement of cardiac performance are also described.

2.1 ENERGY METABOLISM OF THE HEART

Cardiac thermodynamics

Like all biological systems, the heart obeys the laws of thermodynamics. According to the first law, the total amount of energy in an open system remains constant, although the form of energy may change. The second principle of thermodynamics states that a process will occur spontaneously only if it is associated with an increase of entropy (disorder). In biological terms, the living organisms preserve their internal order and development by taking from the surroundings free energy in the form of sunlight (phototrophs / autotrophs) or nutrients (chemotrophs / heterotrophs). An equal amount of energy is returned to the organism's surroundings in the form of heat and entropy (Stryer, 1995; Lehninger, 1993). The relationship is described by the equation:

\[ \Delta G = \Delta H - T \Delta S \]

where  
- G is the free energy in Joules/mol  
- T is the temperature in Kelvin degrees  
- H is the enthalpy, the heat content of the reacting system  
- S is the entropy, a quantitative expression of randomness

G expresses the amount of energy capable of doing work during a reaction at a constant temperature and pressure. If the free energy change, \( \Delta G \), has a negative sign then the reaction can
occur spontaneously and is said to be exergonic. Conversely, in endergonic processes the system gains free energy and \( \Delta G \) is positive. At equilibrium, the concentration of reactants and products remains constant and the rates of forward and reverse reactions are equal. Let us consider for example the simple reaction:

$$A + B \leftrightarrow C + D$$

If we define the standard free energy change (\( \Delta G^\circ \)) as the force driving the system towards equilibrium, then \( \Delta G \) and \( \Delta G^\circ \) are related by the equation.

$$\Delta G = \Delta G^\circ + RT \ln \frac{[C][D]}{[A][B]}$$

where \( R \) is the gas constant, equal to 8.315 J/mol×K. At equilibrium \( \Delta G \) is zero, and so the relationship becomes:

$$0 = G^\circ + RT \ln \frac{[C]_{eq}[D]_{eq}}{[A]_{eq}[B]_{eq}}$$

or

$$\Delta G^\circ = -RT \ln K'_{eq}$$

\( \Delta G^\circ \) and \( K'_{eq} \) (the equilibrium constant) are two physical constants characteristic for each reaction, related by the above simple logarithmic relationship. The standard conditions for a reaction assume that the aqueous medium is buffered to a pH near 7 and the water concentration is 55.5 M. As discussed below, the relationship between \( \Delta G^\circ \) and \( K'_{eq} \) is used by biochemists to calculate metabolite concentrations. If we apply this equation to ATP hydrolysis

$$ATP \rightarrow ADP + Pi + \text{free energy}$$

then

$$\Delta G^\circ = -RT \ln \frac{[ADP][Pi]}{[ATP]}$$
A high [ATP]/[ADP][Pi] ratio is associated with a high value of $-\Delta G_{\text{ATP}}$. This ratio, called the phosphorylation potential, is the thermodynamic driving force for reactions utilising ATP hydrolysis in muscle contraction, ion transport and other vital processes.

A few additional points should be made. Firstly, the free energy change ($\Delta G$) for a reaction is independent of the path by which the reaction occurs. Secondly, enzymes do not affect the point of equilibrium for a reaction, they merely hasten its attainment. In living cells spontaneous reactions take place because the activation energy is lowered by enzymes, their role being to provide 'low-resistance' alternative pathways. (The high activation energy of ATP hydrolysis and its kinetic stability toward nonenzymatic breakdown are the basis for enzyme elimination and the low temperatures used in our assay - described in detail in sections 2.2 and 3.4) Thirdly, standard free energy charges are additive. This means that a thermodynamically unfavourable reaction (endergonic) can be driven by a thermodynamically favourable reaction (exergonic) that is coupled to it. Let us take the following reactions:

\[
\begin{align*}
\text{Glucose} + \text{Pi} & \rightarrow \text{glucose-6-phosphate} + \text{H}_2\text{O} & \Delta G^\circ = 13.8 \text{ kJ/mol} \\
\text{ATP} + \text{H}_2\text{O} & \rightarrow \text{ADP} + \text{Pi} & \Delta G^\circ = -30.5 \text{ kJ/mol}
\end{align*}
\]

They can be summed as:

\[
\text{ATP} + \text{glucose} \rightarrow \text{ADP} + \text{glucose-6-phosphate}
\]

The overall standard free energy is obtained by adding $\Delta G^\circ$ for the two coupled reactions, thus resulting in an exergonic process:

\[
\Delta G^\circ = 13.8 + (-30.5) = -16.7 \text{ kJ/mol}
\]

The above coupled reactions also show the role of ATP as an 'energy currency' of the cells. Before describing ATP, it should be noted that its simple hydrolysis does not achieve energy transfer per se. In the majority of cases the transfer of energy takes place in two steps. Initially part of the ATP molecule, either the phosphoryl group or the adenylate moiety (AMP), is transferred to a substrate or an enzyme, thus raising their free energy content. In a second step, the phosphate-containing moiety transferred in the first place is displaced, generating either Pi, or
AMP. The only notable exception to the mechanism of group transfer is muscle contraction, in which direct ATP hydrolysis is the mechanism for chemo-mechanical transduction.

**ATP production**

This section will describe the properties of high-energy phosphates (HEP) and their production in the myocardium. In 1941 Lipmann and Kalckar anticipated the central role of the ATP-ADP cycle as an energy exchange mode in biological systems. At an ATP content of about 20 nmol/mg dry weight, and at a myocardial O₂ consumption of 5 mmol/h, the heart turns over its entire ATP pool every 4-5 s (Depre, 2000). As a remarkably efficient chemo-mechanical transducer, the heart must adapt quickly to the type of nutrients on offer (Opie, 1998). Its preference to use carbohydrate in the fed state and fatty acids in the fasted state as the major sources of energy has been known for decades. ATP is generated in the myocardium by glycolysis and oxidative phosphorylation. Glycolysis is a simpler but highly integrated process catalysed by cytosolic enzymes. Oxidative phosphorylation is on the other hand a sophisticated mechanism hosted by specialised membrane structures in the mitochondria. The relative efficiencies of the two processes are exemplified by the well-known energy yield: glycolysis produces 2 moles of ATP per mole of glucose, compared to 36 moles of ATP generated by oxidative phosphorylation. For descriptive purposes, it is convenient to group the breakdown of substrates into three stages (Fig. 2.1.1).

**Stage 1** (the intermediary metabolism) - nutrients are transformed into acetyl-CoA.

A very simplified scheme of glycolysis is:

![Glycolysis Scheme](image)

**Stage 2** takes place in the mitochondrial matrix, where acetyl-CoA enters the tricarboxylic acid (Krebs) cycle. The overall reaction of the Krebs cycle is:
Acetyl-CoA + 2H_2O + 3NAD + FAD + ADP + P_i → 2CO_2 + CoA + 3NADH + FADH_2 + ATP

Nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide are highly specialised, soluble cofactors which act as electron acceptors and carriers. In the initial stages 1 and 2, substrate-level phosphorylation has produced comparatively little ATP.

**Figure 2.1.1** The three stages of energy metabolism (from Opie, 1998).

Stage 3 (oxidative phosphorylation, respiratory chain-linked phosphorylation) is concerned with manufacturing virtually all the cellular ATP. The enzyme machinery for this process is situated on the inner surface of the mitochondrial membrane. The substrates are NADH and FADH_2 which have accumulated electrons in the preceding steps. In the well-oxygenated heart, their reducing equivalents are transferred in a closely regulated manner to oxygen, leading to the production of water. Carbon is eliminated in its most oxidised state as CO_2. Under aerobic
conditions 90% of ATP produced comes from oxidative phosphorylation, 5% from glycolysis and 5% from the Krebs cycle.

The high-energy acyl phosphate bond contained in HEPs (R - C - O ~ P)

\[ \text{O} \]

is different from the phosphoester bonds formed with the hydroxyl groups of serine and threonine (R - CH\textsubscript{2} - O - P). The latter low-energy bonds do not participate in energy transfer and are encountered in processes of regulatory phosphorylation. Familiar examples are phospholipase C, protein kinase C, most of the regulatory enzymes of the cardiac metabolism itself, troponin I, and phospholamban. Based on \( \Delta G^\circ \) of hydrolysis, the ~P 'high-energy' phosphate bond is said to be present in adenine nucleotides and other compounds such as creatine phosphate (CP) and phosphoenolpyruvate. Conversely, examples of 'low-energy' compounds are glucose-6 phosphate and glycerol-phosphate (Lehninger, 1993).

The basic energy-producing equation is:

\[
\text{ATP} \rightarrow \text{ADP} + \text{Pi} + \text{energy}
\]

As the workload of the heart increases, oxygen consumption, a good index of the rate of ATP synthesis by oxidative phosphorylation, proportionately increases. Yet [ATP] remains unchanged (Ingwall, 1999). The cellular machinery called to match the rates of ATP synthesis and ATP utilisation is impressive, especially if we consider that the cellular sites of ATP production and utilisation are different. Creatine, a \( \beta \)-amino acid, is synthesised in the liver, kidney and pancreas and supplied to tissues via the bloodstream. In the myocardium up to two thirds of creatine is phosphorylated to CP. The malate-aspartate cycle and the ATP-ADP translocase are means of exchanging large, highly charged molecules, such as NADH and ATP, between the mitochondria and the cytosol. The mitochondrial creatine kinase isoenzyme (MiCK), situated between the inner and outer layers of the mitochondrial wall, is thought to transfer the HEP bond to cytoplasmic CP (Schulze, 2000). In real terms, the end product of mitochondrial metabolism is CP. In the cytosol, CP is instrumental to allocation of ATP to the utilisation sites. The CK isoenzymes catalyse the following reaction in the cytoplasm (with an equilibrium far to the right):

\[
\text{CP} + \text{ADP} \leftrightarrow \text{creatine} + \text{ATP}
\]
thus maintaining the ATP pool at the expense of the abundant CP reservoir. CP has therefore two fundamental roles: (1) energy shuttle, and (2) energy reserve system. Compartmentation of ATP is further substantiated by findings of different properties of ATP resulting from different metabolic pathways. For example, glycogen, situated in the vicinity of the sarcoplasmic reticulum, might provide on-site ATP for the calcium uptake pump (Gordon, 1992). Likewise, evidence is accumulating that, even in normoxic conditions, ATP produced by glycolysis is particularly important for the function of ion channels and membrane pumps (Opie, 1998). The current technology however has not reached the level of sophistication that would permit clinical or even experimental measurement of HEP compounds in functional compartments of the cell.

The regulation of oxidative metabolism is far from clarified, but the key factors seem to be the rate of ADP supply, mitochondrial NAD synthesis and the mitochondrial calcium concentration (Gordon, 1992). Regulation of metabolism also takes place in the cytoplasm, where ATP, ADP and AMP are interconvertible. Adenylate kinase (myokinase) catalyses the reaction:

\[
2 \text{ADP} \leftrightarrow \text{ATP} + \text{AMP}
\]

The ATP resulting in this way can be employed further by myosin ATPase to generate energy. Lack of oxygen stimulates supplementary energy production by breakdown of ATP beyond ADP to AMP. AMP then undergoes irreversible catabolism to inosine and adenosine. These unphosphorylated compounds can leave the cell and attempt to directly restore energy balance by compensatory changes in the form of bradycardia, reduced contractility and coronary vasodilatation. During normoxia, 'salvage' reformation of ATP from ADP is a crucial step in maintaining the adenine nucleotide pool and in preventing loss of purines from the cells (Ingwall, 1999). De novo purine ring synthesis in the mammalian heart is slow and inefficient, and so this mode of HEP replenishment has limited importance (Swain, 1986).

**ATP utilisation**

The heart muscle, just like all other living tissues, provides a milieu in which complex reactions occur at relatively low temperatures and low substrate concentrations. Efficient energy transfer takes place via enzyme-catalysed metabolic pathways, at the centre of which are moiety-conserved cycles. Likely to result from evolutionary selection, these cycles are characterised by relatively unchanged concentrations of participating intermediaries. Essentially they couple the
cellular macro and microcosms (Fig. 2.1.2). Cellular metabolism, the stage in the middle, can itself be described by the notion of inter-related cycles (Fig. 2.1.3, end of chapter 2).

**Figure 2.1.2** Coupling of the circulation and metabolism. The cogwheel mechanism ensures that increased circulation is sustained by increased metabolism and cross bridge cycling (from Taegtmeyer, 2000).

![Systemic Cellular Cross bridge circulation metabolism cycling](image)

Figure 2.1.4 illustrates what proportion of ATP is used for cellular life processes. A few key points on the surgical physiology of the cellular contractile mechanism are presented next.

1. Calcium is pivotal to excitation-contraction coupling. Contraction and relaxation depend on the cyclic concentrations of calcium ions in the cytoplasm (calcium transients). There is anatomic proximity and functional correlation between the L-type calcium channels of the T-tubules and the foot region of the ryanodine receptor in the sarcoplasmic reticulum (SR). Calcium-induced calcium release from the SR is the first step in contraction (Fabiato, 1983).

2. Of the contractile filament proteins, troponin-C is the one responding to calcium transients. Calcium occupancy strengthens its binding to troponin-I, thereby decreasing the inhibitory action of troponin I on actin. A molecular change in tropomyosin also takes place, which finally lifts all barriers between actin and myosin interaction (Rayment, 1993).

3. Crossbridge cycles form between actin and myosin according to a five-step model, at the centre of which is ATP hydrolysis by myosin ATPase (Rayment, 1993). Each cycle produces a power stroke that drives the actin filament along the myosin. The result is muscle fibre shortening in systole. The basic equation for energy liberation is:

\[
\text{MgATP}^2- \rightarrow \text{MgADP}^2- + \text{Pi}^2- + \text{H}^+ + \text{free energy}
\]
The exact charges depend on the cellular pH, and the proton produced is the major source of intracellular acidosis. ADP can: (1) reform ATP via the CK reaction, (2) be further split to form ATP and AMP in the myokinase reaction, or (3) be returned to the mitochondria by the ATP-ADP translocase to stimulate respiration.

**Figure 2.1.4** Turnover of high-energy phosphates in the myocardial cell (adapted from Opie, 1998).

4. The principal mechanism for reducing cytosolic calcium concentration and initiating diastole is calcium uptake by the SR (Opie, 1998). This is an ATP-consuming task performed by the sarco-endo(plasmic) reticulum calcium-ATPase (SERCA). Phospholamban, an integral part of the SR membrane, inhibits SERCA in a regulatory relationship. Beta-adrenergic
stimulation not only increases inotropism, but also through phospholamban improves relaxation (lusitropy). Calsequestrin and calrectulin are negatively charged calcium storage proteins in the SR. Finally, another prime regulator of calcium transients is the cytosolic protein calmodulin, whose four binding sites are occupied by magnesium ions when calcium is absent.

Cardiac energetics in pathologic states

Chronic heart failure

The creatine pool is lower in the failing heart, and also the capacity for ATP resynthesis via the CK reaction is impaired (Nascimben, 1996). Decrease of both CP and C leads to preservation of the CP/C ratio. To a great extent this is an adaptive mechanism, designed to minimise the loss of purine and to preserve the ATP/ADP ratio. The latter is crucial to cell viability, as shown previously in the thermodynamic analysis. A price is paid though for this adaptation: by being unable to adequately replenish ATP from CP, the heart looses the capacity to adapt to increased workload. More recent evidence shows that heart failure is associated with a decrease in both the ATP and the total purine pool, albeit smaller than the changes in the creatine stores (Starling, 1998). Possible mechanisms for this are impaired de novo and salvage ATP synthesis. The precise causal relationships between loss of energy reserve and loss of contractile function deserve more research. Energy supply and contractile function both affect cardiac gene expression (Depre, 2000). The failing heart undergoes a concerted genomic adaptation known as the foetal gene programme. This describes another adaptive change, akin to the foetal heart, whose main feature is an 'energy sparing' rather than 'contractile efficiency' functioning mode. Overall, these concepts extend beyond the physiology laboratory. For example Neubauer and associates have used $^{31}$P magnetic resonance spectroscopy (MRS) for prognostic purposes. They showed that in patients with dilated cardiomyopathy the myocardial CP/ATP ratio is a better predictor of survival than left ventricular ejection fraction and NYHA class (Neubauer, 1997).

Ischaemia

The heart adapts in a different fashion to the acute challenges of ischaemia and reperfusion. There is a continuum of metabolic responses to ischaemia which parallels the continuum in restricted oxygen supply (De Boer, 1983). Among the first defence mechanisms thrown into action is ATP
protection by CP breakdown. The accompanying increase in intracellular inorganic phosphate downregulates contraction. With continued oxygen supply-demand mismatch, the subsequent increase in intracellular acidosis will further impair contractility. As ischaemia progresses, the metabolism switches to anaerobic glycolysis by increased glucose uptake and glycogenolysis. Glycogen breakdown continues until glycolysis is inhibited by its products, this being synonymous with the end of anaerobic ATP production. In the ischaemic myocardium there is a remarkably close correlation between ATP, glycogen and CP content (McElroy, 1989). Once glycolysis ceases, protons, calcium and sodium ions continue to accumulate and the point of ischaemic contracture is reached. This phenomenon occurs at high cytosolic calcium concentrations and low ATP levels. The crossbridges are no longer occupied by ATP and become permanently attached forming the so-called rigor bonds. The high forces generated in the rigor state may lead to tension on the sarcolemma and cytoskeleton, membrane leakage and lethal cell disruption by contraction band necrosis. Clinically, this phenomenon was observed in the operating room and described as the stone heart (Cooley, 1972).

In clinical practice ischaemic insults are rarely as severe as that. The question remains though: can energy levels represent a measure of insult and/or a predictor of functional outcome? In contrast to the theory of metabolic product accumulation, loss of chemical energy is the other important viewpoint for explaining why ischaemic hearts show a spectrum of manifestations from contractile failure to lethal injury. For example, in classic experiments of severe regional ischaemia Jennings and colleagues have shown that 40 minutes of coronary artery occlusion produce a fall in ATP to levels of 2 to 3 μmol/g dry weight (Jennings, 1978). This critical level was associated with irreversible ultrastructural changes. The survival of cells after near-lethal injury depends however on more factors than the ATP content alone. Similarly, attempts have been made to relate the energy content of the cells to whole organ function. It appears that a limited decrease in ATP levels precedes the start of contractile failure by about 10 seconds (Hearse, 1979).

The proponents of the energy deficit theory use several indices. The phosphorylation potential, defined as [ATP]/[ADP][Pi], has already been mentioned and is a good descriptor of cellular energy status. In the well-perfused myocardium normal values are >300mM⁻¹, but doubling of [ADP] and [Pi] at constant ATP levels leads to decreases of this index in the region of 80mM⁻¹ (Ingwall, 1999). Total adenine nucleotide (TAN) content is the sum of ATP, ADP and AMP. This measure is less useful as it is not taking into account the functionally important individual
concentrations of the adenine nucleotides. Finally, the energy charge (EC), defined as \[ \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]} \], is a better indicator than the TAN alone, because it mirrors closely the ATP/ADP ratio. This concept arose from making an analogy between the total adenine nucleotide pool and a chemical storage battery or accumulator cell. While the total amount of material typically remains constant, the chemical energy can be stored and recovered by alteration of the ratio of the components. EC is a linear measure of the metabolic energy stored in the adenine nucleotide system. When only AMP is present EC is 0 and when only ATP is present EC is 1. Actively growing and dividing microbial cells have for example an EC of 0.80-0.95. Cells in the in the stationary phase of growth have an EC of about 0.60 while resting and senescent cells have ratios below 0.5 (Wiebe, 1975).

Other groups have challenged the energy charge theory, showing that myocardial cells can survive with ATP below levels regarded as critical (Owen, 1990). In terms of sublethal dysfunction, a period of 10 minutes of severe ischaemia at 30°C is associated with a decline in intracellular pH, an increase in Pi production and a severe decrease in contractility, all in the presence of preserved ATP stores (Vandenberg, 1993). There is perhaps no need to reconcile the two apparently unrelated theories of response to ischaemia. It is probable that metabolite accumulation and HEP depletion interact to produce diastolic and systolic dysfunction. 'Critical' ATP levels are still difficult to define for several reasons: (1) the duration of ATP depletion, its metabolic provenience and availability in functional compartments are other important variables; (2) the current technology of metabolic assessment is still faced with technical difficulties; (3) catabolism of HEP varies between species and experimental conditions, therefore a single definition of critical levels is insufficient (Minten 1991); (4) adaptive mechanisms are rarely quantified simultaneously.

A more unifying view is gradually emerging. Severe ischaemic insults can induce direct necrosis by the mechanism described, or the more controlled apoptosis (programmed cell death) (Haunstetter, 1998). Necrosis itself is of two varieties. Contraction band necrosis is characterised by hypercontracture (see rigor above) and is more often observed at the borders of a recent infarct. Coagulation necrosis is the more severe form seen in the centre of an evolving infarct. Its name comes from the nuclear clumping and transformation of the myofibrils into amorphous dense bodies, of a coagulated appearance. Apoptosis is a transcriptionally controlled, energy-consuming process in which the sarcolemma retains its integrity. Its hallmark is relative sparing of the mitochondria and endonucleosis, a process whereby genomic DNA is severed at
internucleosomal sites. Lesser degrees of ischaemia will determine adaptation by 'programmed cell survival' (Depre 2000). The three groups of phenomena encompassing resistance to ischaemia in the heart are ischaemic preconditioning, stunning and hibernation, and are discussed below.

Reperfusion

Myocardial reperfusion is defined as the death of myocytes, alive at the time of reperfusion, as a direct result of one or more events initiated by reperfusion. The contribution of apoptosis to myocyte death during reperfusion has not been established in quantitative terms (Depre, 2000). The resulting cellular damage can be reversible or irreversible, depending on the length of ischaemia and on the conditions of reperfusion. Jennings and Hearse discovered the paradoxical situation whereby restoration of blood flow, essential to tissue viability, has the potential of extending the injury beyond that caused by ischaemia itself (Jennings, 1960; Hearse, 1978).

Ischaemic preconditioning was a term coined after the observation that repeated 5-min periods of coronary artery occlusion and reperfusion reduces the size of the infarct produced by a subsequent longer period of coronary occlusion (Murry, 1986). A second window of protection, occurring about 24-96 hours after the initial episode of preconditioning, was also described (Baxter, 1994). Although adenosine is recognised to play a central part in this process, the intracellular signalling steps have not been fully deciphered yet. There is evidence that preconditioning favours anti-apoptotic gene transcription (Baghelai, 1999).

Stunning represents post-ischaemic contractile dysfunction despite restoration of flow and absence of irreversible damage (Heyndrickx, 1975; Braunwald, 1982). In this dissertation, the emphasis of the discussion on ischaemia-reperfusion injury will be on stunning, as this is the most relevant phenomenon in relation to transplantation. Finally, hibernation represents a chronic state of adaptive reduced contractility in underperfused myocardium (Rahimtoola, 1989). The chief feature is reversibility of this state upon revascularisation, although the recovery may range from immediate (Ferrari, 1994) to delayed (Vanoverschelde, 1993). Radionuclide labelled deoxyglucose techniques represent a classic illustration of metabolic methods employed for the assessment of tissue viability in hibernating myocardium (Uren, 1992).
Ischaemia-reperfusion injury is largely responsible for stunning. The mediators of stunning are described in the converging oxyradical and calcium overload hypotheses (Gross, 1999; Piper, 1999). The downstream cascade of events that leads from reperfusion to lethal injury is clearer. Sublethal ischaemia produces cytosolic calcium and sodium overload, along with lowering of cytosolic pH. Mitochondrial re-energisation makes ATP available to the contractile and ion pump mechanisms. During the initial stages of reoxygenation, the circulation of Ca$^{2+}$ between the SR and the cytosol is perturbed. In the presence of osmotic fragility, resulting from Na$^+$ and metabolic products accumulation, the cell is more vulnerable to mechanical stress. Excessive force generation resulting in this milieu may disrupt the cytoskeleton beyond reversibility, and the injury is propagated to neighbouring cells by chemical and physical interaction, namely the gap junctions and the intercalated discs. The heart is equipped with protective shields, some of which are adenosine, the ATP-sensitive potassium channel ($K_{ATP}$) and the sodium-hydrogen (Na-H) antiport exchange system. Their properties are beyond the scope of this summary. A discussion of ischaemia-reperfusion injury is incomplete if it focuses on cardiomyocytes alone. Reperfusion takes place through metabolically and immunologically active vascular conduits. The role of the endothelium in this complex interaction is detailed in section 2.3.

Transplantation

Biochemical energy stores have been used as a measure of outcome in a multitude of transplant studies (Stoica, 2001 - review), but a serial evaluation across transplantation was rarely performed. Our group and others have had a special interest in defining biochemical organ viability in solid organ transplantation. Of the methods available, quantitative birefringence of the muscle was used (Daracott-Cankovic, 1987) or direct quantification of the adenine nucleotides in serial biopsies (Daracott-Cankovic, 1998; Smolenski, 1992). After an initial experience with quantitative birefringence, detailed experiments were undertaken on rat myocardium submitted to warm ischaemia (Daracott-Cankovic, BHF grant PG/93133). A precipitous decline was recorded in the first few minutes for CP and glycogen. The ATP content declined more slowly but after 15 minutes of ischaemia fell rapidly. The level of ADP rose for 15 minutes and thereafter fell, whereas the AMP had a similar pattern but over 25 minutes. Based on simultaneous correlations with birefringence it was then postulated that the fall in the birefringence index observed in increasingly less viable tissue is related to a decrease in the ATP/ADP ratio. This is in agreement with findings of other investigators, who showed that a decreased ATP/ADP can produce impaired muscle contraction by substantial slowing in the cross-bridge kinetics and rigor tension.
(Ventura-Clapier, 1994). Subsequent clinical studies suggested that the energy stores of the left ventricular myocardium correlate with organ function and clinical outcome (Daracott-Cankovic, 1998). Smolenski and colleagues showed that reperfusion is associated with a decrease in ATP and TAN levels for both heart and heart-lung transplantation (Smolenski, 1992). As expected, loss of adenine nucleotides in the myocardium is paralleled by an increase in purine catabolites in serial samples of coronary sinus blood obtained during reperfusion. HEP analysis has been applied to other fields of solid organ transplantation. Postoperative recipient liver function was shown to be inversely correlated to the fall in ATP and TAN levels during the warm ischaemic period (Kamiike, 1988). In summary, HEP levels have been used successfully in transplantation research but, due to the technical complexity of the assay, have not been adopted into clinical practice. The discussion on HEP and transplantation outcomes continues in chapter 4.

2.2 PRINCIPLES OF HIGH-ENERGY PHOSPHATE ANALYSIS

The first law of thermodynamics dictates that the sum of all energies in a closed system must remain constant. In the heart, loss of chemical energy accompanies muscular contraction. If we define the change in chemical energy during contraction as $\Delta E$, the work and heat generated as $W$ and $Q$ respectively, then the above process can be described:

$$\Delta E = W + Q$$

The direct relationship between the chemical energy consumed and the mechanical energy produced by the myocytes made energy substrate assessment an attractive tool for evaluation of contractility potential.

A detailed classification of metabolic working models was made by Jennings and Morgan (Jennings, 1992):

1. Whole heart in vivo
2. Regional ischaemia in vivo
3. Isolated perfused hearts
   - Langendorff
   - Working heart preparation
4. Tissue slices
5 Tissue homogenates
6 Isolated myocytes
7 Isolated mitochondria

In the first three models, the metabolic assessment can be done by one of several methods:

a) Aortic to coronary sinus gradient of metabolites - this method is particularly useful to study the integrated metabolism and to determine the amount of anaerobic activity (e.g. by measuring lactate)

b) Cardiac biopsies

c) Non-invasive methods (e.g. positron emission tomography, $^{31}$P magnetic resonance spectroscopy (MRS))

Privileged access to human myocardial tissue perioperatively and the serial nature of our measurements made cardiac biopsies the method of choice in this project.

**Analysis of high-energy phosphates in myocardial biopsies**

The classification below details the methods available for cardiac sampling, HEP extraction and measurement (Jennings, 1992; Swain, 1986).

**Methods of biopsy**
- Trucut needle
- Vacuum drill
- Whole heart / tissue fragments - pre-cooled Wollenberger tongs

**Cooling agent**
- Isopenthane
- Liquid nitrogen

**Extraction**
- Acid - for adenine nucleotides (ATP, ADP, AMP)
- Alkaline - for pyridine nucleotides (NADH, NADPH)

**Analysis**
- Chromatography
- Enzymatic methods - coupled reactions assessed by
  - luminometry
  - fluorimetry
A general critique of the biochemical method is available elsewhere (Isselhard, 1988; Jennings, 1992). A few important points will be made here, particularly in relation to its potential pitfalls. Firstly, the ratios of HEP concentrations are relative numbers. However, when absolute concentrations are pursued care must be taken in dealing with the tissue reference base (e.g. dry weight vs. wet weight). Secondly, methods measuring CP and ATP calculate the ADP concentration from the CK equilibrium. It should be noted that the in vitro $K_{eq}$ may be different from the one existing in vivo (Heineman, 1992). Thirdly, results from laboratory experiments have to be interpreted cautiously before clinical testing. For example, inter-species differences in the catabolism of HEP may lead to different conclusions from the same experiment (Minten, 1991).

**Principle of the adenine nucleotide assay using the luciferase enzyme system**

Luciferase is an enzyme extracted from the lanterns of fireflies. In the presence of luciferin, oxygen, magnesium and ATP, it catalyses the formation of adenylic-luciferin. The latter is oxidized by atmospheric oxygen to adenylic-oxyluciferin and the process is accompanied by emission of light.

\[
\begin{align*}
(1) \quad \text{ATP} + \text{luciferin} & \xrightleftharpoons{\text{Mg}^{2+}\text{luciferase}} \rightarrow \text{adenyl-luciferin} + \text{pyrophosphate} \\
(2) \quad \text{Adenyl-luciferin} & \xrightarrow{\text{O}_2} \rightarrow \text{adenyl-oxyluciferin} + \text{H}_2\text{O} + \text{light}
\end{align*}
\]

Reaction (1) is reversible and the equilibrium lies far to the right. Reaction (2) is practically irreversible and its product, adenylic-oxyluciferin, is an inhibitor of the luminescent reaction. In the presence of ATP the reaction reaches its maximum rate almost immediately and then decreases as a first order process as ATP is consumed or as inhibitors accumulate. The determination of ATP therefore involves the measurement of the relative intensity of the light emitted within seconds by the reaction mixture.

The wavelength of emitted radiation in the luciferase reaction is 562 nm. The intensity of light depends on the reaction rate of the oxidoreduction step which, in turn, depends on the
concentration of the molecules taking part in the reaction. Commercially available purified luciferase extracts permit stable reaction kinetics, and flash-like light emissions are no longer problematic. Figure 2.2.1 shows a simplified scheme of the ultra-low light detection apparatus. Luminometers are essentially of two types: photon counting and direct current. In the photon-counting variety, while every count is the result of a photon, not every photon is counted. The direct current luminometers are more reliable: they measure electrical current that is maintained by, and is proportional to, the photon flux passing through the photomultiplier tube.

**Figure 2.2.1** Simplified scheme of a photomultiplying luminometer.

The reaction is both sensitive and specific. With purified luciferase extracts, ATP can be detected reliably down to the picomolar concentration; from a specificity point of view, no other ribonucleotide triphosphate can substitute for ATP. Construction of a calibration curve with known amounts of ATP is an obligatory initial step. The method can be easily adapted to measurement of more than one analyte. By using coupled reactions ADP and AMP can be assayed. Myokinase (MK, adenylate kinase) and pyruvate kinase (PK) catalyse ATP formation as described below, with phosphoenolpyruvate (PEP) acting as a phosphate group donor.

\[
\text{AMP + ATP} \quad \xleftrightarrow{\text{MK, Mg}} \quad 2 \text{ADP}
\]

\[
\text{ADP + PEP} \quad \xleftrightarrow{\text{PK, Mg, K}} \quad \text{ATP + pyruvate}
\]

The equilibrium of both reactions is sufficiently far to the right. ADP and AMP are therefore not measured directly, but quantified from the measured amount of ATP that they produce. For this
study we have used a modification of the enzymatic method described by Spielmann et al., further described in detail in chapter 3 (Spielmann, 1981).

Once the calibration curve is obtained, the same reagents and enzymes are used to measure adenine nucleotides in the study samples. There are different methods of HEP extraction from biological material. Briefly, this can be done by boiling the sample in the reaction buffer, or by chemical methods with organic solvents, surfactants or acids. The most important property of an extraction method should be to produce stable extracts. In our study the metabolism was arrested by freezing the sample immediately after biopsy procurement. Before analysis, in order to prevent HEP degradation by ATP-converting enzymes, the samples were ground in perchloric acid (PCA) which degrades enzymatic structures. The advantage of this method is that it results in high yields of ATP, but the disadvantage is that subsequent neutralisation is required to normalise the pH. The luminescence for the ATP, ADP and AMP tubes is measured and the actual molar concentrations are calculated using the calibration curve constructed earlier during the experiment. Absolute values of adenine nucleotides can be obtained by choosing an appropriate reference base. Wet weight is usually employed in chromatography techniques. Dry weight requires preliminary vacuum lyophilisation of the sample. A quartz fibre balance is employed to weigh the sample prior to assaying the substrates. Finally, the chief constituent of dry myocardium is protein, and thus protein can be used as a reference base in the same fashion. It has been shown that dry weight and protein yield comparable result. In the rat heart there are 1.33 mg dry weight per milligram of protein, whereas in the new-born pig heart the figure is 1.39 (Jennings, 1992). In our experiments we evaluated the protein in each biopsy with a modification of the Bradford method, based on the dye-binding principle (Bradford, 1976). The Bio-Rad protein assay revolves around the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range and so, by selecting an appropriate ratio of dye volume to sample concentration, protein can be quantitated accurately. Expressing adenine nucleotides as absolute values requires therefore determination of a reference base for that tissue (dry weight, wet weight or non-collagen protein). Non-collagen protein was determined in all our experiments but, given the potential technical errors introduced by additional measurements of a reference base and also due to the robustness of information provided by the ATP/ADP ratio and the EC, we decided to express the results using these ratios.
2.3 ENDOTHELIAL CELL ACTIVATION

Surgical physiology of the endothelium

The endothelium is a monolayer organ covering an area of several square meters (Tedesco, 1999). It responds to a variety of physical and humoral stimuli with a large repertoire of molecules expressed and secreted. Physiologically it is equipped to take part in vasoregulation, inflammation, immune interaction, coagulation, fibrinolysis, and angiogenesis. In addition to its generic functions, the endothelium has a heterogeneous phenotype and embraces organ-specific roles (Page, 1992; Celermajer, 1997). In the coronary circulation it has adapted to supplying oxygen and energy substrates to the most metabolically active tissue (Opie, 1998). There is increasing evidence that endothelial dysfunction represents an early event that initiates the disease process in atherosclerosis (Lusis, 2000). This is of interest to all those involved in transplantation since the main limiting factor for long-term survival is chronic allograft vasculopathy (Weiss, 1997).

Shear stress from the blood stream is constantly 'sensed' by the endothelium. The physiological vasoregulators responsible for this are NO, endothelin, angiotensin II and prostacyclin (Celermajer, 1997; Anderson, 1999; Stamler, 1999). NO is made by three isoforms of NO synthase (nNOS = 'neuronal', eNOS = 'endothelial', iNOS = 'inducible') and diffuses isotropically through cell membranes (Cannon, 1999). This property of NO makes cause and effect studies particularly difficult. Its vasodilatory effects are mediated through multiple pathways by cGMP, interaction with redox-sensitive potassium channels and inhibition of endothelin (Stamler, 1999). Endothelin, a powerful vasoconstrictor originating in the endothelium, acts as a circulating hormone but also in an auto/paracrine fashion (Weis, 1999). Cardiac NO and endothelin are known to interact with inflammatory effectors (Weis, 1997; Maxwell, 1999). In addition there is compelling evidence that perturbation of NO homeostasis, particularly via redox signalling pathways, is capable of initiating atherosclerosis (Maxwell, 1999; Lusis, 2000).

The endothelium performs many of its functions via surface adhesion molecules. Selectins, integrins and members of the immunoglobulin superfamily are expressed on platelets, leukocytes, endothelia and subendothelial matrix. Coupling of a ligand to its receptor is rarely sufficient to initiate a certain cell program, similar to T cells which require two or more signals to become activated (Rose, 1998). Adhesion molecules interact 'promiscuously' with a range of ligands and a
phenomenon such as leukocyte extravasation or apoptosis depends on a whole repertoire of membrane and subcellular events (Petruzelli, 1999). When the mechanism of leukocyte rolling, adhesion and particularly tissue extravasation is completely characterised, it will be possible to interfere with diapedesis at different points in its pathway (Rabb, 1999).

The interaction between the complement system and the endothelium is bi-directional. The normal endothelium is able to inactivate complement through a range of mechanisms (Tedesco, 1999). On the other hand, activated complement products are able to bind to the endothelium via specific receptors and subsequently promote vasoregulation, inflammation and coagulation, all of which are intimately related processes. The endothelium is physiologically an anticoagulant surface with the contribution of the antithrombin III and the thrombomodulin-protein C-protein S systems (Hancock, 1997). Endothelial dysfunction is associated with varying degrees of antithrombin III and thrombomodulin downregulation. Thrombin is a pleiotropic agent with actions far exceeding the enzymatic serine protease function. It has mitogenic and proinflammatory properties mediated by transmembrane signalling via protease activated receptors (PAR), four types being described to date (Preissner, 2000). The endothelium is capable of expressing different PARs. PAR-2 expression for instance has recently been shown to be protective against ischaemia-reperfusion injury in the rat myocardium (Napoli, 2000). The platelets, no longer seen as 'innocent bystanders', exhibit chemotactic and mitogenic properties (Cicala, 1998). PECAM-1 (CD 31), normally expressed on all endothelia, facilitates interaction with activated platelets (Becker, 2000). The endothelium is also an immunological interface, with antigen-presenting and apoptotic properties (see below) (Rose, 1998; Walsh, 1999).

Noxious stimuli generally elicit a two-stage endothelial response. Constitutive molecules (e.g. ICAM-2) or other proteins which can be mobilised readily from underneath the cell membrane (e.g. P-selectin stored in the Weibel-Palade bodies) are initially involved. This phase (also called type 1 activation) does not involve de novo protein synthesis, as opposed to the second phase (type 2 activation) which is transcription-dependent and takes place after 4 hours or more (Ichikawa, 1997). The mechanism of endothelial activation in cardiopulmonary bypass and in ischaemia-reperfusion has been described elsewhere (Asimakopoulos, 1998; Boyle, 1999; Jordan, 1999). Expression of the more important CAMs in the normal human heart is presented in Table 2.3.1. Entirely normal myocardium is difficult to procure in the clinical setting and so all the studies are limited by the use of suboptimal controls. The description is general, since the
distribution of constitutive and inducible markers varies between arterioles, microcapillaries and venules and between the different chambers of the heart (Page, 1992; Valen, 2000).

**Table 2.3.1** Constitutive phenotype of the cardiac microvasculature in the normal human heart

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-selectin (CD62P)</td>
<td>-</td>
</tr>
<tr>
<td>E-selectin (CD62E)</td>
<td>-</td>
</tr>
<tr>
<td>PECAM (CD31)</td>
<td>++</td>
</tr>
<tr>
<td>VAP-1</td>
<td>++</td>
</tr>
<tr>
<td>ICAM-1 (CD54)</td>
<td>+</td>
</tr>
<tr>
<td>ICAM-2 (CD102)</td>
<td>+/-</td>
</tr>
<tr>
<td>VCAM-1 (CD106)</td>
<td>+/-</td>
</tr>
<tr>
<td>MHC class I</td>
<td>+</td>
</tr>
<tr>
<td>MHC class II</td>
<td>+</td>
</tr>
<tr>
<td>vWF</td>
<td>+</td>
</tr>
<tr>
<td>TM</td>
<td>++</td>
</tr>
<tr>
<td>AT III</td>
<td>++</td>
</tr>
</tbody>
</table>

++ strong, even; + strong but patchy; +/- weak and patchy; - absent expression


**Endothelial preservation in cardiac transplantation**

*The literature*

In order first of all to see how endothelial preservation is represented in the literature, a search of heart preservation-related papers published between 1980 and 1999 in five principal journals was undertaken (Stoica, 2001). The focus of this literature search was on the methods employed in experimental, animal and human studies and on types of outcome measures. All issues of *The Annals of Thoracic Surgery, Circulation, The European Journal of Cardiothoracic Surgery, The*
Journal of Thoracic and Cardiovascular Surgery and The Journal of Heart and Lung Transplantation between 1980 and end of 1999 were searched for papers on preservation methods in cardiac transplantation. The inclusion criteria were all laboratory, animal and human studies focused on heart preservation and storage, and studies applying statistical methods to retrospective series of human cardiac transplantation. The following types of studies and publications were excluded: global ischaemia without preservation, cardioplegia without storage, heart-lung transplantation, multiorgan preservation, retrospective statistical analyses in which preservation was not included as a variable, abstracts, anecdotal and technical reports, and all other correspondence. The methodology of the animal experiments was classified as ischaemia (I), ischaemia-reperfusion (IR) or transplantation (Tx). In animal studies with multiple subgroups the paper was classified after the most complex group (i.e. Tx or IR), the others being considered controls. The measures of outcome were classified as functional, biochemical, morphologic and 'endothelial'. In addition, some human studies had clinical and survival end points.

Two hundred and forty nine publications were identified according to the pre-set criteria. Of these, 206 (83%) reported results of experiments performed on animals and 10 (4%) involved animal tissue only. There were 34 (14%) clinical studies and 9 (4%) studies on human tissue (Fig 2.3.1). Of the 196 animal experiments, over a half (105) were carried out in small animals. Only in a minority (47 papers) of the animal studies was it directly apparent from the title that the results were obtained in animal models. The animal experiments utilised most often a model of global ischaemia with ex vivo reperfusion (121 publications, 49%) (Fig 2.3.2). Endothelial function was used as an outcome measure, alone or in combination with other descriptors, in one paper, only, in the 1980s and in 24 papers in the 1990s (10% in total). The majority of experiments had one (48%) or two (40%) measures of outcome (Fig 2.3.3). Five clinical studies were randomised trials, representing 15% of the clinical research and 2% of all publications identified. A classification of the animal experiments and of the types of outcome measures for all studies is shown in Tables 2.3.2 and 2.3.3.

Table 2.3.2 Types of animal studies in cardiac preservation.

Ischaemia and storage
- Cold ischaemia
- Continuous perfusion

53
Ischaemia, storage and reperfusion

Isolated reperfusion
- Crystalloid
- Blood
  - Autologous
  - Non-autologous
  - Different species

Paracorporeal circulation (support animal)

Transplantation
- Autotransplantation
- Allograft transplantation
  - Isografts
  - Allografts
- Xenotransplantation

Table 2.3.3 Types of outcome measures in animal and human studies.

Biochemical
- Integrated metabolism (aortic to coronary sinus gradient methods)
- High energy phosphate metabolism in the myocardium
  - High performance liquid chromatography (HPLC)
  - Nuclear magnetic resonance (NMR)
- Enzymatic methods
- Isolated mitochondria analysis
- Serum markers (e.g. troponins)

Morphological
- Light microscopy
- Electron microscopy
- Quantitative birefringence
- Morphometric analysis

Functional
- Haemodynamic measurements
  - Direct assessment of pressures/output
Load-independent indices (pressure-volume loops)
Imagistic assessment
Angiography
Echocardiography
Coronary flow
Muscle stand testing

Clinical

Other
Myocardial water content
Intracellular ions measurement
Viability studies in cell cultures
   Trypan-blue staining
   Multiple indicator solution
Blood vessel preparations
   Endothelium-dependent contraction (e.g. acetylcholine)
   Endothelium-independent contraction (e.g. adenosine)

Figure 2.3.1   The distribution of animal and clinical cardiac preservation studies between 1980-1999.

A = animal, AT = animal tissue, H = human, HT = human tissue. Total = 249 publications.
Figure 2.3.2 The distribution of types of preservation research models between 1980-1999, including clinical transplantation.

![Bar chart showing distribution of types of preservation research models between 1980-1999.]

IR, ischemia-reperfusion; TX, transplantation; I, ischemia. Total = 249 publications.

Figure 2.3.3 Distribution of cardiac preservation studies between 1980-1999 depending on number of outcome measures.

![Bar chart showing distribution of cardiac preservation studies between 1980-1999.]

1, one outcome; 2, two outcomes; 3, three outcome measures. Total = 249 publications.

Types of preservation

A report from 1978 on long distance transportation of human hearts for transplantation fuelled the interest of many research groups on the best methods of organ preservation (Thomas, 1978). The
cold storage techniques evolved from two schools of thought. In the first, use of the cardioplegia solutions such as St Thomas' was extended to cardiac preservation (Darracott-Cankovic, 1987). In contrast, the second approach is derived from experience with intra-abdominal organ preservation and is centred on the Euro-Collins and University of Wisconsin (UW) solutions (Konertz, 1998; Stein, 1991). Both these methods have proved successful, despite the fact that the solutions were not specifically designed for cardiac storage during global ischaemia. The rationales of 'intracellular' and 'extracellular' solutions are reviewed elsewhere (Mendler, 1992; Jahania, 1999). The Histidin-Tryptophan-Ketoglutarat (HTK) solution and Celsior were storage media developed with the specific purpose of integrating the principles of solid organ preservation with those of myocardial metabolism (Bretschneider, 1980; Menasche, 1994). These solutions still undergo experimental and clinical trials (Ou, 1999; Wildhirt, 2000). Despite the differences in composition and underlying philosophies, the weight of evidence so far is that the effectiveness of these solutions is closely comparable (Wildhirt, 2000; Garlicki, 1999). Small randomised clinical trials showed that cardioplegia modification, either in the recipient (Carrier, 1996) or in both donor and recipient (Luciani, 1999) may enhance preservation. An option to anoxic storage is aerobic preservation (Copeland, 1973). Continuous perfusion techniques lend themselves to interesting experimental variations, but the lack of clear clinical advantage and the cumbersome addition of equipment during transport makes them virtually absent from clinical practice (Wheeldon, 1992). Variability in preservation strategies is common in clinical practice. A survey showed 167 types of heart preservation solutions to be utilised by 147 transplant centres in the United States (Demmy, 1997). Practice at our institution changed when results of a survey showed that cold storage with cardioplegia solution was associated with an increased risk of deaths (Wheeldon, 1992). Since 1992 we have therefore been using normal saline as the transit milieu. Better cardiac preservation remains an elusive goal, and the safe ischaemic time still does not exceed four hours. With equal short-term outcomes, a certain preservation strategy will only emerge as superior when it prolongs graft survival.

Preservation research

A different perspective for the situation described is obtained if we summarise the factors affecting the cardiac allograft in a transplant operation. By analysing the sequence of events in Fig 2.3.4, it can be seen how even a healthy organ sustains a cumulative injury with consequences ranging from immediate to long term. Recent data from the International Registry of Heart and Lung Transplantation show a clear relationship between ischaemic time and mortality at 1 and 5
years postoperatively (Hosenpud, 1998). Storage has a central position in the time sequence described. However, in experimental models of global ischaemia or transplantation, storage is either studied as a primary variable or is included in a protocol which has another variable as the primary exposure. While the concept of endothelial preservation is evolving, a loose usage of terms tends to confuse issues further. Perhaps the term cardiac preservation should describe the whole strategy to maintain myocardial and endothelial function across transplantation, whereas storage should be reserved for the specific stage in the middle. The events in Fig 2.3.4 illustrate how endothelial injury can be a continuum, both in severity and over time.

**Figure 2.3.4** The initial cumulative injury sustained by the cardiac allograft. (The downstream events resulting from reperfusion with non-autologous blood and the interaction with the recipient's immune system are not depicted.)

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Brain death

| Neurohormonal and hemodynamic instability
| Cardioplegic arrest
| Cold ischemic storage
| Implantation (warm ischemia)
| Reperfusion injury
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The most common publications result by far from small animal work. Many new ideas emerge all the time and are tested in these 'screening' experiments. Only a few dedicated groups follow a research hypothesis through to large animal transplant models and clinical trials. Direct clinical implementation of results obtained in isolated tissue or small animal models sometimes meets
with failure and publication biases help these events to pass largely unknown. An example of perseverance in relating bench work to clinical results is available from Rosenfeldt's group. After an initial favorable experience with UW solution in a rat model, a short series of unexpected clinical failures was encountered (Ou, 1999; Rosenfeldt, 1996). They returned to the isolated rat heart model and demonstrated elegantly how the UW solution looses its protective action at increased temperatures and has deleterious effects on the endothelium (Ou, 1999). This research confirmed once more that the rewarming associated with implantation is a particularly vulnerable phase.

The most common isolated heart models are the beating heart preparation pioneered by Langendorff and the working heart model, in which afterload conditions and the perfusion method can be adjusted (Sutherland, 2000). Crystalloid reperfusion negates the effect of a number of key factors in the reperfusion injury (complement, leukocytes, platelets) (Galinanes, 1990). Reperfusion with autologous blood does not effectively control variables of rejection-induced dysfunction. Many animal studies of ischaemia with or without reperfusion employ live donors and thus exclude the considerable effect of brain death on the heart (Tixier, 1991). This is an important observation, especially when the mode of brain death itself (gradual vs. explosive increase in intracranial pressure) is directly related to the amount of myocardial damage (Shivalkar, 1993). Meanwhile, the exact mechanism of cardiac dysfunction in brain-dead donors remains to be deciphered (Galinanes, 1994; Chiari, 2000; Szabo, 2000). The chain of events is bound to be complex, as Yeh and colleagues showed that brain death alters the left ventricular gene phenotype (Yeh, 1999).

Animal transplantation models provide more physiologic reperfusion but rejection as a variable is generally poorly controlled. In some animal models the number of study subgroups can range from one to ten and it is not readily apparent which ones are study groups and which ones are controls. Steps taken in the study design to eliminate bias are not made explicit or, alternatively, a section on limitations of the study is the exception rather than the rule. Small animal transplant models generally used homozygous subjects but this is not always clear in the protocol. Use of syngeneic subjects is not feasible in large animal models, although attempts to control acute rejection are sometimes noted, by ABO matching for instance (Jeevanandam, 1992). In a murine heterotopic model and an isograft milieu, ischaemia-reperfusion is in itself sufficient to induce CAV, whereas in allografts IR exacerbates the vasculopathy (Wang, 2000). This is in line with the notion that CAV results from subclinical graft injury and is engineered by intricate
immunological mechanisms operating in a milieu of multiple non-immunologic risk factors (Weis, 1997). Controlling immunological variables in transplant models may prove more difficult than at first sight. Hancock points out some pitfalls of using the F344/Lewis rat combination, for which there is no real agreement that it represents a minor antigen mismatch system (Hancock, 1999). Prolonged cardiac preservation time is of course attractive from an immunological point of view. It may confer the benefits of routine prospective HLA matching, as practised in kidney transplantation (Opelz, 1994).

The types of outcome measures for human and animal preservation studies are summarised in Table 2.3.3. Comparisons were undertaken between study groups but rarely between methods to validate new ones (Fremes, 1993). It is prudent to observe that the same measure of outcome applied to different species in the same study can yield different results (Masuda, 1992; Minten, 1991). In spite of inherent limitations, bench research contributed considerably to transplantation biology but less to clinical preservation. There is a strong argument for this type of research: it is unethical to submit patients to poorly tested hypotheses. However, a degree of standardisation in animal experiments is highly desirable to allow meaningful and worthy comparisons. Human studies have a reduced prevalence but are the most likely to bring about more uniformity in practice and to improve results.

New evidence of endothelial activation in cardiac transplantation

Brain death

Two groups independently set up comprehensive experiments in the rat, some including cross-circulation, showing that brain death is a dynamic process associated with endothelial activation and end organ dysfunction. In essence, solid organs upregulate MHC molecules, express selectins and integrins on their endothelia, attract leukocytes in the interstitium and there is also a corresponding increase in circulating activation factors (Takada, 1999; van der Hoeven, 1999; Pratschke, 2000). Pretreatment with selectin antagonists and blockade of T-cell activation resulted in suppression of both T cell and macrophage-related cytokines in end-organs (Takada, 1999). When hearts and kidneys went on to be transplanted, there was accelerated rejection of rat allografts from brain-dead donors compared to controls (Pratschke, 2000; Wilhelm, 2000). In pretransplant biopsies from human cadaveric kidney donors an increase in E-selectin, HLA-DR, ICAM-1 and VCAM-1 was noticed compared to biopsies from living-related donors (Koo, 1999).
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These results may partly explain the higher overall graft survival in living-related clinical kidney transplantation. It is conceivable that similar phenomena accompany human cardiac transplantation. The excellent long-term outcome seen with domino transplantation, both in terms of freedom from CAV and overall survival (Anyanwu, 2002; Anyanwu, 2003), may in fact result from a combination of live donation and short ischaemic time.

Ischaemia

It is useful to separate the discussion of ischaemia and reperfusion in the experimental setting to be able to understand their independent contribution to injury. Pinsky and colleagues elegantly showed how hypoxia alone is sufficient to induce exocytosis of the Weibel-Palade bodies, with expression of vWF and P-sel on to the cell membrane in a time-dependent fashion (Pinsky, 1996). They also demonstrated the key role of P-sel in neutrophil recruitment in a rat isograft model of cardiac transplantation. P-sel null hearts transplanted into wild-type recipients had marked reduction in neutrophil infiltration and increased graft survival compared to wild-type control transplants. Hypoxia alone, if severe and prolonged, produces a significant alteration in endothelial permeability (Ali, 1998). Hypoxia may downregulate basal VCAM-1 and ICAM-1 (Willam, 1999), but the combination of hypoxia and inflammatory stimuli is able to enhance ICAM-1 expression (Zund, 1997; Poston, 1997).

During ischaemia the cell switches to anaerobic metabolism and the energy stores are used for vital cell functions. Hypothermia is an effective strategy to reduce metabolism overall but the duration of cold storage is, of necessity, finite. ATP depletion after prolonged ischaemia, like in the more extensively studied cardiomyocytes, will directly affect the ATP-dependent pumps and lead to alteration of ionic and osmotic gradients across the cell membrane. Catabolism of high-energy phosphates produces hypoxanthine which, upon reperfusion, is one of the most powerful generators of oxygen radicals (Land, 1999). The fate of the cell on reperfusion depends on the extent of ischaemia and the capacity of physiologic scavengers. Lesser degrees of ischaemia, in combination with reperfusion, will create a highly reactive endothelial phenotype.

Reperfusion

The redox state of the vasculature influences gene expression in an adaptive fashion (Kunsch, 1999). Oxygen radicals represent more than noxious stimuli or effectors of bacterial killing in
phagocytic blood cells. At lower levels, commonly referred to as 'oxidative stress', they may function as second messengers in intricate mechanisms of signal transduction and transcription control. Nuclear factor κB (NF-κB) and AP-1 (activator protein-1) are the most extensively studied redox-sensitive transcription factors. It is unlikely that oxygen radicals directly activate the transcription factors. The probability is that activation takes place via protein phosphorylation pathways. NF-κB regulates a multitude of cytoprotective genes and, due to its central position, might be ideally suited to therapeutic inhibition (Boyle, 1999; Feeley, 2000). Some of the convergent pathways of IR injury are mentioned below.

Collard and colleagues showed in a human umbilical vein endothelial cell preparation that prolonged hypoxia produces NF-κB translocation to the nucleus which further increases during reoxygenation (Collard, 1998). In addition it appears that the ensuing protein synthesis leads to a neo-epitope expression in the membrane followed by iC3b complement deposition and activation via the classical pathway. The activation of complement in IR is multifactorial, especially in transplantation, but far from innocuous (Tedesco, 1999; Baldwin, 2000).

Neutrophils are the main bloodstream mediators of IR, and their recruitment is greatly facilitated by phenotypic changes in the ischaemic tissues. The ischaemic myocardium is able to secrete a variety of leukotactic substances, including TNF-α, IL-8, IL-6, platelet activating factor, complement and leukotrienes (Jordan, 1999; Kupatt, 1999). IR can rapidly induce selectin ligand expression on to the surface of cardiomyocytes and cultured endothelial cells (Seko, 1996). ICAM-1 is unaffected by hypoxia in the coronary endothelium, but reoxygenation produces marked upregulation via a NF-κB-mediated pathway. NO is able to reduce reoxygenation-specific ICAM-1 expression, probably by diminishing oxidative stress (Kupatt, 1997). By using NF-κB decoy oligonucleotides, it was possible to block ICAM-1 upregulation and neutrophil adhesion to rodent coronary endothelium (Kupatt, 1999). Feeley et al employed the same method of NF-κB inhibition in a transplant experiment and demonstrated reduced endothelial adhesiveness and a decrease in acute and chronic allograft rejection (Feeley, 2000). In a murine cardiac isograft model, which avoids allore cognition bias, the positive feedback loop established between ICAM-1 and IL-1 after reperfusion may be pivotal to primary graft failure (Wang, 1998). Jaakkola and colleagues performed a study on ischaemic and reperfused human myocardium showing the distribution of salient CAMs and their role in leukocyte binding (Jaakkola, 2000). Cardiopulmonary bypass (CPB) and reperfusion with non-autologous blood are the two other factors adding to the complexity of the IR phenomenon in cardiac transplantation. It is thus
conceivable, although not demonstrated that the reperfused donor heart has a higher inflammatory load than native hearts in conventional cardiac surgery.

**Allorecognition and acute rejection**

All the microvascular and small vessel endothelial cells constitutively express MHC class II antigens (Rose, 1998), further upregulated by IR along with MHC class I (Land, 1999). Endothelial cells are able to cause allo-stimulation of T cells, which is strongly suggestive of their antigen-presenting ability (Rose, 1998). The dendritic cells are the main antigen presenters in T-cell allorecognition and their activation may take place in the donor even before organ procurement (Takada, 1999; Land, 1999). Recipient T cells and donor dendritic cells are normally separated by the endothelial monolayer. The endothelium however is far from inert after IR and has the ability to specifically interact with T lymphocytes by VCAM-1 or Fas ligand (FasL) for example (Walsh, 1999; Kokura, 2000). Fas (CD95/APO-1) is a 'death receptor' expressed on most cell types, including leukocytes. On the other hand FasL, a member of the type II membrane proteins like TNF-α, is restricted in its expression to immune privileged sites (e.g. eye, testis) and the endothelium. The Fas-FasL system has a physiological role in lymphocyte apoptosis by limiting antigen-activated lymphocyte extravasation. This function diminishes in TNF-mediated inflammation (Walsh, 1999). Modulation of FasL in target transplant tissue has so far lead to conflicting results but will undoubtedly receive increasing attention (Martinez, 1999; Thomas, 2000). How acute rejection and episodes of infection, particularly with cytomegalovirus, subsequently lead to repeated ECA is described elsewhere (Baldwin, 2000; Salom, 1998).

**Protective mechanisms and the role of apoptosis**

In response to an insult, a complex intracellular dialogue decides upon life or death of individual cell. Apoptosis (programmed cell death) has been compared and contrasted to necrosis. Recent biochemical studies have however weakened this contrast, since apoptotic and non-apoptotic pathways of death interact at multiple levels (Saraste, 2000). Apoptosis can proceed through type I pathways (mitochondrial-independent, Fas-dependent) or type II pathways (mitochondrial-dependent). The Bcl-2 family of genes contains pro-apoptotic (e.g. Bax) and anti-apoptotic members and preferentially controls type II pathways. Bcl-2 and Bcl-XL are involved in cytoprotection both through direct effects but also through NF-κB inhibition and downregulation of pro-inflammatory genes (Badrichani, 1999). Ischaemia alone induces cell death by necrosis,
whereas the lethal effect of ischaemia-reperfusion is achieved by both apoptosis and necrosis, with neutrophils possibly playing a central role (Zhao, 2000). Evidence is emerging that apoptosis is involved in peri-transplant pathology. Apoptotic pathways are activated in dysfunctional donor hearts (Birks, 2000), but also in acute and chronic rejection (Szabolcs, 1998; Xu, 2001).

Ultimately, the fate of individual cells depends on a fine balance between death signals and protective mechanisms. Heat shock proteins for example are a group of 'molecular chaperones', the induction of which is associated with enhanced mechanical and endothelial function after cardiac ischaemia. The coronary endothelium is the main site of 70 kD heat shock protein induction in the rat heart (Amrani, 1998). The heme oxygenases (HO) are proteins involved in reducing oxidative stress, with induction of HO-1 having protective effects in terms of reduced leukocyte adhesion in vitro (Hayashi, 1999).

Manipulation of anti-apoptotic pathways, although limited in its early experimental success, is an exciting therapeutic option for the future (Yaoita, 2000). Before that, however, the contribution of apoptosis to cardiovascular pathology has to be more clearly elucidated (Feuerstein, 2000). There are several problems in achieving this. First of all, apoptotic pathways have multiple checkpoints and redundancies. By using one method in isolation, it is very difficult to ascertain that a particular cell is committed to death (Saraste, 2000). Secondly, even when apoptosis is diagnosed with reasonable certainty, its contribution to overall organ dysfunction is difficult to quantify. It is not clear whether apoptosis induces dysfunction by loss of tissue mass or if other mechanisms are at work (Feuerstein, 2000). Finally, apoptosis of noncardiomyocytes is an even more obscure phenomenon.

In the immediate postoperative period the functional consequences for the transplanted heart can range from subclinical to lethal as a result of acute allograft failure or rejection. Acute pump failure is not necessarily a consequence of energy store depletion and it has been recognised in unpredictable fashion even in association with domino donation or short ischaemic times. A no reflow phenomenon in the microcirculation has long been implicated in the pathogenesis of the syndrome (Manciet, 1995). In a series of animal experiments Guyton's group demonstrated how the right ventricular microvasculature is affected more than in the left ventricle by the sequence of cold storage and reperfusion. (Murphy, 1997). Compelling laboratory and clinical evidence is available to suggest that iNOS too is implicated in donor organ failure. NO mediates profound vasodilatation in the allograft resistance vessels, and this haemodynamic profile creates the
premise for tissue oedema and muscular stiffness (Skarsgard, 2000). This could be the basis for
the observed mRNA iNOS expression in human allografts with impaired left ventricular
performance (Lewis, 1996; Paulus, 1997). For all the above reasons, we have not aimed in the
current dissertation to quantify apoptosis in donor organ failure. Our objective is to characterise
the pattern of expression of pro-apoptotic and anti-apoptotic markers, which will be described
with the generic term of 'stress markers', and to correlate their expression with allograft function.

**Chronic rejection**

This is mediated by a combination of immune and alloantigen independent factors (Weis, 1997;
Häyry, 1998). The endothelium is kept in a chronic state of low key inflammation and mediates
proliferative events that take place in the surrounding smooth muscle and matrix. Serum levels of
TNF-α, P-sel and VCAM-1 were persistently elevated in patients followed up for up to 2 years
after transplantation (Andreassen, 1998). Most of the proinflammatory genes already mentioned
are expressed in CAV, including evidence of Fas-mediated cytotoxicity (Häyry, 1998; Koskinen,
1997; Dong, 1996). In a rat model of accelerated CAV, P-sel and VCAM-1 were significantly
upregulated during acute and chronic rejection (Koskinen, 1997). A unifying view is now
emerging according to which the perioperative phase, the acute and the chronic rejection
represent a continuum of events (Land, 1999; Halloran, 1997). It is based in turn on a novel
approach to the immune response, which is not primarily triggered by recognition of non-self but
by danger and tissue destruction in a wider sense. In an attempt to separate the immune factors,
Wang et al showed that IR injury may on its own be sufficient to induce CAV in isografts, and
that CAV is greatly accelerated in an allograft milieu (Wang, 2000). In addition, cyclic AMP
pulse therapy during preservation was able to inhibit CAV but the mechanism remains unknown.
Interestingly, acute and chronic rejection also depend on the cytokine and growth factor
genotypes, a good example being the reduced incidence of CAV in donor or recipients who are
low producers of transforming growth factor beta (Densem, 2000). The recipient may also
influence the degree of allograft loss by production of anti-endothelial and anti-HLA antibodies,
although the precise mechanism is not yet deciphered (Dunn, 1992; Suciu-Foca, 1991; Fredrich,
1999). Very probably, the individual genotype affects all phases of peritransplant ECA.

Early endothelial dysfunction appears to be transitory in terms of vasomotion (Sabate, 2000), but
is predictive of development of CAV at 1 year postoperatively (Davis, 1996). As a result many
efforts are directed towards the enhanced inflammatory state post-transplant and towards
predicting CAV before it is clinically apparent (Weis, 1999; Andreassen, 1998; Wildhirt, 2000; Labarrere, 2000). A relationship was discovered between endothelial vasomotion and perturbation of endothelin physiology early after transplantation (Weis, 1999). Another study from the same group in Munich showed that 26% of their patients had microvascular endothelial dysfunction within 1 month of transplantation and postulated that abnormalities in the NO synthesis pathways and increased levels of circulating cytokines might be partly responsible (Wildhirt, 2000). To date it is unclear whether iNOS has a protective or a causative role in the development of CAV (Cannon, 1999). Other tissue or serum markers of CAV have been investigated. For example, a procoagulant phenotype in the allograft microvasculature is predictive of CAV (Labarrere, 2000). The therapeutic options in CAV are very limited and the outcome is universally fatal (Weis, 1997). Prevention and slowing down the progression of disease appear therefore as the best strategies. In this sense, lipid-lowering agents are known for their direct antioxidative effect on the endothelium and are routinely given after transplantation (Andreassen, 1998).

In conclusion, by expressing only a small proportion of its genome, the endothelium is a highly specialised monolayer. In response to injury however the endothelial phenotype will rapidly change. It is then possible to transfer inflammation from the blood stream to the parenchyma. The overall organ response may vary across a wide range and will depend on the fine balance between injury and protective factors. The importance of the individual genotype is increasingly recognised. In the case of cardiac allografts, conditions exist for uninterrupted endothelial insult which starts after brain death and continues, with variable intensity, through to chronic rejection. However, the acute phase changes are insufficiently described to date in clinical cardiac transplantation. Perhaps a clinical stage is approached in which rejection is no longer viewed as a 'medical' and often relentless disease. By considering new treatments in the operating room, surgeons may be able to improve the overall outcome of cardiac transplantation.

In this dissertation I aim to describe the pattern of endothelial activation in brain-dead and live donors in relation to ischaemia, reperfusion and in the first few months after cardiac transplantation. The markers studied will be P-selectin (type 1 activation), E-selectin and VCAM-1 (type 2 activation), thrombomodulin (anticoagulant phenotype), Bcl-2, Bax, Hsp-70 and iNOS (stress markers).
2.4 PRINCIPLES OF ENDOTHELIAL STUDY

Research and clinical methods

The more common methods of studying endothelial morphology and function are presented in table 2.4.1. Extrapolation of experimental research to clinical practice requires a cautious and methodical approach. Here is just one example. As opposed to the coronary microcirculation of humans, rodents (and also the human umbilical vein) do not express MHC class II constitutively on the endothelium (Rose, 1998). This feature may make it easier to suppress rejection in rodent experiments.

Table 2.4.1 Experimental and clinical modalities of endothelial assessment

**Experimental**
- Contraction/relaxation studies - vascular rings
  - Endothelium-dependent (e.g. acetylcholine)
  - Endothelium-independent (e.g. adenosine)
- Phenotype/activation/adhesion studies
  - e.g. human umbilical vein
- Transplantation
  - Isografts
  - Allografts
    - Wild type
    - Transgenic / Knock-out

**Clinical**

**Invasive**
- Intracoronary studies
  - Quantitative angiography
  - Doppler flow measurement
  - Intravascular ultrasound
- Transcardiac gradient of soluble molecules
- Cardiac biopsy
  - Intraoperative (transmural)
  - Postoperative (endomyocardial)
Non-invasive
Positron emission tomography
Limb studies
Soluble plasma molecules

The principles of immunolocalisation

**Immunohistochemistry**

Coons and colleagues first used this method in 1940, when they attempted to localise antigens and antibody responses related to tissue pathogens in infected and immunised animals (Coons, 1941). They employed direct and later indirect, fluorescence-based methods. The poor storage qualities of frozen sections, along with insufficient morphological detail and background fluorescence stimulated the interest for more research. The principles of the *direct* and *indirect* methods are explained below, both of which involve specific antibodies.

Antibodies belong to the group of proteins called immunoglobulins. They are synthesised by plasma cells in a highly specific manner in response to antigen exposure. A well-determined site on the antibody named paratope links, usually in non-covalent fashion, to a given site on the antigen called epitope. The specificity of the immune reactions is well known and represents the major advantage of immunochemical techniques. For example, at the submolecular level monospecific antisera can differentiate between macromolecules containing the optic isomers of a single aminoacid. The same antibody, with epitope specificity for a given antigen, can be produced in five major variants called classes: immunoglobulin G (IgG), IgA, IgM, IgD, and IgE. IgG constitutes cca. 80% of the serum immunoglobulin and is the most frequently used in immunochemical techniques. Polyclonal antibodies are produced by different plasma cells and they react with different epitopes on the antigen against which they are raised. The rabbit is the animal used most often for their production. A variable dose of antigen, depending on its immunogenicity, is usually injected subcutaneously and blood containing the polyclonal serum is later harvested according to strict protocols. Affinity chromatography can be used to further isolate the antigen-specific antibodies. Monoclonal antibody production involves raising an antibody against a specific antigenic epitope. For reasons of economy, mice are used almost exclusively for the production of monoclonal antibodies. After an immune response has been
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achieved, B cells from lymph nodes or spleen are harvested and fused with nonsecreting mouse myeloma cells under specific conditions. The hybrid cells (hybridoma) are propagated either by transplantation into the peritoneal cavity of syngeneic mice, or in culture medium. Monoclonal antibodies are more expensive to produce but the specificity they confer to particular applications is a great advantage.

Antibody titre, incubation time and temperature are tightly interwoven in their effect on the quality of staining (Boenisch, 1989). These factors can be changed independently or, as is more often the case, in complementary fashion, to improve staining. The practical goal is to achieve optimal specific staining with minimal interference from the background. The antibody titre in immunohistochemistry is defined as the highest dilution of an antiserum which results in optimal specific staining with the least amount of background. This highest dilution is determined primarily by the absolute amount of specific antibodies present and by their affinity. The latter can be defined loosely as the time required to reach equilibrium with the tissue antigen. Correct antibody dilutions are generally determined in small pilot experiments in which a fixed incubation time has been selected. On paraffin sections optimal dilutions are not so frequently signalled by a peak in staining intensity alone, but rather by strong specific staining in the presence of minimal background (maximal signal-to-noise ratios). Dilutions are usually expressed as the ratio of the more concentrated stock solution to the total volume of the desired dilution. Mixing one part of the stock solution with nine parts of diluent, giving a total of ten parts, makes a 1:10 dilution. Higher titres of antibodies (and higher affinities) allow for shortening of the incubation time. In practice however the incubation time is set first before determining the optimal antibody dilution, a period of 30-60 minutes being the most widely used. Equilibrium in antigen-antibody reactions is reached more quickly at 37°C, but most incubations are performed at room temperature.

Immunoenzymatic staining methods utilise enzyme-substrate reactions to convert colourless chromogens into coloured end products. The subsequent discussion will describe the principles of immune enzymology and staining using as an example the techniques employed in our experiments: peroxidase-based avidin-biotin methods. The catalytic action of enzymes (E) is described by the two step equation, in which S and P are substrate and product respectively:

\[ E + S \rightleftharpoons ES \text{ complex} \]
\[ ES \rightarrow E + P \]
Enzymes utilised in immunohistochemistry should generally fulfil a number of criteria:

- Available in highly purified form and relatively cheap
- Activity not abolished by conjugation (covalent or noncovalent binding)
- Stable in solution
- Minimal interference with specific antigen-related staining from endogenous enzyme activity
- Products of enzymatic reaction readily detectable and stable

The most frequently used enzymes are peroxidase extracted from the horseradish plant (Nakane, 1967), alkaline phosphatase from the calf intestine, glucose oxidase from *Aspergillus niger* and beta-galactosidase from *Escherichia coli*. Peroxidase can be attached to other proteins either covalently or noncovalently. We used a two-step conjugation procedure to avidin. Peroxidase has an iron-containing heme group (hematin) as its active site and in solution is coloured brown. The hematin of peroxidase first forms a complex with hydrogen peroxide and then causes it to decompose resulting in water and atomic oxygen. The complex formed between the enzyme and excess hydrogen peroxide is catalitically inactive and in the absence of an electron donor (e.g., chromogenic substance), is reversibly inhibited. Quenching of endogenous peroxidase activities is achieved by excess hydrogen peroxide in the absence of an electron donor. There are several electron donors which, upon being oxidised, become insoluble, coloured products called chromogens. We used DAB (3'-diaminobenzidine tetrahydrochloride) which forms a brown end product highly insoluble in alcohol and other organic solvents. Oxidation of DAB also causes polymerisation, resulting in the ability to react with osmium tetroxide, and thus increasing its staining intensity and electron density.

The choice of staining method depends on type of specimen investigated, degree of sensitivity required, processing time and costs involved. In general there are direct and indirect methods with further sub-variations (Boenisch, 1989). In the *direct* technique an enzyme-labelled primary antibody reacts with the antigen in the tissue. Subsequent use of substrate and chromogen concludes the sequence (fig. 2.4.1). Although this method is cheap and quick, it involves one antibody only and produces little signal amplification; it is now used only rarely. In the *indirect* method an unconjugated primary antibody binds to the tissue antigen. An enzyme-labelled secondary antibody direct against the primary antibody is then applied, followed by the substrate-
chromogen solution (fig. 2.4.2). A three-step indirect method also exists. The advantage of the indirect technique is much increased sensitivity. Third, there is an unlabelled antibody method. This employs a preformed soluble enzyme-antienzyme immune complex and is one of the most sensitive techniques available. The staining sequence is as follows: unconjugated primary antibody, secondary antibody, soluble enzyme-anti-enzyme complex, substrate solution (fig. 2.4.3). The primary antibody and the antibody of the enzyme immune complex must be made in the same species so that the secondary antibody can link the two together. The technique is named after the particular enzyme complex used. For example, the PAP method utilises a peroxidase-antiperoxidase complex, whereas APAAP employs the alkaline phosphatase-antialkaline phosphatase combination. Finally there are the avidin-biotin techniques, which we also used in our study. Biotin is a vitamin and coenzyme available in a wide variety of tissues and avidin is a protein with four binding sites for biotin. The two methods currently in use are the avidin-biotin complex (ABC) and the labelled avidin-biotin (LAB) technique (fig. 2.4.4). They both require biotinylation, which is a mild process of covalently attaching biotin to a 'link' antibody. Open sites on avidin from the avidin-biotin complex or enzyme-labelled avidin then bind to the biotin on the link antibody. The biotinylated antibody does not have to be added in excess since free Fab sites are not needed for binding. The sensitivity of the indirect immunoperoxidase method is such that antigen detection became possible even in archival formalin-fixed paraffin-embedded material (Byron, 1997).

Technical aspects

Fixation is the process whereby tissues are preserved in a reproducible and life-like fashion. Fixatives prevent autolysis by inactivating lysosomal enzymes and inhibit the growth of bacteria and moulds. Furthermore, fixatives stabilise the cells and tissues to protect them from the rigors of subsequent processing and staining. In performing their protective role, fixatives denature proteins by coagulation, by forming additive compounds or by a combination of the two. Enzyme inactivation takes place by a conformational change which actually affects all the proteins. Paradoxically, the protective effect introduces a degree of artefact. Most fixative solutions will therefore also include chemicals with a stabilising effect on the proteins. Biopsy tissue preservation is accomplished by snap freezing or paraffin embedding. Cryostat sections have better antigen preservation than paraffin sections but the price is loss of resolution and morphological detail. We used paraffin embedding of formaldehyde-fixed myocardial tissue. Formaldehyde fixes by addition, reacting primarily with basic aminoacids to form cross-linking
methylene bridges. This means that there is relatively low permeability to macromolecules and that the structures of intracytoplasmatic proteins are not significantly altered. When applying monoclonal antibodies to formalin-fixed, paraffin-embedded tissue the following three considerations are important:

- Does paraffin processing (e.g. overheating) destroy the epitope under investigation?
- Does formaldehyde react with the epitope under investigation?
- Does it react with adjacent aminoacids causing conformational changes?


In order to unmask the tissue antigen, proteolytic enzymes (e.g. trypsin) have been used with varying success. These methods give good results with some antigens but they are dependent on the type and length of fixation. In early 1990s a different approach to antigen retrieval was introduced, involving heat application (Pileri, 1997; Byron, 1997). Heat is commonly applied in a microwave oven or in a pressure cooker. The mechanism by which this approach works is not completely defined but it appears to involve to involve temperature disruption of calcium coordinate complexes (Morgan, 1997). This approach turned out to be superior to enzyme pretreatment and permitted analysis with antibodies previously considered unsuitable for use in
routinely processed material. Unmasking however is not always necessary, as it depends on the antigenic site one wishes to study.

**Figure 2.4.2** Two-step indirect method. Enzyme-labelled secondary antibody reacts with primary antibody bound to tissue antigen (reproduced from Boenisch T. Staining methods. In: Naish SJ, ed. Immunochemical staining methods. Carpinteria: Dako, 1989).

Controls are required for the validation of immunohistochemistry results. Reagent controls are generally performed by the manufacturer. Procedure controls involve reagent substitution and tissue controls. Reagent substitution is usually performed for the primary antibody, which is the most critical. Negative tissue controls do not contain the relevant tissue marker and ascertain specificity. Positive tissue controls contain the target protein - control for loss of sensitivity is particularly important for tumours, when staining intensity frequently varies with the degree of tumour differentiation. Internal ('built-in') tissue controls have the advantage that they eliminate procedure-related variables. The marker is contained in the target tissue and in adjacent normal elements, occasionally obviating the need for positive controls.
Figure 2.4.3  Soluble enzyme immune complex method. Preformed complex reacts with secondary antibody. Primary antibody and antibody of enzyme immune complex must be made in the same species (reproduced from Boenisch T. Staining methods. In: Naish SJ, ed. Immunochemical staining methods. Carpinteria: Dako, 1989). PAP = peroxidase anti-peroxidase, APAAP = alkaline phosphatase anti-alkaline phosphatase.
Figure 2.4.4 Avidin-biotin methods. Preformed avidin-biotin enzyme complex (ABC) or enzyme-labelled avidin (LAB) reacts with biotinylated secondary antibody (reproduced from Boenisch T. Staining methods. In: Naish SJ, ed. Immunochemical staining methods. Carpinteria: Dako, 1989).

2.5 TOWARDS INTEGRATED MEASUREMENT OF FUNCTION

The discrepancy between haemodynamics and tissue function

The presence of ventricular dysfunction alone accounts for up to 25% of all hearts rejected for transplantation during formal donor heart assessment, and possibly for the turning down of countless potential donors prior to even formal assessment (Satchitananda, 2001). The large number of successful transplants using hearts with borderline or poor function suggest that our ability to determine intrinsic ventricular dysfunction in brainstem dead donors is limited (Stoica, 2001).

An explanation for this may arise from the methods of donor ventricular functional assessments currently in use. These rely on direct observation of ventricular wall motion, the use of ejection indices to describe ventricular performance and the use of surrogate markers of ventricular dysfunction such as inotropic requirement (for which no consensus exists between individual
transplant units) (El Oakley, 1996). Echocardiographic studies have suggested that isolated wall motion abnormalities are often transitory and do not correlate well with post-transplant function (Dujardin, 2001). Ejection indices are also limited within the varied loading conditions associated with brainstem death, because of their dependence on cardiac loading conditions. All manner of adverse cardiac loading conditions are in fact encountered in the brainstem dead donor. If we are to improve the quantity of organs without compromising quality we should therefore be able to define and determine irreversible ventricular dysfunction in these patients. Our investigations concentrated on the donor right ventricle as there is evidence to suggest that brainstem death preferentially induces right ventricular dysfunction, and also because persistent right ventricular dysfunction is associated with significant early postoperative mortality.

Tissue markers of donor cardiac function are not available in clinical practice. Invasiveness of cardiac sampling and processing difficulties are the main obstacles in establishing a reliable test. Serum markers are potentially more attractive. Higher levels of troponins I and C have recently been found to be associated with hearts which were either turned down for transplantation or had poor function in the recipient (Potapov, 2001). The assumption is that tissue loss from the donor heart leads to reduced contractility. Is it perhaps possible to find tissue markers which predict poor function before tissue loss occurs?

**Load-independent indices of function**

The Frank-Starling principles of ventricular adaptation can be extrapolated from the isolated strip of myocardium to the intact isolated and perfused ventricle and to the ventricle in situ. In the latter situation however it becomes very difficult to separate intrinsic contractile function from the effects of preload and afterload. To ascribe an observed change in shortening to a change in contractility, the other determinants of pump function (preload, afterload and heart rate) have to be kept constant. This requirement makes ejection phase parameters, such as stroke volume, ejection fraction, or the first derivative of the left ventricular pressure (dP/dt\text{max}), unreliable measures of myocardial contractility. One way in which intrinsic contractility can be measured more accurately is by describing the pressure-volume (P/V) relationship for each cardiac cycle (Kass, 1987). In the P/V diagram the volume plotted on the x axis is related to the myocardial fibre length, and the pressure plotted on the y axis corresponds to the generated force. A diagrammatic representation of a cardiac cycle is shown in Fig. 2.5.1.
Figure 2.5.1  Schematic of left ventricular pressure-volume relationship. The points represent:
A = end diastole; B = end of isovolumic contraction (ejection begins); C = end systole (aortic valve closes); D = end of isovolumic relaxation (mitral valve opens). ESPVR = end-systolic pressure volume relationship; EDPVR = end-diastolic pressure volume relationship; Ees = end systolic elastance (a measure of chamber distensibility); V0 = ‘dead volume’ of the left ventricle (this volume cannot be measured clinically, it is a concept relating to the theoretical volume at which the left ventricle would generate no pressure). In the physiological range, the end-systolic and end-diastolic points of the variably loaded beats fall along single lines, the ESPVR and EDPVR respectively. The slope of ESPVR (Ees) describes how stiff the ventricle is at the end of systole, in other words how sensitive ejection it is to increases in afterload. Together with the position of ESPVR (V0), Ees can describe the contractile state of the ventricle in a less load-sensitive fashion. Similarly, EDPVR is an indicator of the passive chamber stiffness. An increase in contractility will shift the ESPVR upwards and leftwards.
In the isolated heart, the easiest method of generating P/V loops is by using a isovolumic preparation. Preload can be varied by changing the volume of saline contained in a balloon introduced in the left ventricle. In the heart in situ, preload can be changed by intermittently occluding the inferior vena cava or, if the chest is closed, with the aid of a balloon placed in the inferior vena cava. The pressure and volume variables are recorded with the aid of the conductance catheter (see below). There are however practical and theoretical concerns in using the end-systolic P/V relationship (ESPVR) as a measure of ventricular contractility. Specifically, by varying loading conditions within the physiological range in order to define ESPVR, contractility changes may take place. In other words, the relationship between P and V may be intrinsically non-linear. Preload recruitable stroke work (PRSW) is another relation derived from variably loaded P/V loops which is more ‘immune’ to loading variability. Stroke work (SW) is the external work performed by the left ventricle. It is calculated as the area of the P/V loop but it can be loosely approximated as the product of stroke volume and mean arterial pressure. During caval occlusion the relationship between SW and V_{ED} (end-diastolic volume) is highly linear and insensitive to afterload in the physiological range (Feneley, 1992). The slope of the SW-V_{ED} relationship (denoted as M_w) is a measure of myocardial contractility which has less variability than Ees. However, in a study in patients, both the ESPV and SW-V_{ED} relationships showed high and comparable linearity and both responded to dobutamine by marked increases in Ees and M_w respectively (Feneley, 1992). When the same concepts were applied to the right ventricle, it was noticed that the RV P/V loop is triangular in shape, unlike the familiar square or rectangle obtained from the LV, this being a reflection of the different loading conditions of the two ventricles (Fig. 2.5.2) (Redington, 1988). The method was subsequently well validated for the RV and found to be useful in many applications in adults and children (White, 2000).

Pressure-volume recordings are clinically obtained with the conductance catheter. The conductance catheter has multiple electrodes (usually 8 or 10, equally spaced), with an incorporated micromanometer. The catheter is positioned such that it traverses the long axis of the ventricle. An alternating 30 μA, 20 KHz current is passed across the ventricular cavity from the proximal to the distal electrode. The conductance (the inverse of resistance) between each pair of intra-ventricular electrodes is calculated. Conductances from the electrode pairs are summed and converted to volume using a signal conditioner. The measured volume has to be corrected for parallel conductance, i.e. the fact that some of the induced current flows outside the blood in the ventricular cavity (ventricular wall, adjacent ventricle, pericardium) (Baan, 1992). The left ventricular volume at time t corrected for parallel conductance is:
\[ V(t) = \left(1/\alpha\right)(V_m(t) - \alpha V_c) \]

where \( V_m(t) \) is the uncorrected volume of the ventricle, \( V_c \) is the volume offset from parallel conductances and \( \alpha \) is a correction factor (calculated by comparing the stroke volume measured by the conductance catheter with that derived from thermodilution catheters).

They are in turn given by

\[ V_m(t) = L^2 \rho G_m(t), \]

and

\[ V_c = L^2 \rho G_\rho \]

where \( G \) is the sum of measured conductances, \( L \) is the inter-electrode distance and \( \rho \) is the blood resistivity (measured before each acquisition of pressure volume data).
Figure 2.5.2  Pressure-volume loop recordings from the heart of a brain-stem dead patient. Note the typical right ventricular and left ventricular morphology of the pressure-volume relationships. With the addition of stress testing (e.g. by dopamine infusion, not shown in the figure) the contractile reserve can also be evaluated. (courtesy of Drs D K Satchithananda and P A White).
Figure 2.1.3  The energy transducing myocardial machinery (adapted from Vogt, 1999).
CHAPTER 3

METHODS

This chapter outlines the clinical and laboratory methods used in the current project.

3.1 PATIENT SELECTION

Study design

The project objectives listed in section 1.4 were prospectively evaluated in a clinical study which aimed to enrol consecutive donors and recipients. The recruitment period was from 1 December 1999 to 1 June 2001. Our research protocol was approved by the Huntingdon Local Research Ethics Committee (LREC ref. no. 99/362). The transplant recipient agreed to performance of measurements during the donor and the recipient operation, and to follow up studies where appropriate. The recipients read the information sheet whilst still on the transplant waiting list. More details on the sequence of measurements are given in section 3.3. Our research protocol was also presented to the local and regional donor co-ordinators. In spite of the absence of guidelines from the British Transplant Society, in all cases we sought consent for the study from the donor family, via the donor co-ordinator. Special attention was paid to patient confidentiality. Throughout the study we insured that tissue collection conformed to accepted models of ethical research (Stone, 2000; Satchithananda, 2000; Ezekiel, 2000).

Inclusion criteria

(a) The intraoperative study
- All heart and heart-lung transplants in which the donor and recipient patients gave consent. Donor studies were done in all consented cases where possible, irrespective of subsequent use of the organs for transplantation. In this way information was acquired about substandard donor hearts. Potential domino donors in which no recipient was identified for the domino heart were included where possible.

(b) The postoperative study
- All heart transplants in which the donor and recipient patients gave consent (e.g. some recipients consented only to the intraoperative study).
All imported hearts (i.e. retrieved by other centres) in which the recipient had consented to the study were included.

**Exclusion criteria**

- Donor hearts for which the donor families did not give consent for research.
- Irritable hearts prone to arrhythmia and hypotension, unstable donors in general where any delay would jeopardise safe procurement of other solid organs. In such cases incomplete sets of results were obtained.
- Technically difficult recipient surgery (redo operations, prolonged ischaemic time, bleeding and any high-risk cases in general).
- Heart-lung recipients - intraoperative (for the conductance catheter only) due to uncontrollable variables introduced by the pulmonary circulation.
- Heart-lung recipients - postoperative (for the conductance catheter and the biopsy study) due to the absence of routine postoperative right heart catheterisation.
- Exported hearts - only the conductance catheter was performed selectively, as other transplant centres and their recipients would be unaware of our research protocol.

Sixty-nine donor hearts were studied in total (including 6 domino, see below). These organs provided tissue material for the results presented in chapters 4-6.

**Controls**

In order to evaluate the influence of brain death, we included two groups of controls. The first was a domino group, in which live patients receiving heart-lung transplants were donating their own hearts onwards for cardiac transplantation. In addition, a second group of patients undergoing elective non-transplant cardiac surgery were included as baseline controls.

The first control group (domino) was formed of 6 patients with cystic fibrosis and a mean age of 24 years. In 3 of the cases suitable recipients were identified and domino donation proceeded at Papworth Hospital. These hearts had serial biopsies during the operation and afterwards, for rejection surveillance. The other 3 patients did not become domino donors because of lack of suitable recipients. However we obtained permission to biopsy their native heart before explantation for the purpose of the study.
The second group was formed of 9 patients who agreed to donate right atrial tissue at the time of their coronary revascularisation in 1999. The mean age was 59 years (range 45-74) and none of the patients had congestive cardiac failure. Biopsies were processed after following the appropriate protocols of the Papworth Hospital Tissue Bank.

3.2 SURGICAL MANAGEMENT AND CARDIAC SAMPLING

Donor management

In the Papworth regional retrieval zone the donor coordinator usually initiates the hormonal substitution treatment. In the absence of absolute contraindications to organ donation the Papworth organ retrieval team will then travel to the regional donor hospital and perform a comprehensive donor assessment. The team also comprises an anaesthetist, primarily in charge of haemodynamic management, and a technician/perfusionist who assists with monitoring, intravenous infusions, plegic solutions etc. The author was also part of the retrieval team for all the donor cases enrolled in this study and assessed regionally.

<table>
<thead>
<tr>
<th>Medication</th>
<th>Bolus</th>
<th>Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tri-iodothyronine</td>
<td>4 µg followed by 3 µg/h</td>
<td></td>
</tr>
<tr>
<td>ADH</td>
<td>1 unit followed by 1-4 units/h</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>Infusion to maintain normal blood glucose (minimum of 1 unit/h)</td>
<td></td>
</tr>
</tbody>
</table>

A dose of 500 mg methylprednisolone was also given routinely during the donor operation. Before taking blood samples for culture antibiotic prophylaxis was administered: vancomycin 1 g and meropenem 1g.

The donor infusions and the inotropes/venesction were titrated to achieve the following haemodynamic targets:
CHAPTER 3

<table>
<thead>
<tr>
<th>Measure</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial pressure</td>
<td>&gt; 60 mmHg</td>
</tr>
<tr>
<td>Central venous pressure</td>
<td>&lt; 12 mmHg</td>
</tr>
<tr>
<td>Pulmonary capillary wedge pressure</td>
<td>&lt; 12 mmHg</td>
</tr>
<tr>
<td>Systemic vascular resistance</td>
<td>800-1200 dynes/sec/cm²</td>
</tr>
<tr>
<td>Cardiac index</td>
<td>&gt; 2.4 L/min/m²</td>
</tr>
<tr>
<td>Left ventricular stroke work</td>
<td>&gt; 15 mg-m/beat/m²</td>
</tr>
<tr>
<td>Dopamine infusion</td>
<td>&lt; 10 µg/kg/min</td>
</tr>
</tbody>
</table>

The ventilator was generally set to deliver a high tidal volume of 12-15 ml/kg with a positive end-expiratory pressure of 5 cm H2O. A minimum inspired oxygen concentration (FiO2) compatible with good oxygenation was maintained. The following figures were used as a guide for suitable heart-lung blocks after resuscitation:

- FiO2 60% > 43 kPa
- FiO2 100% > 55 kPa

Before sternotomy the surgeon performed a fiberoptic bronchoscopy. A sterile specimen was retained for microbiology and a set of arterial blood gases was obtained after the procedure. After opening the chest the usual visual and palpatory assessment of the thoracic organs was undertaken. With both pleural spaces widely opened the surgeon gently massaged any atelectatic areas while the anaesthetist was manually inflating the lungs. The SGC and arterial blood gas measurements were repeated once more before stopping the circulation, and on these readings the decision of using the organs was finally based. In terms of cardioplegia we used 1 litre of St Thomas' 2 crystalloid solution infused under pressure in the aortic root. Pneumoplegia was preceded by a prostacyclin infusion directly into the pulmonary artery over 10-15 minutes (Parry, 1999). Its role is to vasodilate the pulmonary bed and so to ensure maximal distribution of the plegic perfusate. The initial dose was 5 ng/kg/min, increased to 20-40 ng/kg/min over 10 minutes, provided there was no significant hypotension or tachycardia. The extracellular-type of pulmonary preservation fluid had the following composition (depending on the patient's weight, exemplified below for a 70-kg subject):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor blood</td>
<td>450 ml</td>
</tr>
<tr>
<td>Citrate-phosphate-dextrose</td>
<td>63 ml</td>
</tr>
<tr>
<td>Ringer’s solution</td>
<td>700 ml</td>
</tr>
</tbody>
</table>
CHAPTER 3

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin 20%</td>
<td>200 ml</td>
</tr>
<tr>
<td>Mannitol 20%</td>
<td>100 ml</td>
</tr>
<tr>
<td>Heparin</td>
<td>10,000 units</td>
</tr>
<tr>
<td>Total volume</td>
<td>1523 ml</td>
</tr>
</tbody>
</table>

Before thoracic organ explantation the anaesthetist withdrew the central catheters. Cardiectomy was then performed in standard fashion (Icenogle, 1989).

**Recipient management**

The donors and recipients were matched for blood group and size (within 10% of height).

**Anaesthesia**

All patients had a double-lumen central line and a Swan Ganz sheath before induction of anaesthesia. Induction was normally carried out with fentanyl and propofol and anaesthesia was generally maintained with propofol at 3.5 mg/kg/h. Antibiotics were in the form of gentamycin 160 mg and flucloxacillin 1 g and a dose of 500 mg methylprednisolone was also administered.

**Cardiopulmonary bypass**

A standard circuit for open heart surgery was used, incorporating a roller pump (Cobe, Gloucester, UK), the Dideco D903 Avant reservoir (Mirandola, Modena, Italy) and membrane oxygenators of different makes. The circuit was primed with 1.15 L of Hartmann's solution, 350 ml 10% mannitol and 5000 IU heparin. Anticoagulation and reversal of anticoagulation were achieved in conventional fashion with bovine lung heparin and protamine sulphate respectively. Aprotinin (bolus of 2,000,000 units) followed by continuous infusion of 500,000 IU/h) was occasionally for heart-lung cases and heart transplant cases with previous sternotomy. Leukocyte filter use was at the discretion of the operating surgeon. Moderate hypothermia was generally employed (32-34°C) and blood pressure while on CPB was maintained with metaraminol. The average bypass temperature and any anti-inflammatory measure (i.e. trasylo, leukocyte filter or both) were subsequent taken into account for data interpretation.
Organ implantation

The heart and heart-lung blocks were implanted with standard techniques described previously (Jamieson, 1989). A double atrial cuff technique was generally used for cardiac transplantation and topical cooling was not normally employed at implantation. The sequence of anastomoses for HTx was generally left atrium, right atrium, pulmonary artery and aorta, with aortic unclamping at the end of implantation. The left heart was decompressed and deaired through the left ventricular apex biopsy site and additionally with the use of a left heart vent placed in the aortic root (14G, Medtronic DLP, Minneapolis, Minnesota). In a stable haemodynamic environment, separation from CPB was routinely performed on infusions of dopamine at 2-5 mg/kg/min and isoprenaline at 0.005-0.01 mcg/kg/min. The isoprenaline infusion was occasionally continued beyond 48 hours in the presence of bradycardia. In addition, it was routinely given on the ward to patients with a transpulmonary gradient >10 mm Hg, who also had a right heart catheterisation at 1 week postoperatively. Due to the invasive nature of our study, we avoided submitting the patients to further unnecessary risks. A Swan-Ganz catheter was not therefore routinely floated in the recipient, but it was duly resorted to in the presence of the slightest clinical suspicion of graft dysfunction to establish the diagnosis and to guide therapy.

Immune suppression

All patients had triple therapy with steroids, cyclosporine and azathioprine. An intravenous infusion of methylprednisolone (500 mg over 30 minutes) was started immediately after reperfusion. Antithymocyte globulin was employed for all heart-lung recipients (1 dose on CPB followed by 2 additional doses on days 1 and 2 postoperatively, depending on the T cell response) and for heart transplant patients with preoperative renal dysfunction (creatinine >150 mmol/l) or in acute renal failure on day 1 postoperatively.

Cardiac sampling

The sequence of biopsies and measurements in a typical case of heart procurement, implantation and follow up is illustrated in fig. 3.2.1. The following biopsies were performed in chronological order:

1 - on assessing the donor heart with the chest open
• biopsies for HEP - RV and LV
2 - after splanchnic dissection, before cross-clamping the aorta
• biopsies for HEP and ECA - RV and LV
3 - on arrival at Papworth, before implantation - marking end of cold ischaemia
• biopsies for HEP - RV and LV
4 - after implantation, before removing the aortic cross-clamp - marking end of warm ischaemia
• biopsies for HEP and ECA - RV and LV
5 - after 10 minutes of reperfusion, with the patient still on cardiopulmonary bypass
• biopsies for HEP and ECA - RV and LV
6, 7, 8 - 1 week, 3 months and 6 months postoperatively, during the routine follow-up right heart catheterisation in HTx recipients
• biopsy for EGA - RV only

Figure 3.2.1 The sequence of intraoperative and postoperative myocardial biopsies. Conductance catheter studies were performed where possible between time points 1 and 2 and after reperfusion.

<table>
<thead>
<tr>
<th>Donor op.</th>
<th>Cold isch. time</th>
<th>Implantation</th>
<th>Reperfusion</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6-8</td>
</tr>
</tbody>
</table>

Intraoperative biopsies 1-5 were obtained by the operating surgeon using Tru-cut Temno biopsy needles size 16G/9cm (Allegiance, Illinois, USA) (fig. 3.2.2). The biopsy site was identical in all cases: at the apex of the left ventricle and on the anterior wall of the right ventricle, avoiding coronary vessels. Prior to sampling, purse strings were placed on the biopsy site using 5-0 prolene sutures with pericardial pledgets. Any amount of bleeding, usually minimal, was well controlled by snuggers applied to the sutures. The purse string was tied at the end of the sampling sequence.

The ECA biopsy was taken first each time and placed on a sterile piece of cardboard. Outside the sterile operative field the biopsy was placed in a formalin container for transport to the pathology laboratory. The HEP biopsy was taken next and within 10 seconds the biopsy needle was immersed in a liquid nitrogen flask on a side table. Two to three minutes after the freezing, the tissue on the needle was transferred into pre-labelled cryotubes which were then returned to the
storage flask. Heart transplant recipients also had endomyocardial right ventricular ECA biopsies. This was done in conventional fashion with the Caves bioptome during routine right heart catheterisation at 1 week, 3 months and 6 months postoperatively.

Figure 3.2.2 The 16 G Temno trucut needle with a core of myocardial tissue.

3.3 CONDUCTANCE CATHETER TECHNIQUE

Whenever consent for research was available in an organ destined for a recipient at our centre, a RV conductance catheter study was performed. Ventricular functional assessment was only carried out in donors with stable circulation, once catecholamine support was minimised and loading optimised. Recipient studies were carried out after separation from cardiopulmonary bypass. They were performed in selected cases who were sufficiently stable for an invasive study. Recipients of heart-lung blocks were excluded due to the unknown variables introduced by the

1 Courtesy of Dr DK Satchithananda. For abbreviations see also section 2.5
donor lungs at reperfusion. All studies were performed under general anaesthesia, in the expiratory phase of respiration. After the initial evaluation, dopamine at 5mcg/kg/min was used to test the contractile reserve.

A 7 Fr 8-electrode conductance catheter with incorporated solid state pressure transducer (Millar Instruments Inc., Houston, Texas) was inserted via a small introducer sheath in the RV infundibulum, below the level of the pulmonary valve. Catheters used had total inter-electrode distances of between 5-7 cm. Individual catheters were sized to fit the maximum intra-ventricular cavity using external RV dimensions as a guide. Optimal placement of the catheters was determined by the presence of co-ordinate pressure and segmental volume signals with appropriate phase relationships. The resultant pressure volume loops were recorded at steady state, during parallel conductance determination, and during preload variation. Preload variation was achieved by transient inferior vena caval snaring over at least 5 consecutive cardiac cycles. All results are averaged from at least 2 measurements. The conductance signal was generated and processed in a Sigma-5 DF unit (Leycom, Leiden, Netherlands). The amplified pressure signal, the derived volume and the ECG were fed directly into a Viking computer. The volume and pressure signals were sampled at 250 Hz and transferred through a 12 bit 16 channel A to D converter into custom-made dedicated software. The parallel conductance was calculated using the saline injection technique described by Baan (Baan, 1992). Typical P/V loops obtained in our patients are shown in Fig. 2.5.2.

Calculation of $\alpha$, $V_c$ and systolic functional indices were performed offline. $V_c$ was calculated from intersection of the end systolic and the end diastolic points following injection of 7ml of 10% saline at steady state conditions. Diastole was defined by the R wave of the ECG and systole was defined by the maximum pressure/volume for each cardiac cycle. ESPVR was produced by linear regression of consecutive systolic points in families of pressure volume loops obtained during caval occlusion. The PRSW slope was calculated from a plot of SW against $V_{ed}$ for these individual loops.

3.4 HIGH ENERGY PHOSPHATES ANALYSIS

The enzymatic method employed involved construction of a calibration curve using known amounts of adenine nucleotides (Spielmann 1981). Provided the calibration was satisfactory, the
assay proceeded on the same day to analysis of biopsy samples. The samples were ground in liquid nitrogen to which 1 mL of 5% perchloric acid was added for extraction. After thawing, the mixture was homogenised and then centrifuged. The supernatant was used for determination of HEP in the nanomolar range. The protein pellet was used to measure the amount of non-collagen protein in the biopsy sample. Results are also available as absolute values in nanomoles of adenine nucleotides per milligram of protein.

**Determination of high energy phosphates**

**Stock solutions**

**TRIS-EDTA buffer**: 0.1 M TRIS-base (Sigma, Steinhelm, Germany) (6.055 g), 2 mM EDTA dipotassium salt (Sigma, Steinhelm, Germany) (372.2 mg), dissolved in 500 mL of double-distilled deionised water.

**Reaction buffer**: 0.1 M TRIS-base (6.055 g), 2 mM EDTA dipotassium salt (372.2 mg), 9 mM MgCl₂ (Sigma, St Louis, MO) (914.9 mg), 5 mM KCl (Sigma, St Louis, MO) (186.4 mg), 0.3 mM phosphoenolpyruvate (Sigma, Gillingham, Dorset, UK) (30.9 mg), dissolved in 500 mL of double-distilled deionised water.

The buffer solutions are then titrated with glacial acetic acid at 25°C to a pH of 7.80. For TRIS buffers this corresponds to a pH of 7.6 at 37°C, which is the temperature of the pyruvate kinase and myokinase reactions. Buffers are made fresh every week and are kept overnight at 4°C to avoid bacterial contamination.

**Standard HEP solution**: 1 mM ATP (55.11 mg), 1mM ADP (42.72 mg), 1 mM AMP (49.92 mg) (all from Sigma, Steinhelm, Germany), dissolved in 100 mL TRIS-EDTA buffer. Aliquots of 1mL are made and stored at -70°C for up to 3 months until required for analysis.

**Enzyme preparation**

Enzymes of the purest specification were used from Sigma-Aldrich (Steinhelm, Germany).
Pyruvate kinase (PK) suspension (type II, from rabbit muscle) with 4.6 mg/mL and 540 u/mg, stored at 4°C, was used. Aliquots of 40 μL were spun at 8,000 rpm for 5 minutes on the day of the assay. Each pellet was re-dissolved in 1 mL and 0.9 mL of reaction buffer, for ADP and AMP measurement respectively, the final concentration of the enzyme corresponding to 99.3 units/mL.

Myokinase (MK) (from chicken muscle, 2500 units, 2290 units/mg) came in solid form. After resuspension in 1 mL of reaction buffer, aliquots of 0.1 mL were made and stored at less than 0°C for up to two weeks. One aliquot of 0.1 mL was added to the 0.9 mL tube of PK, the final concentration of PK and MK in this combination being 99.3 units/mL and 250 units/mL respectively.

The ATP assay mix comes in lyophilised form and contains firefly luciferase, luciferin, MgSO₄, EDTA, DTT and BSA in a Tris buffer (Sigma, Steinhelm, Germany). The mixture was reconstituted with 5 mL of double-distilled deionised water filtered through a bacterial filter to avoid contamination. Aliquots of 0.5 mL and 0.8 mL were made and stored at less than 0°C. The Eppendorf tubes were kept wrapped in aluminium foil on ice at all stages to avoid light desensitisation.

Before incubation with the substrate, the PK and MK were dialysed at 4°C for 60 minutes against the reaction buffer. This was undertaken in order to eliminate the remaining ammonium sulphate from the PK solution. To optimally preserve the enzyme activity and reduce the effect of inadvertent bacterial contamination all enzymes were kept in Eppendorf tubes on ice at all stages of the assay.

Calibration

Precautions were taken to treat the samples and the standards identically. The calibration steps were:

- Serial dilutions of the HEP standard solution at 1, 5, 10 and 30 μM. These were made in 1 mL of with TRIS buffer and a blank with TRIS only was also used.
- Addition of 1 mL of 7.1 % perchloric acid solution to all tubes.
- Neutralisation with predetermined amount of 0.75 M KOH solution (usually between 0.9 and 0.92 mL KOH per millilitre of PCA). This step brings the pH back to 7.6 at 37°C.
Centrifugation at 12,000 rpm for 5 min.

Further 1:10 dilution in TRIS buffer for all standards. This step helps to calibrate over a wide range of dilutions while avoiding overload on the luminometer scale.

Incubation for all standard dilutions

ATP: 10 μL standard + 10 μL reaction buffer

ADP: 10 μL standard + 10 μL PK

AMP: 10 μL standard + 10 μL PK-MK

All measurements were done in triplicate. The reaction was carried out in LP3 luminometry tubes, incubated in a water bath at 37°C for 30 minutes.

Addition of 40 μL of TRIS buffer to each tube to a final volume of 60 μL.

Addition of 15 μL of luciferase to each tube, vortex mixture for oxygenation and immediate reading on the luminometer (LKB-Wallac Model 1251, Lorton, VA 22079). The final ratio of luciferase to volume of reaction was 1:5, as recommended by the manufacturer.

The results of a typical calibration curve are shown in fig. 3.4.1. Linear correlation was considered satisfactory when R^2 > 0.985. Enzyme activity was acceptable if ADP:ATP ratio was above 0.7:1 in the HEP stock solution.

Sample preparation

At 4°C in the cold room the samples were ground in liquid nitrogen in separate ceramic mortars. One millilitre of 7.1% PCA was added to each mortar before the evaporation of all liquid nitrogen. The mixture was allowed to thaw and was immediately transferred into Eppendorf tubes. The HEP extraction continued on the spinning wheel for 30 minutes. The samples were then centrifuged for 5 minutes at 12,000 rpm. The supernatant was used for the HEP assay and the pellet was re-dissolved in 1M NaOH for subsequent protein assay. One millilitre of TRIS buffer was added to all samples and steps 3-8 of the calibration process were repeated.

Calculation

This has to take into account the fact that ADP and AMP were not measured directly by this assay, but after conversion into ATP in the presence of PK and PK-MK respectively. In other words, the luminometer captures photons resulting from ATP only. The total amount of ATP in a given reaction tube results from all three adenine nucleotides, depending on the enzyme mixture.
used. The method of Spielmann and colleagues emphasises this important point. Their calibration graph has three different scales on the x axis, corresponding to the ATP measured in the three types of reactions (figure 3.4.2). A typical example of calculation from a biopsy of rat myocardium is presented in figures 3.4.3 and 3.4.4.

**Figure 3.4.1** An example of a curve obtained in the author’s experiments (read in conjunction with figures 3.4.2 and 3.4.3).

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>Average</th>
<th>Average-0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.1</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>1</td>
<td>120.5</td>
<td>112</td>
<td>107.4</td>
</tr>
<tr>
<td>5</td>
<td>562.4</td>
<td>568.8</td>
<td>570.4</td>
</tr>
<tr>
<td>10</td>
<td>1054</td>
<td>1079</td>
<td>1078</td>
</tr>
<tr>
<td>30</td>
<td>3369</td>
<td>3336</td>
<td>3350</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>ADP</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
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<td>5</td>
<td>4.6</td>
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<td>1</td>
<td>228.7</td>
<td>265.5</td>
<td>240.2</td>
</tr>
<tr>
<td>5</td>
<td>1189</td>
<td>1190</td>
<td>1213</td>
</tr>
<tr>
<td>10</td>
<td>2346</td>
<td>2159</td>
<td>2214</td>
</tr>
<tr>
<td>30</td>
<td>6662</td>
<td>6311</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>AMP</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.1</td>
<td>10.8</td>
<td>10.5</td>
</tr>
<tr>
<td>1</td>
<td>351.9</td>
<td>323.9</td>
<td>391.4</td>
</tr>
<tr>
<td>5</td>
<td>1588</td>
<td>1649</td>
<td>1516</td>
</tr>
<tr>
<td>10</td>
<td>3142</td>
<td>3126</td>
<td>3001</td>
</tr>
<tr>
<td>23</td>
<td>9034</td>
<td>9220</td>
<td>9129</td>
</tr>
</tbody>
</table>

The three curves correspond to:
- a) standard + reaction buffer for determination of ATP
- b) standard + PK for determination of ATP resulting from ATP and ADP
- c) standard + PK-MK for determination of ATP resulting from ATP, ADP and AMP
The PK and MK are rarely working 100% and so the ATP measured in the sets of tubes a, b and c is not in the ratio of 1:2:3. To correct for this the ADP:ATP and the AMP:ATP transformation ratios are calculated for each calibration. This is done by dividing the corresponding coefficients of the equations a, b and c. In this case for example

\[ \frac{216.64}{111.56} = 1.94 \]
\[ \frac{303.02}{111.56} = 2.71 \]

These figures are used for 'calibrating' the x axis and for calculating the exact amount of ATP in the sets of tubes a, b and c.

**Figure 3.4.2** The ideal calibration curve, when the enzymes are working 100% (from Spielmann, 1981).

![Ideal Calibration Curve](image)

**Figure 3.4.3** Example of HEP calculation in a sample of rat myocardium.

<table>
<thead>
<tr>
<th></th>
<th>Average</th>
<th>Blank</th>
<th>Average -0</th>
<th>ATP conc.</th>
<th>True conc.</th>
<th>ATP/ADP</th>
<th>EC</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>999.6</td>
<td>1076.3</td>
<td>987.7</td>
<td>1021.2</td>
<td>1.1</td>
<td>1020.1</td>
<td>9.2</td>
</tr>
<tr>
<td>ADP</td>
<td>1454.3</td>
<td>1547.3</td>
<td>1484.3</td>
<td>1495.3</td>
<td>4.6</td>
<td>1490.7</td>
<td>12.93</td>
</tr>
<tr>
<td>AMP</td>
<td>1833.3</td>
<td>1901.4</td>
<td>2093.4</td>
<td>1942.7</td>
<td>10.4</td>
<td>1932.3</td>
<td>16.96</td>
</tr>
<tr>
<td>TAN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16.96</td>
</tr>
</tbody>
</table>

ATP in the ATP tubes is calculated from the 1st equation of the calibration curve as:
ATP (uM) = (1020.1 + 6.749)/111.56 = 9.20

ATP in the ADP tubes is calculated from the 2nd equation of the calibration curve as:
ATP (uM) = 1.94*(1490.7 - 46.053)/216.64 = 12.93

ATP in the AMP tubes is calculated from the 3rd equation of the calibration curve as:
ATP (uM) = 2.71*(1932.3 - 35.339)/303.02 = 16.96

The amount of ADP (uM) is therefore 12.93 - 9.2 = 3.73
and
the amount of AMP (uM) is 16.96 - 12.93 = 4.03

The energy charge is calculated as (ATP + 1/2ADP)/(ATP + ADP + AMP)
All concentrations are in micromoles.

**Figure 3.4.4** Protein assay calibration curve and calculation of HEP absolute values.

<table>
<thead>
<tr>
<th>Conc.</th>
<th>Average</th>
<th>Conc.</th>
<th>Average</th>
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</thead>
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<td>0</td>
<td>722</td>
<td>0</td>
<td>731</td>
</tr>
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<td>936</td>
<td>2</td>
<td>943</td>
</tr>
<tr>
<td>4</td>
<td>1069</td>
<td>4</td>
<td>1091.67</td>
</tr>
<tr>
<td>6</td>
<td>1194</td>
<td>6</td>
<td>1246.67</td>
</tr>
<tr>
<td>8</td>
<td>1372</td>
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<td>1380</td>
</tr>
<tr>
<td>10</td>
<td>1482</td>
<td>10</td>
<td>1488.33</td>
</tr>
<tr>
<td></td>
<td>740</td>
<td>2</td>
<td>943.67</td>
</tr>
<tr>
<td></td>
<td>952</td>
<td>4</td>
<td>1091.67</td>
</tr>
<tr>
<td></td>
<td>1284</td>
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<td>1380</td>
</tr>
<tr>
<td></td>
<td>1497</td>
<td>10</td>
<td>1488.33</td>
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<tr>
<td></td>
<td>731</td>
<td></td>
<td>1488.33</td>
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<tr>
<td></td>
<td>943.67</td>
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<td>1246.67</td>
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<td>1091.67</td>
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<td>1380</td>
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<tr>
<td></td>
<td>1380</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

rat sample 1070 1045 1066 1060.33

5 uL sample were used per cuvette

The protein concentration in the sample is calculated as follows.

From the calibration equation an absorbance of 1060.3 corresponds to 3.83 ug protein in that cuvette.
5 μL rat sample has ... 3.83ug protein
1000 μL (1mL) ... x
x=(1000*3.83)/5=766 ug protein

From the adenine nucleotide assay we know the concentration of ATP (9.2 uM, see fig. 3.3.4)
1 L extract has ... 9.2 uM ATP and ... 766 mg protein
therefore x = 9.2/766=0.01201 uM ATP per mg protein
which is equivalent to 12.01 nM/mg protein
ADP, AMP, and TAN are calculated in exactly similar fashion.

**Determination of non-collagen protein**

After adding NaOH on the protein sample the mixture was homogenised by vortexing followed by sonication. Standard stock solution of bovine serum albumin was made fresh each week in the concentration of 1 mg/mL and further diluted 1:10 in NaOH for the assay. The BioRad reagent was diluted 1:5 with distilled water. The readings were done in triplicate and the contents of the cuvettes were as follows:

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>Albumin 1:10 μL</th>
<th>NaOH 1M (μL)</th>
<th>BioRad (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>100</td>
<td>900</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>80</td>
<td>900</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>60</td>
<td>900</td>
</tr>
<tr>
<td>6</td>
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<td>8</td>
<td>80</td>
<td>20</td>
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<tr>
<td>10</td>
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<td>0</td>
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<tr>
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<td>95</td>
<td>900</td>
</tr>
<tr>
<td>Sample n</td>
<td>5</td>
<td>95</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>
Reproducibility

Rat myocardial tissue was used repeatedly in the pilot phase of the study to determine the optimum conditions for the assay. Five white male Wistar rats were used for procurement of fresh myocardial tissue. The animals were 70 days old and the average weight was 402.8 grams. Humane care of the animals was provided following the requirements of The Animals (Scientific Procedures) Act, 1986. The rats were sacrificed by a rapid intraperitoneal injection of barbiturate (pentobarbitone sodium BP, 400 mg in 2 ml volume). Immediately after cessation of heart beats the chest was opened and the sternal plate removed. A small artery forceps was applied across the atrioventricular groove and the ventricular mass was excised. The ventricles were opened, any remaining blood was washed away with normal saline solution and the myocardial tissue was freeze clamped, all within 2 minutes. The disc of myocardial tissue was fragmented in liquid nitrogen, placed in cryotubes and stored in liquid nitrogen at -196°C for subsequent analysis.

In serial experiments the following sources of error were identified and controlled:

- Micropipette calibration.
- Luminometer calibration.
- Bacterial contamination of buffers and enzymes.
- Degradation of standard HEP solution at temperatures above -50°C.
- Faulty luminometry tubes (e.g. cracked at the bottom).
- Condensation in the luminometry tubes at 37°C in the water bath.
- Luciferase mixture thaw-freeze cycles.
- Loss of neutralisation power of the 0.75M KOH stock solution.
- Sample grinding error.

Separate controlled experiments were therefore done to determine the following:

- Optimal dilutions of standards in terms of sample size and luminometry scale.
- Luminescence decay of samples and standards.
- Optimal ratio of luciferase to final volume of reaction.
- Optimal pH at incubation and variations with temperature.

The pH at incubation was found to be the most critical part of the assay set up. PK and MK work maximally at a pH of 7.60, and become easily inhibited at a different pH, to the extent that there
is positive inhibition of the reaction and the internal reproducibility of triplicates is also lost. Difficulty was initially encountered in providing this milieu for the 20 μL volume of reaction at incubation. A representative experiment to deal with this problem is presented below.

**Experiment to ensure optimal pH at incubation**

Neutralisation of a mixture of PCA and TRIS buffer was undertaken at 37°C with a bigger volume. Seven millilitres of 7.1% PCA were mixed with 7 mL of TRIS buffer and warmed up to 40°C in an incubator. Rapid titration with warm 1M KOH solution was then undertaken using a Hanna HI-9321 pH bench meter with dual TRIS electrode and temperature probe. The volume of KOH required for neutralisation is expressed as percentage of the PCA/TRIS volume.

<table>
<thead>
<tr>
<th>Volume of 1M KOH (%)</th>
<th>pH</th>
<th>t°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>4.24</td>
<td>39.2</td>
</tr>
<tr>
<td>65</td>
<td>6.02</td>
<td>38.4</td>
</tr>
<tr>
<td>66</td>
<td>6.76</td>
<td>37.8</td>
</tr>
<tr>
<td>67</td>
<td>7.15</td>
<td>37.3</td>
</tr>
<tr>
<td>68</td>
<td>7.42</td>
<td>36.9</td>
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<tr>
<td>69</td>
<td>7.62</td>
<td>36.4</td>
</tr>
<tr>
<td>70</td>
<td>7.78</td>
<td>35.9</td>
</tr>
</tbody>
</table>

It can be seen how the volume of KOH and the temperature interact significantly to produce wide variations in the pH. The volume of KOH required to produce an ideal pH at 37°C is 69%. This volume was subsequently used for the experiment in the microlitre range. This experiment was repeated once a week and each time one of the reagents was made fresh. 1M KOH was replaced with 0.75M KOH to avoid saturating the solution. Subsequently it was found that titration at room temperature (25°C) based on pH conversion tables for TRIS buffers (Sigma) is sufficiently accurate.

**Results on rat myocardium**

Results on non-ischaemic rat myocardium are presented in table 3.4.1 The values are slightly lower than previously published results on rat myocardium using enzymatic methods (Larrieu, 1987; Tan, 1993; Toshima, 1992). The 0.70 value for energy charge is subnormal, but still
compatible with viable tissue. The most likely explanation for this is related to difficulties in grinding large myocardial fragments and non-uniform adenine nucleotide extraction with PCA. The coefficient of variation of less than 10% for EC shows good reproducibility of the enzymatic assay. The much higher coefficient of variation (45%) obtained for the absolute value of ATP (nM/mg protein) reflects the additional error introduced by a second assay to determine the tissue reference base. As a result, the preferred method of expressing adenine nucleotide content in this dissertation is by ATP/ADP ratio and EC.

Results on human myocardium

At the end of the assay learning curve Dr. T. Kealey provided unused myocardial biopsies from human donor hearts transplanted at Papworth Hospital in 1997. These were transmural biopsies obtained from the left ventricular apex with a Temno needle. The average values for ATP and TAN of 18.9 and 27.2 nM/mg protein respectively fall within the range previously reported by this laboratory and by other groups using enzymatic methods (Flameng, 1986; Alkhulaifi, 1994).
Table 3.4.1  Serial experiments on rat myocardium and coefficient of variability of the assay.

<table>
<thead>
<tr>
<th>Rat myocardium procured on 08/05/00</th>
<th>Experiments performed in July-August 2000</th>
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<table>
<thead>
<tr>
<th></th>
<th>EC</th>
<th>AVE</th>
<th>STDEV</th>
<th>SEM</th>
<th>CV</th>
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<tr>
<td>rat 1</td>
<td>0.657</td>
<td>0.75 e</td>
<td>0.65</td>
<td>0.72</td>
<td>0.59</td>
</tr>
<tr>
<td>rat 2</td>
<td>0.725</td>
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<td>0.76</td>
<td>0.69</td>
<td>0.65</td>
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<tr>
<td>rat 3</td>
<td>0.67</td>
<td>0.752</td>
<td>0.79</td>
<td>0.69</td>
<td>0.72</td>
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<tr>
<td>rat 4</td>
<td>0.649</td>
<td>0.639</td>
<td>0.65</td>
<td>0.703</td>
<td>0.74</td>
</tr>
<tr>
<td>rat 5</td>
<td>0.736</td>
<td>0.5829</td>
<td>0.56 e</td>
<td>0.71</td>
<td>0.71</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ATP nM/mg prot</th>
<th>AVE</th>
<th>STDEV</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat 1</td>
<td>10.57</td>
<td>7.49 e</td>
<td>17.4</td>
</tr>
<tr>
<td>rat 2</td>
<td>19.62</td>
<td>7.96</td>
<td>14.2</td>
</tr>
<tr>
<td>rat 3</td>
<td>12.06</td>
<td>5.79</td>
<td>13.5</td>
</tr>
<tr>
<td>rat 4</td>
<td>18.79</td>
<td>10.03</td>
<td>4.53</td>
</tr>
<tr>
<td>rat 5</td>
<td>12.05</td>
<td>6.69</td>
<td>4.44</td>
</tr>
</tbody>
</table>

Abbreviations:
- EC = energy charge
- AVE = mean
- STDEV = standard deviation
- SEM = standard error of the mean
- CV = coefficient of variation
- e = technical error, eliminated before analysis
- n/a = not available (not done), rat myocardium replaced with human myocardium on these occasions

CV was calculated as (STDEV / AVE)*100

3.5  ENDOTHELIAL CELL ACTIVATION ANALYSIS

Immunohistochemistry protocols

The following steps describe the immunohistochemistry protocol for paraffin-processed specimens.
- Dewax sections and take to water through graded alcohols.
- Perform antigen retrieval if needed (see below).
- Equilibrate slides in phosphate buffered saline (PBS) at pH=7.60 buffer for 5 min.
- Hydrogen peroxide for 5 min to block endogenous peroxidase.
- Wash in running tap water for 5 min.
- Wash in PBS 3 x 5 min.
- Incubate in normal serum for 10 min.
- Drain normal serum from sections and apply primary antibody for 30 min.
- Wash in PBS 3 x 5 min.
- Incubate in secondary antibody for 30 min.
- Wash in PBS 3 x 5 min.
- Incubate with ABC complex for 30 min.
- Wash in PBS 3 x 5 min.
- Apply DAB chromogen for 5 min.
- Wash sections well in running tap water.
- Counterstain with Carazzi's haematoxylin for 1 min.
- Differentiate in tap water for 5 min.
- Dehydrate, clear and mount sections in DPX or other permanent mountant.

This ABC complex immunocytochemistry technique was automated using the Dako Techmate 500 X-Y autostainer (Dako, Glostrup, Denmark), which is based on capillary action. All reagents but the PBS (Oxoid, Oxford, UK) are a component part of the Techmate kit.

**Antigen retrieval**

*Enzyme digestion antigen retrieval*

Prior to the standard protocol the sections were incubated with Dako Proteinase K for 10 min. In certain cases this was followed by microwaving (see table 3.5.1).

*Citrate buffer antigen retrieval*

Eight hundred mL of citrate buffer were added to a microwave dish and the pH was adjusted to 6.0. The slides were placed into this solution and allowed to equilibrate, after which microwaving (2 cycles of 5 mins.) and staining proceeded in standard fashion.
**EDTA buffer antigen retrieval**

Prior to microwaving (2 cycles of 5 mins.) and staining the slides were placed for 5 min. in an EDTA-based unmasking solution with pH=8.0.

**Sodium bicarbonate antigen retrieval**

The slides were allowed to equilibrate in a solution of sodium hydrogen bicarbonate and Tween 20 detergent (Merck, Poole, UK) at pH=8.0. Microwaving (2 cycles of 2 1/2 mins.) and staining followed as per routine. A 750-watt scientific microwave was used (Euroserv, Gloucester, UK).

**Reproducibility and expression of results**

Sections were initially stained with haematoxillin and eosin and examined for their general histological appearance. To ensure that the endothelium is intact even in the absence of ischaemic changes, all biopsies were stained with CD31, a marker of endothelial integrity. CD31 was also used to count all the vessels on individual sections. This assisted in evaluating P-sel, the adhesion molecule with the highest expression in our study (for a description of the principle see Tanio, 1994). The biopsies stained with monoclonal antibodies were then examined by an independent observer who was blinded to the patient's identity and to the side and timing of the biopsy. A second independent histologist randomly checked slides for all tissue markers to ensure inter-observer agreement, which was excellent.

For individual vessels the staining for adhesion molecules was defined as present or absent. Early in the study it was noticed that all biopsies tend to have more prevalent staining on the non-muscularised post-capillary venules. When staining of the non-muscularised vessels was very widespread, there was a general tendency to have a parallel increase in the staining of muscularised pre-capillary arterioles. All muscularised and non-muscularised vessels on all sections were counted and labelled as positive or negative. Since the number of muscularised vessels is relatively small on most sections obtained with the 16G Trucut needle, it was decided not to include these vessels in the final count. Results for adhesion molecules are therefore expressed as percentage of non-muscularised positive vessels. Thrombomodulin (Thr) expression was scored on a scale ranging from 0 to 5, with 5 representing staining of all microcapillaries and 0 being complete Thr depletion. In all studies we encountered Thr expression ranging from 0 to 5. In the presence of positive tissue controls, which showed that the monoclonal antibody was working well, we did not study a parallel patient control group for Thr. The stress markers (iNOS, ...
hsp70, Bcl-2 and Bax) were expressed as either present or absent on myocytes, muscularised vessels and non-muscularised vessels. In addition, the intensity of myocyte staining was expressed on a scale ranging from 0 (absent) to 3 (maximum).

3.6 STATISTICS

Definition of variables

For all patients studied in this project donor organ failure in the recipient was defined as follows. Right heart failure was diagnosed if all the conditions below were satisfied: RA > 15 mm Hg, CI < 2.5 L/min/m², PCWP < 10 mm Hg, urine output < 1 mL/kg/h and the need for special therapies (one or more of the following: IABP, pulmonary vasodilators, 3 or more inotropes). Global graft failure was diagnosed in the presence of borderline haemodynamics (CI < 2.5 L/min/m², PCWP > 15 mm Hg) and the need for special therapies as defined above. Rejection was defined according to the accepted criteria of the International Society of Heart and Lung Transplantation (Billingham, 1990).

Statistical methods

Summarising data (descriptive statistics)

Quantitative (continuous) variables are expressed using the mean and standard deviation. When the distribution is skewed the median and interquartile range are used. Qualitative (categorical) data are expressed as percentages or rates. When the assumptions of the t test and the central limit theorem were violated data transformations (e.g logarithmic or geometric) were performed. Non-parametric tests (which do not make any assumptions about the shape of the distribution) were used where appropriate.

Analysing the data (inferential statistics)

Statistical testing was performed using the SPSS 9.0 software (Chicago, Illinois). The tests used for a particular comparison are described with each table/graph as appropriate. The critical value for statistical significance was defined as a two-sided p<0.05. Exact p values are provided for
each comparison, but confidence intervals are also given whenever possible. Details of different types of analysis of variance (ANOVA) and post hoc comparisons are provided where necessary.
### Table 3.5.1  Monoclonal antibodies specifications and technical details.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Antibody type</th>
<th>Antibody source</th>
<th>Antigen retrieval</th>
<th>Dilution used</th>
<th>Company</th>
<th>Clone no.</th>
<th>Positive control</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombomodulin</td>
<td>monoclonal</td>
<td>mouse</td>
<td>Not required</td>
<td>1:200</td>
<td>DAKO, Ely, Uk</td>
<td>M 0617</td>
<td>Normal lung</td>
<td>yes</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>monoclonal</td>
<td>mouse</td>
<td>MW citrate + enzyme</td>
<td>1:10</td>
<td>Immunotech (Coulter), BP 177-13276, Marseille, France</td>
<td>1244</td>
<td>Tonsil</td>
<td>yes</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>monoclonal</td>
<td>mouse</td>
<td>MW EDTA + enzyme</td>
<td>1:25</td>
<td>PharMingen (Becton Dickinson), <a href="http://www.pharmingen.com">www.pharmingen.com</a></td>
<td>65111A</td>
<td>Tonsil</td>
<td>yes</td>
</tr>
<tr>
<td>P-selectin (CD62P)</td>
<td>polyclonal</td>
<td>rabbit</td>
<td>MW sodium bicarbonate + enzyme</td>
<td>1:100</td>
<td>PharMingen (Becton Dickinson), <a href="http://www.pharmingen.com">www.pharmingen.com</a></td>
<td>09361A</td>
<td>Tonsil</td>
<td>yes</td>
</tr>
<tr>
<td>CD31 (PECAM-1)</td>
<td>monoclonal</td>
<td>mouse</td>
<td>MW sodium bicarbonate + enzyme</td>
<td>1:30</td>
<td>DAKO, Ely, Uk</td>
<td>JC 70</td>
<td>Placenta</td>
<td>yes</td>
</tr>
<tr>
<td>E-selectin</td>
<td>monoclonal</td>
<td>mouse</td>
<td>MW EDTA + enzyme</td>
<td>1:20</td>
<td>Vector, Peterborough, UK</td>
<td>NCL-ESEL</td>
<td>Tonsil</td>
<td>yes</td>
</tr>
<tr>
<td>Hsp70</td>
<td>monoclonal</td>
<td>mouse</td>
<td>MW citrate + enzyme</td>
<td>1:50</td>
<td>Labvision, Peterborough, Uk</td>
<td>#MO931</td>
<td>Tonsil</td>
<td>yes</td>
</tr>
<tr>
<td>Bax</td>
<td>monoclonal</td>
<td>mouse</td>
<td>MW EDTA+ enzyme</td>
<td>1:50</td>
<td>TCS Biologicals Ltd, Bucks, Uk</td>
<td>Bax-1</td>
<td>Tonsil</td>
<td>yes</td>
</tr>
<tr>
<td>iNOS</td>
<td>monoclonal</td>
<td>mouse</td>
<td>MW EDTA+ enzyme</td>
<td>1:400</td>
<td>Labvision, Peterborough, UK</td>
<td>#MO621</td>
<td>Normal lung</td>
<td>yes</td>
</tr>
</tbody>
</table>

Negative controls were performed by omission of the primary antibody. This is effective if the antibody has been characterised by extensive titre runs and numerous positive and negative controls whilst setting the antibody up.
CHAPTER 4
HIGH-ENERGY PHOSPHATES ANALYSIS

This chapter presents and discusses results on the energy metabolism in clinical cardiac transplantation.

4.1 PATIENTS AND METHODS

Fifty-two hearts were studied with serial biopsies from both ventricles at the following time points:

1 – on initial assessment in the donor
2 – before explantation
3 – at the end of cold ischaemic time
4 – at the end of warm ischaemic time
5 – after 10 minutes of reperfusion

The biopsies are named R1, L1, R2, L2 etc. and details of the organs studied are given in the flow chart below.

Flow diagram chapter 4 The patients enrolled in the biochemical study.

55 patients
  52 heart donors  3 routine AVR patients (controls)
   6 unused hearts  46 transplantable hearts (3 domino)
   2 poor function  4 CAD
                     43 transplanted (1 domino)  3 not used (2 domino)
                     30 HTx¹  13 HL/Tx

¹ HTx: Heart Transplantation
AVR, aortic valve replacement; CAD coronary artery disease; HTx, heart transplant; HLTx, heart-lung transplant.

† because of lack of suitable recipient.

‡ 29 patients followed-up, 1 recipient excluded as a result of refusing to consent to invasive studies during organ implantation (donor data were however available in this case).

There were 6 unusable hearts, 2 of which were turned down on grounds of poor function and the other 4 with palpable coronary artery disease. Twenty hearts in which we did not have access to the donor, either because the organ was imported (n=8) or consent for research in the donor was not obtained (n=12), had recipient studies only (from biopsy 3 onwards). Of 26 usable hearts in which we did donor studies 3 were domino, but 2 of them were not further transplanted because of lack of suitable recipients. Another 2 usable hearts from brain-dead donors were not followed up because of lack of suitable recipient and no consent for research in the recipient respectively. A total of 42 transplants were therefore followed up (29 heart and 13 heart-lung transplants). In order to facilitate a comparison of brain vs. live donors, 3 patients undergoing routine aortic valve replacement were also included as live controls. Seventeen donors and 5 recipients also had an assessment with the RV conductance catheter. The mean age of patients donating usable hearts (n=46) was 37.9±10.9 years. The mean ischaemic time for the transplants performed (n=42) was 184.2±28.5 minutes.

Results are shown as the ATP/ADP ratio and EC. These ratios appear to be an excellent and reproducible descriptor of the biochemical energy reserve in the myocardium (see sections 2.1 and 2.2). The distribution for ATP/ADP was positively skewed and for EC was negatively skewed. After logarithmic transformation of ATP/ADP and 1-EC, a normal distribution was obtained and the t test for paired and unpaired observations was applied as appropriate. Results are expressed as geometric means with 95% confidence intervals. Discrete variables were compared with Fisher’s exact test. To reduce bias from repeated measurements in the same patients, mixed effects ANOVA was performed with stage as a fixed effect and patient and repeated measures contrast as random effects. The donor organ failure sub-analysis was performed with fixed effects one-way ANOVA and Wilcoxon’s test. For all ANOVAs, Scheffé’s post-hoc correction was applied to adjust for multiple testing. A p value of less than 0.05 was considered statistically significant.
4.2 RESULTS

Brain dead vs. live patients

A comparison was made between 23 hearts from brain-dead donors used for transplantation and 6 hearts from live patients (3 domino donors and 3 controls undergoing elective aortic valve replacement). There was no statistically significant difference between biopsies from brain dead and live patients (figure 4.2.1).

Trend over time and RV vs. LV

Only hearts used for transplantation from brain-dead donors were included in this analysis (n=41). The values for ATP/ADP and EC are shown in table 4.2.1 and figures 4.2.2 and 4.2.3. There were no statistically significant differences between RV and LV at any time point, but important time-dependent changes were seen in both ventricles. Energy stores were generally preserved until the end of cold ischaemia. The RV however seems predisposed to a drop in HEP levels during the procurement operation with subsequent replenishment during cold storage.

An important decrease in energy stores for both RV and LV was observed during warm ischaemia. Between R3 and R4 for example the ATP/ADP fell from a mean of 5.27 to 2.57 (p<0.001) and the EC fell from 0.88 to 0.82 (p<0.001). Length of warm ischaemic time did not correlate with percentage fall in energy stores between time points 3 and 4 (r=0.14, p=0.24 for ATP/ADP, and r=0.18, p=0.14 for EC). Similarly, warm ischaemic time did not correlate with percentage recovery of energy stores at reperfusion (between biopsies 4 and 5) in either the RV or the LV. After 10 minutes of reperfusion the energy stores did recover, albeit to below the levels recorded at the end of cold ischaemic time (Table 4.2.1). A separate analysis was performed to examine the pattern of metabolic recovery in patients with donor heart failure compared to those with normal allograft function.

Allograft failure

Forty-two patients had follow-up studies with biopsies extending into the reperfusion period, and eleven of those developed donor organ failure. There were 5 cases of RV failure and 6 cases of global failure, with 1 fatal case in each subgroup. The mean value of HEP in L4 and R4 was not
significantly different between the three subgroups (figure 4.2.4). Dysfunctional organs however showed marked variations at reperfusion and overall failed to replenish their energy stores. Patients with RV dysfunction for example had lower mean ATP/ADP in R5 compared to R4 (p=0.25). The clinical risk factors and the putative protective factors (i.e. trasylol administration to the recipient) were examined to ensure that they were equally distributed between patients with and without allograft failure (table 4.2.2). An accumulation of risk factors was observed in the allograft failure group. The average ischaemic time was longer (207 vs. 176 min., p=0.01) and there was a trend towards donors being still on inotropes at the time of organ retrieval. The allograft failure group also contained 2 patients mechanically bridged to transplantation. When the expression of endothelial adhesion molecules and thrombomodulin was compared at reperfusion and at 1 week postoperatively, the two groups were not significantly different.

Table 4.2.1 Variation of energy stores over time. Results are expressed as geometric mean (95% confidence interval). Energy stores at time point 5 are lower than at time point 3 (incomplete recovery): in the RV p=0.001 for ATP/ADP, p=0.07 for EC, and in the LV p=0.003 for ATP/ADP and p=0.01 for EC.

| Time point | ATP/ADP | | | EC | | | |
|---|---|---|---|---|---|---|
| | RV | LV | RV | LV | RV | LV |
| 1 | 3.89 (3.02, 5.01) | 4.14 (3.39, 5.05) | 0.87 (0.84, 0.89) | 0.87 (0.84, 0.88) |
| 2 | 3.13 (2.31, 4.25) | 4.45 (3.51, 5.62) | 0.81 (0.76, 0.85) | 0.87 (0.84, 0.89) |
| 3 | 5.48 (4.55, 6.10) | 4.26 (3.72, 4.88) | 0.88 (0.86, 0.89) | 0.88 (0.86, 0.89) |
| 4 | 2.68 (2.28, 2.92) | 2.08 (1.83, 2.35) | 0.82 (0.80, 0.84) | 0.79 (0.76, 0.80) |
| 5 | 3.82 (3.25, 4.49) | 3.18 (2.80, 3.62) | 0.85 (0.83, 0.87) | 0.84 (0.82, 0.85) |
### Table 4.2.2 Prevalence of clinical risk/protective factors and profile of endothelial activation in the normal function group and in the allograft failure group.

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Normal function (n=31)</th>
<th>Allograft failure (n=11)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor age</td>
<td>37.6 (13.3)</td>
<td>41.4 (12.3)</td>
<td>0.42</td>
</tr>
<tr>
<td>Cardiac index (L/min/m²)</td>
<td>3.62 (0.75)</td>
<td>3.52 (0.68)</td>
<td>0.75</td>
</tr>
<tr>
<td>Ischaemic time (min)</td>
<td>176.0 (32.2)</td>
<td>207.2 (39.6)</td>
<td>0.01</td>
</tr>
<tr>
<td>Warm ischaemic time (min)</td>
<td>65.2 (18.6)</td>
<td>64.2 (17.3)</td>
<td>0.87</td>
</tr>
<tr>
<td>Transpulmonary gradient (mmHg)</td>
<td>8.85 (3.3)</td>
<td>9.0 (4.3)</td>
<td>0.92</td>
</tr>
<tr>
<td>Temperature on bypass (°C)</td>
<td>31.8 (2.9)</td>
<td>30.8 (2.7)</td>
<td>0.35</td>
</tr>
<tr>
<td>L3 ATP/ADP*</td>
<td>5.03 (2.53)</td>
<td>4.43 (2.81)</td>
<td>0.70</td>
</tr>
<tr>
<td>R3 ATP/ADP*</td>
<td>5.96 (2.73)</td>
<td>5.11 (2.07)</td>
<td>0.64</td>
</tr>
<tr>
<td>Brain dead donors (%)</td>
<td>30 (97%)</td>
<td>11 (100%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Heart transplants</td>
<td>20 (65%)</td>
<td>9 (82%)</td>
<td>0.45</td>
</tr>
<tr>
<td>Donors on inotropes at retrieval</td>
<td>18 (58%)</td>
<td>10 (91%)</td>
<td>0.07</td>
</tr>
<tr>
<td>Imported organs</td>
<td>5 (16%)</td>
<td>3 (27%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Recipient on ventricular assist</td>
<td>0</td>
<td>2 (18%)</td>
<td>0.06</td>
</tr>
<tr>
<td>preoperatively</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocyte filter</td>
<td>5 (16%)</td>
<td>1 (9%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>13 (42%)</td>
<td>5 (46%)</td>
<td>1.00</td>
</tr>
<tr>
<td>R5 Psel (% capillaries)*</td>
<td>80.4 (23.2)</td>
<td>82.1 (17.7)</td>
<td>0.79</td>
</tr>
<tr>
<td>R5 VCAM-1 (% capillaries)*</td>
<td>55.1 (26.1)</td>
<td>46.7 (28.2)</td>
<td>0.54</td>
</tr>
<tr>
<td>R5 Esel (% capillaries)*</td>
<td>55 (32)</td>
<td>39.3 (23.9)</td>
<td>0.21</td>
</tr>
<tr>
<td>R5 Thr*</td>
<td>1.9 (1.3)</td>
<td>2.6 (1.2)</td>
<td>0.35</td>
</tr>
<tr>
<td>R6 Psel (% capillaries)*</td>
<td>77.4 (18.2)</td>
<td>63.6 (19.5)</td>
<td>0.27</td>
</tr>
<tr>
<td>R6 VCAM-1 (% capillaries)*</td>
<td>41.8 (30.9)</td>
<td>19.6 (23.9)</td>
<td>0.17</td>
</tr>
<tr>
<td>R6 Esel (% capillaries)*</td>
<td>29.5 (32.2)</td>
<td>32 (18.2)</td>
<td>0.83</td>
</tr>
<tr>
<td>R6 Thr*</td>
<td>1.8 (1.4)</td>
<td>2.6 (1.1)</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Expressed as mean (standard deviation) or absolute value (percentage). The t test or the Mann-Whitney test* were used for continuous variables and Fisher's exact for categorical variables. Thr depletion is expressed on a scale of 0 to 5, with 5 representing maximum (normal) expression.
Figure 4.2.1  Comparison between myocardial energy stores of brain-dead and live patients. Shown as geometric mean (95% CI). A. ATP/ADP (p=0.70 for RV and p=0.12 for LV); B. EC (p=0.84 for RV and p=0.06 for LV).
Figure 4.2.2  ATP/ADP variation across transplantation. Shown as geometric mean (95% CI). * significant variation from previous measurement on the R side (p<0.005), † significant variation from previous measurement on the L side (p<0.001), ‡ significant difference between time points 5 and 3 (p=0.001 for RV, p=0.003 for LV).

Figure 4.2.3  Energy charge variation across transplantation. Shown as geometric mean (95% CI). * significant variation from previous measurement on the R side (p<0.03), † significant variation from previous measurement on the L side (p<0.001), ‡ significant difference between time points 5 and 3 (p=0.07 for RV, p=0.01 for LV).
**Figure 4.2.4** High-energy phosphates before and after reperfusion in patients with good allograft function compared with patients with donor organ failure. **A.** Grafts with normal function improve their ATP/ADP ratio in both R (*p=0.04, Mann-Whitney test) and L ventricles (†p=0.01). ANOVA shows that ATP/ADP has a significant change between ischaemia and reperfusion overall (p=0.01 for RV, p<0.001 for LV) and that this change is different for the 3 subgroups on the right but not on the left (p=0.04 RV; p=0.35 LV). However, the average ATP/ADP ratio is not statistically different for the 3 subgroups at either time point (p=0.37 RV; p=0.29 LV). **B.** Recovery of EC is statistically significant only in the LV of good-functioning hearts (†p=0.05). EC demonstrates a similar pattern of variation, with significant change at reperfusion overall (p=0.01 for RV and p=0.002 for LV). The change was not statistically significant between subgroups (p=0.28 RV; p=0.85 LV) and the mean value too was not different at either of the time points (p=0.75 RV; p=0.33 LV).
**Functional correlations**

Only 3 of these recipients were on the customary combination of low-dose isoprenaline and dopamine at the time of recording PV data. (This is because the PV recordings were done immediately after discontinuing extracorporeal circulation but often before starting inotropic support.) There was no correlation between cardiac index, as determined by the Swan-Ganz catheter in the donor (n=48, after excluding 4 hearts with palpable coronary artery disease), and mean HEP contents at time point 1. (The HEP values of R1 and L1 were averaged to increase the power of this correlation.)

Of the 17 hearts with biochemical and P/V assessments, 15 were subsequently used for transplantation. Five of these hearts had follow-up studies in the recipient immediately after separation from cardiopulmonary bypass. There were 6 donors on inotropes at the time of retrieval, including the 2 patients whose hearts were turned down on functional grounds. The results for P/V measurements are shown in table 4.2.3 below and the statistical correlations with HEP are shown in table 4.2.4 at the end of the chapter. No correlation was found between HEP and any of the functional indices, apart from a weak correlation with EDPVR (table 4.2.4).

**Table 4.2.3** Results of pressure-volume loop measurements and high-energy phosphate stores in 17 hearts from brain-dead donors.

<table>
<thead>
<tr>
<th>Study no.</th>
<th>ATP/ADP</th>
<th>EC</th>
<th>CI</th>
<th>dp/dt max</th>
<th>dp/dt min</th>
<th>ESPVR</th>
<th>EDPVR</th>
<th>PRSW</th>
<th>Ea/Es</th>
<th>Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.01</td>
<td>0.91</td>
<td>2.7</td>
<td>242</td>
<td>195</td>
<td>0.35</td>
<td>0.13</td>
<td>10.6</td>
<td>1</td>
<td>yes</td>
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<tr>
<td>2</td>
<td>7.06</td>
<td>0.87</td>
<td>4.2</td>
<td>453</td>
<td>355</td>
<td>0.45</td>
<td>0.18</td>
<td>28.89</td>
<td>1.3</td>
<td>yes</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
<td>0.91</td>
<td>2.9</td>
<td>203</td>
<td>100</td>
<td>0.33</td>
<td>0.09</td>
<td>13.57</td>
<td>2</td>
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<tr>
<td>4</td>
<td>1.74</td>
<td>0.79</td>
<td>3.1</td>
<td>479</td>
<td>294</td>
<td>0.24</td>
<td>0.02</td>
<td>15.87</td>
<td>1</td>
<td>yes</td>
</tr>
<tr>
<td>5</td>
<td>4.67</td>
<td>0.86</td>
<td>3.3</td>
<td>319</td>
<td>194</td>
<td>0.38</td>
<td>0.02</td>
<td>18.13</td>
<td>0.8</td>
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<tr>
<td>6</td>
<td>4.65</td>
<td>0.84</td>
<td>3.6</td>
<td>334</td>
<td>229</td>
<td>0.46</td>
<td>0.08</td>
<td>16.88</td>
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<tr>
<td>7</td>
<td>3.41</td>
<td>0.83</td>
<td>4.5</td>
<td>277</td>
<td>195</td>
<td>0.19</td>
<td>0.08</td>
<td>19.35</td>
<td>0.4</td>
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</tr>
<tr>
<td>8</td>
<td>7.4</td>
<td>0.91</td>
<td>4.2</td>
<td>319</td>
<td>194</td>
<td>0.13</td>
<td>0.06</td>
<td>9.79</td>
<td>0.2</td>
<td>yes</td>
</tr>
<tr>
<td>9</td>
<td>2.79</td>
<td>0.87</td>
<td>3.4</td>
<td>265</td>
<td>131</td>
<td>0.31</td>
<td>0.05</td>
<td>15.37</td>
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<tr>
<td>10</td>
<td>4.2</td>
<td>0.84</td>
<td>3</td>
<td>387</td>
<td>228</td>
<td>0.83</td>
<td>0.09</td>
<td>26.33</td>
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<tr>
<td>11</td>
<td>3.72</td>
<td>0.86</td>
<td>3.2</td>
<td>277</td>
<td>97</td>
<td>0.22</td>
<td>0.04</td>
<td>14.1</td>
<td>0.5</td>
<td>yes</td>
</tr>
<tr>
<td>12</td>
<td>2.62</td>
<td>0.83</td>
<td>2.8</td>
<td>426</td>
<td>822</td>
<td>0.43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>4.56</td>
<td>0.86</td>
<td>3.3</td>
<td>177</td>
<td>122</td>
<td>0.16</td>
<td>0.05</td>
<td>8.25</td>
<td>1</td>
<td>yes</td>
</tr>
<tr>
<td>14</td>
<td>2.29</td>
<td>0.73</td>
<td>3.1</td>
<td>231</td>
<td>88</td>
<td>0.18</td>
<td>0.03</td>
<td>8.23</td>
<td>0.4</td>
<td>yes</td>
</tr>
<tr>
<td>15</td>
<td>7.78</td>
<td>0.88</td>
<td>4.1</td>
<td>330</td>
<td>135</td>
<td>0.33</td>
<td>0.05</td>
<td>16.8</td>
<td>1</td>
<td>yes</td>
</tr>
</tbody>
</table>
The units of measurement are CI (L/min*m²), dP/dt_max (mm Hg/s), dP/dt_min (mm Hg/s) and PRSW (g*m). The other variables are adimensional. Blank fields represent missing data.

None of the 5 patients with follow-up intraoperative studies developed donor organ failure. dP/dt_max improved between retrieval and reperfusion (p=0.01) and there was a trend towards improved ATP/ADP and dP/dt_min (table 4.2.5, end of chapter). The ATP/ADP ratio at reperfusion was inversely correlated with dP/dt_max (r=-0.93, p=0.02) but positively correlated with PRSW (r=0.90, p=0.03). The detailed results for these 5 patients are presented in table 4.2.6 (end of chapter).

4.3 DISCUSSION

Brain-dead vs. live donors

Brain death is not associated with depleted myocardial energy stores. This is in agreement with findings of other groups in animal and in human studies (Bittner, 1994; Starling, 1991). The finding of a trend towards lower energy stores in the LV of live patients may be due to the reduced number of observations. It is also recognised that patients with ventricular hypertrophy have reduced ATP levels in the subendocardium (Peyton, 1982), and there were 3 patients with ventricular hypertrophy in the live group. (It would have been desirable to include more than 5 domino hearts, but because we were unable to achieve that we had to resort to live controls. Inevitably, the 3 patients undergoing aortic valve replacement had LV hypertrophy. The reason for enrolling these patients was that the operation was done with a LV apical vent and so biopsies at the LV apex were ethically permissible. No statistical correction was justified for a small group of live patients made up of 3 dominos and 3 patients undergoing aortic valve replacement. This methodological flaw is however recognised.) Although animal experiments and clinical observations suggest that the RV is more vulnerable in a brain death environment, our results show that a deficiency in the energy metabolism is not the underlying mechanism for this
phenomenon. A comparison of cardiac ECA between brain-dead and live patients is presented in Chapter 5.

**Overall trend**

Not only are the energy stores in the RV and LV comparable at the baseline, but they tend to evolve in parallel across transplantation. The adenine nucleotides in the RV tend however to diminish during organ procurement. This may be due to isolated episodes of pulmonary hypertension, at the moment of brain death or afterwards. In individual patients it is possible that direct adrenal stimulation by the abdominal retrieval team contributes to intermittent catecholamine discharges. This hypothesis is purely speculative. During brain death however the left atrial pressure exceeds the pulmonary pressure, temporarily halting the entire pulmonary capillary blood flow (Novitzky, 1987; Van Trigt, 1995). The right ventricle is physiologically not equipped to withstand such a formidable increase in afterload. Perhaps this initial injury contributes to the impaired RV contractile performance reported after brain death (Bittner, 1999) and to the variations in RV HEP seen in our study. Although the β-adrenergic pathway appears to be intact after brain death (Van Trigt, 1995), it is not clear if the observed increase in the tissue catecholamine concentration further affects function (Mertes, 1994). Whatever the mechanism of the initial HEP variation in the RV, at the end of cold storage both ventricles have normal energy stores. Strictly from a metabolic point of view, they seem equally well prepared for the subsequent strain imposed by warm ischaemia and reperfusion.

The fall in energy stores during organ implantation is a recognised phenomenon. It has been previously reported in liver transplantation. Furthermore, in liver transplantation there seems to be an inverse correlation between recovery of energy stores and length of warm ischaemic time (Kamiike, 1988). We were unable to show a correlation between warm ischaemic time and the variation of HEP contents during either cardiac implantation or at reperfusion. Topical cooling of the heart during implantation has been used very selectively at our institution in recent years but these results suggest that some form of protection may have to be reconsidered. Recovery of energy stores after reperfusion requires more than 10 minutes. This in agreement with the results of Smolenski, who showed that HEP levels after 30 minutes of reperfusion are still below levels at the end of cold ischaemia (Smolenski, 1992). These authors however did not comment on the clinical outcome of the 20 patients studied.
Allograft failure

Compared to recipients who had an uneventful haemodynamic course, patients who went on to develop donor organ failure did not have lower HEP levels at the end of cold ischaemia (table 4.2.2). Also, the HEP levels at time point 2, i.e. after optimisation of the donor circulation and before harvesting, were not statistically different (results not shown). Our findings are comparable to those of Starling et al, who demonstrated no relationship between HEP levels at the end of cold ischaemia and allograft function in the postoperative period (Starling, 1991). Furthermore, in our study HEP levels before and after reperfusion are insignificantly different in patients with and without subsequent allograft dysfunction (figure 4.2.4).

Failure to increase energy stores at reperfusion may be predictive of donor organ failure, but this finding did not reach statistical significance due to the marked variability in HEP values at time point 5. However, this hypothesis deserves further testing. It would be particularly helpful for the operating surgeon to have a test which is predictive of donor organ failure, such that prophylactic treatments (e.g. inhaled nitric oxide) are started as early as in the operating room. Until such an instrument is available caution should be exercised in avoiding an accumulation of clinical risk factors in individual cases. Given the increased inflammatory load of the donor heart, it was interesting to see that endothelial activation was not different either between the two groups (table 4.2.2). Perhaps donor organ failure, whether temporary or fatal, results from a more complex combination of intrinsic contractile dysfunction and variable loading conditions, particularly in the pulmonary bed. Finally, although marked variations in HEP levels at reperfusion challenge the robustness of this assessment method, it is reassuring to see that other groups noticed the same phenomenon (Starling, 1991 – discussion).

Correlation with function

A dissociation between energy metabolism and contractile function was demonstrated in the human donor heart. HEP levels in brain-dead donors did not correlate with the cardiac index measured by thermodilution or with most RV load-independent parameters. ATP/ADP in the RV of 17 donors however had a statistically significant correlation with the load-independent PRSW (table 4.2.3). In the 5 patients with follow-up studies all biochemical and haemodynamic parameters improved after reperfusion, but only 3 of the variables were in the range of statistical significance (table 4.2.4). This improvement took place despite implanting the heart in a
circulation with a much higher pulmonary vascular resistance (259.2 vs. 96.2 dyne-sec/cm$^5$, $p=0.01$). Since none of the 5 patients developed donor organ failure, perhaps removal of the heart from the brain-death environment was sufficient to ensure better function in these cases. However, this is a very cautious conclusion based on small numbers. Some of the observed improvement may actually be due to the regular low dose of inotropic treatment used in 3 of the recipients at the time of recording.

Other groups performed an analysis of the metabolic state of the donor heart using $^{31}$P MRS (Van Doddenburgh, 1996). A correlation was found between the energy content (expressed as CP/ATP) and the cardiac index at 1 week postoperatively, but not in the early postoperative period. Spectroscopic methods are in addition limited in their ability to be repeated in the perioperative period. In contrast, adenine nucleotides (but not creatine phosphate) lend themselves to measurement in endomyocardial biopsies (Regitz, 1992; Bøtker, 1994). In a more general context, there is ongoing debate in areas of experimental and clinical cardiology on the relationship between HEP levels and contractile function (Bashore, 1987; Taegtmeyer, 1985; Jennings, 1985; Regitz, 1992). The weight of evidence suggests that HEP levels are preserved over a wide range before systolic function is affected. In practical terms, as we have seen in this study, normal levels of adenine nucleotides in cardiac tissue do not exclude functional derangement. In fact they tell us little about possible perturbations in ATP turnover or utilisation by the contractile apparatus. Recent results show that HEP do not diffuse through the cytoplasm in homogenous fashion. On the contrary, the myocardial sarcoplasm is a sum of microdomains with restricted diffusion and preferential channelling of substrates to enzymes (Kaaasik, 2001). The efficiency of ATP utilisation remains for the moment beyond the possibilities of clinical testing. Taken together, these results suggest that the fate of the HEP pool at reperfusion remains incompletely described in transplantation, particularly in relation to clinical end points.

**Usable vs. unusable hearts**

Transplant surgeons would much appreciate a marker of intrinsic donor heart function, particularly for borderline organs. In clinical studies reporting outcomes of donor hearts, a non-quantifiable variable is the amount of brain death-induced cardiac dysfunction. Earlier work from the Papworth group showed convincingly that hearts with an impaired birefringence index (a laboratory measure of biochemical and contractile function) have poor early and late clinical outcome (Darracott-Cankovic, 1989). However, those observations were made before introducing
routine instrumentation of all donor hearts with a pulmonary flotation catheter in 1990. It is conceivable that some of the organs from those earlier reports were very borderline and were functioning in a suboptimal haemodynamic milieu. Some may have been turned down by Swan-Ganz catheter criteria, which could explain the magnitude of the initial birefringence observations. Later work on rat myocardium submitted to warm ischaemia established that a low ATP/ADP ratio is the most likely explanation for a decrease in myocardial birefringence (Dr Darracott-Cankovic, unpublished observations). When the ATP/ADP index was measured in serial LV biopsies in clinical heart transplantation, a correlation with the clinical outcome was observed but a more detailed haemodynamic characterisation of recipients was not made in that study (Daracott-Cankovic, 1998).

Our current study group contained 6 patients whose hearts were turned down from donation and only 2 of those were rejected on functional grounds. We feel that any donor hearts excluded from donation by structural disease (e.g. ventricular hypertrophy, palpable coronary artery disease etc.) should also be excluded from comparisons of function. At time point 1 the mean ATP/ADP in the 2 hearts unsuitable on functional grounds was lower than in used organs (3.28 vs. 4.14 in the LV, and 2.75 vs. 3.89 in the RV). With such small numbers it is however impossible to judge how good HEP levels are at detecting unsuitable organs. The 2 poorly functioning hearts were also failing on conventional Swan-Ganz haemodynamic criteria and on the basis of inotropic load too.

The above example illustrates the point that HEP levels may become a sensitive marker of cardiac function only when the heart is failing clinically, despite all resuscitative efforts. This notion is in agreement with recent experimental reports, in which the reduced afterload and coronary perfusion were shown to be significantly involved in brain death-induced cardiac dysfunction (Szabo, 2001). From the clinicians standpoint this implies that, within the practice of donor optimisation, a more robust index of intrinsic cardiac function will have to be identified and used in future studies.
Table 4.2.4 Pearson’s correlation coefficients between right ventricular HEP values and P/V parameters in brain-dead donors (n=17) and recipients (n=5) at time points 1 and 5 respectively.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Brain dead donors (n=17)</th>
<th>Recipients (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP/ADP</td>
<td>4.23 (1.38)</td>
<td>3.65 (1.35)</td>
</tr>
<tr>
<td>EC</td>
<td>0.85 (0.03)</td>
<td>0.86 (0.49)</td>
</tr>
<tr>
<td>CI (L/min)</td>
<td>3.36 (0.74)</td>
<td>N/a</td>
</tr>
<tr>
<td>dP/dtₘₙₜₜ (mm Hg/s)</td>
<td>310.6 (66.4)</td>
<td>315.2 (114.5)</td>
</tr>
<tr>
<td>dP/dtₘᵦₜₜ (mm Hg/s)</td>
<td>219 (98)</td>
<td>189.6 (77.2)</td>
</tr>
<tr>
<td>ESPVR</td>
<td>0.43 (0.23)</td>
<td>0.41 (0.23)</td>
</tr>
<tr>
<td>EDPVR</td>
<td>0.067 (0.03)</td>
<td>0.076 (0.04)</td>
</tr>
<tr>
<td>PRSW (g/m)</td>
<td>15.7 (4.0)</td>
<td>16.3 (5.9)</td>
</tr>
<tr>
<td>Ea/Es</td>
<td>0.95 (0.41)</td>
<td>1.10 (0.57)</td>
</tr>
</tbody>
</table>

The only values in the range of statistical significance are *p=0.06, ^p=0.02, and ^p=0.03. Cardiac index measurement by thermodilution was not available in 4 of 5 recipients because measurement was not indicated clinically.

Table 4.2.5 High-energy phosphates and P/V parameters between time points 1 and 5 in 5 donor-recipient pairs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Brain dead donor</th>
<th>Reperfusion</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP/ADP</td>
<td>3.65 (1.35)</td>
<td>5.28 (2.77)</td>
<td>0.08</td>
</tr>
<tr>
<td>EC</td>
<td>0.86 (0.49)</td>
<td>0.86 (0.49)</td>
<td>0.93</td>
</tr>
<tr>
<td>dP/dtₘₙₜₜ (mm Hg/s)</td>
<td>315.2 (114.5)</td>
<td>683.2 (206.7)</td>
<td>0.01</td>
</tr>
<tr>
<td>dP/dtₘᵦₜₜ (mm Hg/s)</td>
<td>189.6 (77.2)</td>
<td>453.4 (179.7)</td>
<td>0.08</td>
</tr>
<tr>
<td>ESPVR</td>
<td>0.41 (0.23)</td>
<td>0.52 (0.10)</td>
<td>0.44</td>
</tr>
<tr>
<td>EDPVR</td>
<td>0.076 (0.04)</td>
<td>0.08 (0.021)</td>
<td>0.89</td>
</tr>
<tr>
<td>PRSW (g/m)</td>
<td>16.3 (5.9)</td>
<td>22.2 (5.4)</td>
<td>0.21</td>
</tr>
<tr>
<td>Ea/Es</td>
<td>1.10 (0.57)</td>
<td>1.54 (0.59)</td>
<td>0.29</td>
</tr>
<tr>
<td>PVR (dynes·s·cm⁻⁵)</td>
<td>96.2 (10.2)</td>
<td>259.2 (59.5)*</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Expressed as mean (SD) and compared with the paired t test. The recipient PVR is the preoperative value obtained at right heart catheterisation.
Table 4.2.6  Detailed results for five hearts studied with pressure volume-loops in the donor and the recipient. The high-energy phosphates shown are from biopsies taken on donor optimisation and 10 minutes after reperfusion.

<table>
<thead>
<tr>
<th>Study no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP/ADP</td>
<td>5.01</td>
<td>9.35</td>
<td>4.5</td>
<td>6.11</td>
<td>1.74</td>
</tr>
<tr>
<td>EC</td>
<td>0.91</td>
<td>0.88</td>
<td>0.91</td>
<td>0.85</td>
<td>0.79</td>
</tr>
<tr>
<td>dP/dt(_{\text{max}}) (mm Hg/s)</td>
<td>242</td>
<td>329</td>
<td>203</td>
<td>746</td>
<td>479</td>
</tr>
<tr>
<td>dP/dt(_{\text{min}}) (mm Hg/s)</td>
<td>195</td>
<td>371</td>
<td>100</td>
<td>712</td>
<td>294</td>
</tr>
<tr>
<td>ESPVR</td>
<td>0.35</td>
<td>0.67</td>
<td>0.33</td>
<td>0.48</td>
<td>0.29</td>
</tr>
<tr>
<td>EDPVR</td>
<td>0.13</td>
<td>0.08</td>
<td>0.09</td>
<td>0.09</td>
<td>0.03</td>
</tr>
<tr>
<td>PRSW (g·m)</td>
<td>10.6</td>
<td>30.1</td>
<td>13.6</td>
<td>23.5</td>
<td>15.8</td>
</tr>
<tr>
<td>Ea/Es</td>
<td>0.9</td>
<td>3.0</td>
<td>1.0</td>
<td>2.5</td>
<td>0.6</td>
</tr>
<tr>
<td>PVR (dynes·s·cm(^{-2}))</td>
<td>92</td>
<td>248</td>
<td>98</td>
<td>408</td>
<td>109</td>
</tr>
</tbody>
</table>
CHAPTER 5  
ENDOTHELIAL CELL ACTIVATION

This chapter presents and discusses results of endothelial activation in clinical cardiac transplantation.

5.1 PATIENTS AND METHODS

Forty-four donor hearts were studied with serial biopsies from both ventricles at the following time points:

1 - on initial assessment in the donor  
4 - at the end of warm ischaemic time  
5 - after 10 minutes of reperfusion  
6 - at 1 week postoperatively  
7 - at 1 month postoperatively  
8 - at 3 months postoperatively

In order to rationalise the number of biopsies on the donor heart, tissue sampling at time points 2 and 3 was omitted for the endothelial analysis. The postoperative biopsies were obtained from heart transplant patients only, who undergo rejection surveillance by routine endomyocardial biopsies. Since acute rejection is known to be associated with ECA, all follow-up biopsies with rejection grade greater than 1 in the ISHLT classification were eliminated from the time trend analysis (There were 8 biopsies in total belonging to 8 different patients; 5 of them had rejection grade > 2 and in the other 3 there was insufficient tissue for diagnosis).

The biopsies are named R1, L1, R4, L4 etc. The patients enrolled in the study are described in a tree diagram on the next page. Ten hearts were not considered suitable for transplantation based on structural disease (e.g. palpable coronary plaques) (n=4) or poor function (n=6). The former were excluded from further comparisons as we felt that structurally diseased organs would be a source of bias. The mean age of the 34 accepted donors was 36.9±11.2 years (range 12-61). When consent for research was not granted by the donor family (n=5) or when organs were retrieved by other teams outside our zone (n=6) a donor biopsy was not taken. There were 3 live local donors.
who proceeded to cardiac donation in domino fashion but for 3 other domino donors a suitable recipient was not found at the time of operation (they were however included in the live vs. brain-dead comparison). All 6 domino donors suffered from cystic fibrosis. Thirty-one transplants were performed: 25 heart transplants, using a standard biatrial cuff technique, and 6 heart-lung transplants. Mean ischemic time was 196.6±39.9 minutes. In terms of follow-up, 19 hearts were studied with 1-week biopsies, 17 at 1 month and 15 at 3 months respectively. The control tissue was right atrial appendage from 9 patients who had undergone coronary revascularisation at Papworth Hospital in 1999. Only in 8 brain-dead donors we had a common dataset of biopsies for endothelial activation and pressure-volume loop recordings. Because we were analysing four different histological markers we felt that correlation with a multitude of conductance catheter variables would be statistically flawed ('fishing for significance'). As a result, functional recovery is analysed in terms of allograft failure, as defined in section 3.6.

Flow diagram chapter 5  
The patients enrolled in the endothelial activation study.

```
44 patients  
  ┌─────┐  
  │     │  
  │10 unused hearts 34 transplantable hearts (6 domino)├──┐  
  │      │  
  │6 poor function 4 CAD├──┐  
  │      │  
  │31 transplanted (3 domino) 3 not used * (3 domino)├──┐  
  │      │  
  │25 HTx 6 HLTx├──┘  

* because of lack of suitable recipient.

CAD coronary artery disease; HTx, heart transplant; HLTx, heart-lung transplant.
```

The ECA data were not normally distributed even after logarithmic transformation. Unless otherwise indicated, data are expressed as median ± interquartile range and were analysed with non-parametric methods. Multiple comparisons between groups for adhesion molecules were carried out with the Kruskal-Wallis test. The chi-square test was used for simple comparisons between groups and the median test for Thr expression analysis. The relationship between certain outcomes (e.g. donor organ failure) and expression of ECA markers was analysed with Mann-
Whitney's test. Correlations were explored with Pearson’s method. A p value of less than 0.05 was considered statistically significant.

5.2 RESULTS

Histological pattern

Haematoxillin-eosin staining was adequate in all sections (panel 5-1 – for histology panel please refer to end of chapter 5). CD31 was expressed in all biopsies, demonstrating that the endothelium was viable (panel 5-2). Adhesion molecules showed variable expression. When they were upregulated there was a pattern of presence on both muscularised and non-muscularised vessels (panel 5-3), however capillary and arteriolar staining were counted separately on each section (panel 5-4). P-sel was present on a high proportion of vessels in most biopsy specimens. Conversely, E-sel and VCAM-1 reached a peak of approximately 50% at reperfusion. Neutrophil infiltrates were observed only very rarely and were not quantified further. Thr showed varying degrees of depletion in the capillary bed (panel 5-5 and 5-6).

The influence of brain death

A comparison was made between domino hearts and hearts from BD donors (figure 5.2.1). In relation to control tissue, both groups of hearts used for transplantation have upregulation of adhesion molecules, particularly P-sel and VCAM-1, and depletion of Thr (figure 5.2.2).
Figure 5.2.1. Adhesion molecule expression in donor hearts. Data shown as percentage of positive vessels, median ± interquartile range.

(A) P-selectin. Significant difference between transplanted hearts and controls (*RV p=0.003, and **LV p=0.01). Significant difference between brain dead used and unused donors (§RV p=0.001, and §§LV p=0.005).
(B) VCAM-1. The median values for controls and for unused hearts from brain dead donors were 0 (not shown). Significant difference between transplanted hearts and controls (*RV p=0.01, and **LV p=0.01). Also difference between brain dead used and unused donors ($RV p=0.004$, and $§§LV p=0.06$).

(C) E-selectin. No statistically significant difference between transplanted hearts and controls, also no difference between brain dead used and unused donors.
Figure 5.2.2  Thrombomodulin expression in cardiac microcapillaries across transplantation. Expressed as median and interquartile range on a scale of 0 to 5 (p=0.30 for overall LV variation and p=0.02 for RV).

When usable hearts from brain-dead donors were compared with hearts rejected on functional grounds, it was found that the latter have less P-sel and VCAM-1 upregulation (figure 5.2.1). The median VCAM-1 expression in LV capillaries was 7% for used hearts and 0% in unused hearts (p=0.06, Mann-Whitney’s test), whereas the values for the RV were 14% and 0% respectively (p=0.004). In unused donor hearts from brain-dead donors there was a trend towards Thr depletion (score 1 vs. 2 for LV, p=0.10; and score 0.5 vs. 1 for RV, p=0.08). There were no statistically significant differences for L-sel.

Trend over time and RV vs. LV

All hearts used for transplantation (from either live or brain-dead donors) were studied for the time trend of ECA (figures 5.2.2 and 5.2.3). The descriptive statistics did not suggest that there was a significant difference between the RV and the LV at any of the intraoperative time points. P-sel is markedly expressed in all donors and remains elevated throughout transplantation. The expression tends to diminish in the first 3 months postoperatively but remains supra-normal.
VCAM-1 and E-sel are upregulated too in all donors. As opposed to E-sel, the expression of VCAM-1 is diminished at the end of the ischaemic period, but both adhesion molecules are markedly upregulated at reperfusion (figure 5.2.3). The amount of decrease in VCAM-1 between time points 1 and 4 inversely correlated with the length of total ischaemic time ($r=-0.51$, $p=0.03$ for LV; $r=-0.49$, $p=0.06$ for RV) but did not correlate with the warm ischaemic time. Both VCAM-1 and E-sel normalise gradually in the postoperative period. Finally, Thr is depleted in cardiac donors, with no difference between brain-dead and live donors. It tends to recover at the end of ischaemia but there is however further depletion after reperfusion and this appears to worsen in the postoperative period.

**Allograft failure**

In order to examine whether ECA is predictive of donor organ failure, the data for 31 recipients were compared: 18 had normal allograft function and 13 had donor organ failure (6 had isolated RV failure and 7 had global dysfunction). There was no relationship between donor organ failure and expression of adhesion molecules or depletion of Thr in serial intraoperative biopsies of RV and LV (comparisons made at time points 1, 4 and 5). This lack of correlation was also observed when postoperative RV biopsies were compared between patients with and without donor organ failure (time points 6 to 8). Since this preliminary analysis was negative and also in view of the multiple testing performed, a subanalysis of biopsy side vs. failure side was not performed. As in the HEP study, there was a trend towards accumulation of clinical risk factors in the donor organ failure group (table 5.2.1). All patients who developed donor organ failure received hearts which were still on inotropes at the time of retrieval ($p<0.001$) and had a longer mean ischaemic time ($p=0.01$).

**The effect of an anti-inflammatory intervention**

Of the 31 recipients studied, 12 received aprotinin (Trasylol). It was noticed that in comparison with those who did not receive trasylo, this group had lower levels of VCAM-1 ($p=0.05$) and E-sel ($p=0.07$) in L5 but not in R5. Furthermore, in the trasylo group there was a blunted response in VCAM-1 expression between L4 and L5 ($p=0.05$, compared to the no-trasylol group; the VCAM-1 levels of the two groups at time 4 were not statistically different). The putative anti-ECA effect of trasylo was however not preserved in R6 biopsies at 1 week and did not involve P-sel or Thr at either of time points 5 or 6.
Figure 5.2.3  Percentage of non-muscularised vessels positive for adhesion molecules across transplantation. Expressed as median and interquartile range.

A. P-selectin (p=0.55 for overall LV variation and p<0.001 for RV). * Significant difference compared to controls (p=0.01 for LV and p=0.002 for RV).

![Graph of P-selectin](image)

B. VCAM-1 (p<0.001 for overall variation in the RV and LV). * Significant difference compared to controls (p=0.01 for LV and p=0.004 for RV).

![Graph of VCAM-1](image)
C. E-selectin (p<0.001 for overall LV variation and p=0.001 for RV).

![E-selectin graph]

**Table 5.2.1** Prevalence of risk factors in the normal function group and in the allograft failure group.

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Normal function (n=18)</th>
<th>Allograft failure (n=13)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor age</td>
<td>36.8 (15.4)</td>
<td>39.5 (13.3)</td>
<td>0.61</td>
</tr>
<tr>
<td>Donor cardiac index (L/min/m²)</td>
<td>3.74 (0.95)</td>
<td>3.48 (0.69)</td>
<td>0.49</td>
</tr>
<tr>
<td>Ischaemic time (min)</td>
<td>177.6 (45.6)</td>
<td>222.8 (47.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>Warm ischaemic time (min)</td>
<td>66.2 (24.1)</td>
<td>68.3 (22.8)</td>
<td>0.80</td>
</tr>
<tr>
<td>Transpulmonary gradient (mmHg)</td>
<td>8.3 (2.9)</td>
<td>9.1 (3.9)</td>
<td>0.61</td>
</tr>
<tr>
<td>Temperature on bypass (°C)</td>
<td>32.2 (2.6)</td>
<td>29.9 (3.9)</td>
<td>0.06</td>
</tr>
<tr>
<td>Brain dead donors (%)</td>
<td>15 (83%)</td>
<td>13 (100%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Heart transplants</td>
<td>14 (78%)</td>
<td>11 (85%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Donors on inotropes at retrieval</td>
<td>6 (33%)</td>
<td>13 (100%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Imported organs</td>
<td>1 (6%)</td>
<td>5 (39%)</td>
<td>0.06</td>
</tr>
<tr>
<td>Recipient on mechanical support preoperatively</td>
<td>1 (6%)</td>
<td>3 (23%)</td>
<td>0.28</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>7 (39%)</td>
<td>5 (39%)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Expressed as mean (standard deviation) or absolute value (percentage). The t test was used for continuous variables and Fisher’s exact for categorical variables.
5.3 DISCUSSION

This study showed that donor hearts exhibit ECA which is further enhanced by transplantation but is not predictive (for the markers studied) of donor organ failure. Since rejection was such a rare event in our series we were unable to correlate it with peritransplant ECA.

The influence of brain death and domino transplantation

Expression of adhesion molecules in this study is in agreement with previously published results for the normal human heart (see table 2.3.1) and also with experimental research in brain death (Takada, 1999; Pratschke, 2000). Furthermore, this is to our knowledge the first study to provide a serial characterisation of ECA in clinical cardiac transplantation. Of the three adhesion molecules studied, only E-sel changes did not reach statistical significance in brain-dead used and unused donors (figure 5.2.3). In addition, long-term follow-up of soluble adhesion molecules in cardiac transplants recipients showed a similar pattern of variation (see below) (Andreassen, 1998). This suggests that E-sel has a limited role in mediating allograft inflammation in the acute or chronic stages of cardiac transplantation, and so it is a less attractive target for intervention. However, our results show unequivocally that hearts used in clinical transplantation have both type 1 (P-sel) and type 2 endothelial activation (VCAM-1).

Adhesion molecules upregulation in kidneys from brain-dead donors but not in living-related donors has recently been reported (Koo, 1999). In contrast to that study, it was surprising to see that domino hearts (from living-unrelated donors) had ECA at levels comparable to organs from brain-dead patients. Since all of the domino donors were cystic fibrosis sufferers with recurrent chest infection, this observation may be due to the inflamed intrathoracic environment. With small numbers of patients it is impossible to say if baseline inflammation in the donor heart negates the effect of live donation to any extent. In analysing the clinical outcomes of domino transplantation, our group has not been able to show a benefit in terms of acute rejection, infection, early and medium-term survival and 2-year freedom from CAV (Oaks, 1994; Smith, 1996; Luckraz, 2002). An inflammatory phenotype at the outset and subsequent allotransplantation possibly underlie the observed lack of benefit. Moreover, the advantage of a reduced ischaemic time is lost when domino hearts are exported (Oaks, 1994). The domino operation (presented in section 1.3) may have to be re-examined in the light of these findings. It is possible that the best use of a heart-lung block is double lung donation to a cystic fibrosis or
emphysema sufferer and separate cardiac donation. Heart-lung transplantation is still the best option for adult congenital heart disease (Waddell, 2000; Stoica, 2001), but such recipients are by definition not suitable for domino donation. It is likely therefore that the trend towards less frequent domino donations will continue (Anyanwu, 1999).

There is no immediate explanation for the reduced expression of adhesion molecules in the unusable donor hearts compared with hearts used for transplantation. It should be emphasized again that the unused organs included in this comparison did not have palpable coronary artery disease and so the chance of flow-limiting ischaemia was minimal. Although unused hearts had an endothelial phenotype which is close to normal, the myocyte phenotype was markedly different (see chapter 6). A higher degree of leukocyte extravasation could have been responsible for this observation, but on reinspection of the histology there was no striking difference between the two groups in this respect (As a result, a formal evaluation of tissue leukocyte infiltration was not performed. Furthermore this would have been technically difficult due to the small size of the biopsy). A further limitation of the biopsy method is that it represents a snapshot in a long time frame. Although in the experimental setup type 2 ECA has been associated with more leukocyte extravasation (Takada, 1998; Van der Hoeven, 2001), it is interesting to note that clinical investigators demonstrated more ECA in kidneys from brain-dead donors but not higher levels of leukocyte extravasation (Koo, 1999). The mechanism and consequences of leukocyte trafficking in transplantation deserve further testing. In a review of endothelial pathophysiology in transplantation Karimova and Pinsky point out that, as far as ECA is concerned, there is a fine dividing line between physiological endothelial activation and endothelial dysfunction (Karimova, 2001). From this angle, it may be that unused donor hearts lack a physiological adaptation imposed by the environment of brain death.

**Trend over time and RV vs. LV**

P-sel was upregulated throughout clinical cardiac transplantation and persisted post-operatively in the absence of rejection. As with the other adhesion molecules in our study, there was however a lack of correlation with allograft function. Experimental studies have not yet clarified the role of P-sel in transplantation. The literature suggests that in both physiological and pathological processes there is a degree of signalling redundancy. Although receptor blockade was successful in reducing the inflammatory load in peripheral organs after brain death (Takada, 1998), the presence of P-sel in donor tissues does not limit allograft survival nor does increase chronic
rejection (Raisky, 2001). Hypoxia is known to increase NF-κB-dependent transcription whereas hypothermia tends to blunt this response (Verrier, 1996). In our study VCAM-1 expression was inhibited during cold storage but E-sel expression increased. The most interesting finding is the 10-fold upregulation of VCAM-1 at reperfusion. De novo VCAM-1 synthesis takes 4-5 hours and, since biopsies 4 and 5 are separated by less than 15 minutes, it follows that a high proportion of VCAM-1 observed at reperfusion must have been pre-formed. Perhaps VCAM-1 is inserted in the cellular membrane following a two-hit model of injury, when the tissue receives a further insult at reperfusion. At that point in time the expression of VCAM-1 and E-sel is comparable (figure 5.2.3).

Adhesion molecules in the cardiac vasculature returned to near-normal levels in the first 3 months after transplant but remained elevated in the case of P-sel in the absence of acute rejection. The significance of this finding is unclear. As discussed in 2.3, ECA is a central element in acute and chronic rejection. Andreassen and colleagues found that soluble P-sel and VCAM-1 remain at high levels for up to 2 years post-transplant whereas soluble E-sel is not elevated during follow-up. There are however few data linking serum adhesion molecules to their shedding from the cardiac endothelium post-transplant. In a heterogeneous group of cardiac transplant biopsies P-sel expression was very sparse (Steinhoff, 1991). Further studies are therefore required to establish the relationship between adhesion molecules and chronic rejection. There is now little doubt that subclinical inflammation persists in many transplant recipients. For example, those who go on to develop early allograft vasculopathy have markedly higher levels of serum C-reactive protein (Pethig, 2000).

Our study showed a reduction in Thr expression across transplantation. The small size of the specimens precluded unfortunately an evaluation of other anticoagulant pathways, such as antithrombin III. In an experimental model of porcine liver transplantation, Thr depletion correlated well with ischaemic liver injury, possibly through apoptosis of sinusoid endothelial cells (Ejiri, 2001). Thr reduction, observed at the outset in our study, appears to improve during cold ischaemia but accentuates further at implantation. The fact that Thr variation did not reach statistical significance in the LV is probably due to the lack of follow-up beyond time point 5 (fig. 5.2.4). Thr depletion, which accentuated post-transplant (fig 5.2.4), may be a risk factor for allograft vasculopathy (Labarrere, 2000) but more follow-up is required to replicate this finding in our patients.
Although expression of adhesion molecules showed important time-dependent changes, no
difference was seen between the two ventricles. Birks et al showed that increased TNF-α
expression in the donor RV was related to subsequent development of RV failure (Birks, 2000).
Furthermore, the same group at Harefield showed that myocardial levels of TNF-α and IL-6 in
usable donor hearts were lower than in unused hearts but higher than in patients with advanced
cardiac failure (Birks, 2000). The LV was not biopsied in their study. As the authors point out, it
is difficult to speculate to what extent an inflammatory marker, whether measured in a tissue
lysate or histologically, has a cause and effect relationship with donor organ failure.

**Allograft failure and anti-inflammatory interventions**

The current study showed no correlation between endothelial phenotype, either intra- or
postoperatively, and allograft failure. One possible explanation is that the majority of hearts used
for clinical transplantation have ECA and this is compatible with satisfactory allograft function.
The endothelium releases neutrophil chemoattractants in cardiac transplantation (Oz, 1995), but
how this influences allograft functions is still to be elucidated. As shown in section 2.3, the
endothelium is an extremely complex structure and ‘endothelial activation’ is possibly a
misnomer. There is very probably a delicate chain of events between the presence or absence of a
surface molecule and execution of a cell programme. True activation may require binding of
several adhesion molecules, similar to co-stimulation of lymphocytes which involves more than
one signal. The influence of the individual genotype is once again emphasised. Increased
production of TGF-β in the allograft is associated with diastolic dysfunction, early vasculopathy
and decreased survival (Aziz, 2001). In order to improve clinical outcomes there is a need for
more studies in the acute phase of cardiac transplantation. Although the ability of the capillary
bed to support flow upon reperfusion appears pivotal to efficient recovery of pump function
(Manciet, 1995), the tissue phenotype associated with minimum ‘no reflow’ is still to be
described.

Aprotinin has recently been shown to reduce leukocyte extravasation in vitro in dose-dependent
fashion (Asimakopoulos, 2000). Our patients received aprotinin in a random fashion, based on a
history of previous sternotomy and on the surgeon’s preference beyond this recognised
indication. In this context the anti-inflammatory effect observed in our study should be
interpreted with caution. First of all, it is not known if aprotinin administered according to our
protocol reached the anti-inflammatory threshold. Secondly, the effect was found only for E-sel
and VCAM-1 in the LV, whereas the RV and Thr did not benefit. Given the symmetry of ECA in both ventricles across transplantation, the likelihood is that this is a chance finding. In summary, the anti-inflammatory effect of aprotinin in cardiac transplantation needs further evaluation in an experimental setup. The role of other anti-inflammatory interventions is discussed in a broader context in chapter 7.
5.1 Haematoxylin- eosin staining of myocardial biopsy.

5.2 Uniform CD31 staining of arterioles, venules and capillaries.

5.3 Abundant endothelial staining with VCAM-1 on both arterioles and capillaries.

5.4 P-selectin present on capillary but absent on arteriole. This is the same section as in panel 5.2.

5.5 Intense and uniform staining of microcapillaries with Thrb (score 5).

5.6 Scanty and discrete Thrb staining (score 1).
CHAPTER 6
CELLULAR STRESS MARKERS

This chapter presents and discusses results of myocardial and endothelial ‘stress markers’ in clinical cardiac transplantation.

6.1 PATIENTS AND METHODS

Seventeen hearts were studied with serial biopsies from both ventricles at the following time points:

1 – on initial assessment of the donor
4 – at the end of warm ischaemic time
5 – after 10 minutes of reperfusion
6 – at 1 week postoperatively.

The patients enrolled in the study are described in a tree diagram on the next page. Five hearts without palpable coronary artery disease were turned down on functional grounds and the other 12 were transplanted (3 as domino). Ten donors were studied by our team and the other 2 organs were imported and followed up by us. The mean age of the 10 donors studied was 38.5±11.5 years. The organs were implanted as 10 heart and 2 heart-lung transplants, with an average ischaemic time of 187.2±35.5 minutes.

To rationalise the number of biopsies on the donor heart, tissue sampling at time points 2 and 3 was again omitted for this study. Postoperative biopsies were obtained from the 10 heart transplant patients at 1 week. Only 3 patients had evidence of rejection graded as IA according to the ISHLT classification. Four of the patients in this study had donor organ failure. In two of these cases the complication was rapidly fatal and the 1-week biopsy was replaced with a post-mortem biopsy obtained within 24 hours. Endothelial staining was labelled as present or absent on the arterioles and on non-muscularised vessels, whereas myocyte staining intensity was graded from 0 (absent) to 3. Control tissue from non-transplant patients was not available for this component of the study. For the same reasons outlined in the previous chapter (section 5.1) functional recovery is analysed in terms of allograft failure, as defined in section 3.6.
Comparisons were made with Pearson’s chi squared and Fisher’s exact tests and statistical significance was attributed when p was less than 0.05.

Flow diagram chapter 6

The patients enrolled in the stress markers study.

17 patients (3 live donors)

5 unused hearts (poor function) 12 transplanted hearts (3 domino donors)

10 HTx 2 HLTx

HTx, heart transplant; HLTx, heart-lung transplant.

6.2 RESULTS

Histological pattern

Haematoxillin-eosin staining showed normal tissue architecture, with no ischaemic injury or indirect, histological evidence of apoptosis. Expression of stress markers in the peritransplant phase is summarised in table 6.1. Hsp70 and bcl-2 were generally absent in donor cardiac tissue in biopsies 1, 4 and 5, but bcl-2 appeared occasionally on intravascular and perivascular lymphocytes (panel 6-1; histology panel 6 is at the end of chapter 6). (Hsp70 was detectable intraoperatively in only 2 hearts. In 1 case it was expressed weakly in the endothelium and myocardium of L1/R1 biopsies but not subsequent to that. In the other patient it was strongly expressed in end-ischaemia and reperfusion biopsies.) Hsp70 was absent on the endothelium but present in all myocytes with variable intensity at 1 week (panel 6-2). In contrast, iNOSs stained both the endothelium and the myocytes in all peri- and postoperative biopsies (panel 6-3 and -4). Similarly, bax stained positive on all myocytes, most capillaries and some arterioles in all biopsies (panel 6-5 and -6). The 3 domino donors did not differ markedly in their expression of the above markers. A notable difference was the absence of bax in L1/R1 biopsies. Only 3 hearts stained positive for bax in donor biopsies and they were all from brain-dead patients.
Table 6.2.1  Summary of expression of stress markers on endothelium and on myocardium in peritransplant biopsies.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Intraoperative</th>
<th>Postoperative 1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endothelium</td>
<td>Myocytes</td>
</tr>
<tr>
<td>Bel-2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bax</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Hsp-70</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>INOs</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Trend over time and RV vs. LV**

The time-dependent variation was statistically significant for both endothelium and the myocardium. Bax, the marker for which an endothelial variation was seen over time, had a tendency to be upregulated in muscularised (p=0.06) and non-muscularised vessels (p=0.03) (Figure 6.2.1). The intensity of myocyte staining for iNOs increased from grade 2 to 3 during transplantation in both RV and LV (p=0.02) (Figure 6.2.2). Similarly, the intensity of myocyte staining for bax increased from grade 1 to 3 over time in both ventricles (p<0.001) and remained statistically significant when RV 1-week biopsies were included (p=0.004) (Figure 6.2.3).

**Figure 6.2.1**  Expression of Bax on RV endothelium. Data shown as percentage of cases with positive/negative staining at each time point.

(A) Muscularised vessels, p=0.06.
(B) Non-muscularised vessels, \( p=0.03 \).

![Bax bar chart](image)

**Figure 6.2.2** Perioperative RV myocyte staining for iNOS. All cases stained positive and data are shown as percentage of cases ranging from 1 (low intensity) to 3 (high intensity). The intraoperative variation includes time points 1 to 5 and is statistically significant \( (p=0.02) \). The intraoperative LV myocyte staining had an identical pattern \( (p=0.02) \). When 1-week results are included the overall variation is not statistically significant \( (p=0.34) \).
**Figure 6.2.3** Perioperative RV myocyte staining for Bax. All cases stained positive and data are shown as percentage of cases ranging from 1 (low intensity) to 3 (high intensity). The intraoperative variation includes time points 1 to 5 and is statistically significant (p<0.001). The intraoperative LV myocyte staining had an identical pattern (p<0.001). When 1-week results are included the overall variation remained statistically significant (p=0.004).

---

**Donor organ failure**

There was no relationship between expression of iNOs, bax and hsp70 in the endothelium or the myocardium in biopsies 5 or 6 and subsequent development of donor organ failure. None of the 3 domino recipients developed allograft failure.

**Unused donor hearts**

Like in used donors, hsp70 and Bcl-2 were absent from all unused donor hearts. There was no difference in endothelial staining for bax between used and unused donors. Compared to transplanted hearts, unused donors had less endothelial staining but more prominent myocyte staining for iNOs (Figures 6.2.4 and 6.2.5). Similarly, unused donors had significantly more intense bax staining of myocytes (Figure 6.2.4). It is difficult to draw conclusions about the influence of brain death on the markers studied, since we only had 3 domino donors. On simple inspection of their data the pattern of expression does not seem to be different from those of brain dead donors. Occasionally the expression was somewhat surprising (the only donor with bax
myocyte staining higher than grade 1 in both ventricles was a domino – figure 6.2.3). Our conclusions therefore describe an average set of organs used in clinical transplantation.

**Figure 6.2.4** Expression of Bax and iNOS in RV myocytes of used (n=10) vs. unused (n=5) donor hearts. Data shown as percentage of cases with positive/negative staining.

(A) Bax, p=0.002. The pattern in the LV was similar (p=0.01).

(B) iNOS, p=0.004. The pattern in the LV was similar (p=0.004).
Figure 6.2.5  Expression of iNOS in RV capillaries in used (n=10) vs. unused donor hearts (n=5). Data shown as percentage of cases with positive/negative staining, p=0.02. LV data showed a similar pattern (p=0.09).

6.3 DISCUSSION

Histological pattern and the influence of brain death

This study analysed the transplanted human heart for the expression of NO, an important endothelial mediator, and of hsp70, a cytoprotective protein. By staining for the bax-bcl regulatory pair, we also wanted to assess the apoptotic balance perioperatively. The emerging role of apoptosis in transplantation and the rationale for studying the above ‘stress markers’ were discussed in section 2.3. Our results essentially show that clinical cardiac transplantation is associated with activation of bax and iNOS in both ventricles. The endothelium and the myocytes exhibit an apoptotic phenotype which appears to be largely unopposed by expression of bcl-2 or hsp70. One important limitation of our study is that apoptosis was not studied by other methods and documented as such (Saraste, 2000). It is again due to the small biopsy size that we were unable to perform multiple assays for apoptosis, such as DNA fragmentation tests or caspase quantification in tissue lysate.
Before discussing our results further, it is important to note the conclusions of Birks and Yacoub. They showed that donor hearts have an elevation of both TNF-α and IL-6, which was particularly marked in organs with poor left ventricular function (Birks, 2000). Proinflammatory cytokines expressed at such high levels may account directly for donor heart dysfunction and also for triggering subcellular defence responses. TNF-α is mentioned in this context not only in relation to its capacity to induce apoptosis but also because it is able to induce the other markers studied by us, hsp70 and iNOS. Thus, the second phase of TNF-mediated contractile impairment is thought to be mediated by NO produced by the inducible isoform (Meldrum, 1998).

Heat shock, or stress, proteins are families of highly conserved molecules that afford cytoprotection by intracellular assembly, folding and translocation of oligomeric proteins. The stress response is a universal adaptation that follows not only elevation in temperature but also oxidative stress, cytokine exposure, etc. (Pockley, 2001). Hsp70 is not constitutively present in the heart, but in heat-shocked rats hsp70 is predominantly induced in the endothelium (Amrani, 1998; Leger, 2000). There is a wealth of experimental studies showing a role for the hsp family of proteins in protecting transplanted organs against the ischaemia-reperfusion injury (Pockley, 2001). We have not found reports of hsp70 immunohistology in the brain dead donor. In postoperative biopsies from 15 patients (obtained at an unspecified time after transplant), Baba and colleagues showed that hsp70 was generally expressed by the myocytes but not by the endothelium (Baba, 1998). Our results show that the perioperative stress is not sufficient for early induction of hsp70 in the heart, with expression taking place sometime between reperfusion and 1 week postoperatively.

The present study was not designed to evaluate the prevalence of apoptosis in clinical transplantation, its contribution to allograft failure or its mechanism: via transmembrane (type I) or mitochondrial (type II) signals. In the quest for a marker of allograft function, we were mainly interested to see what the balance of regulatory proteins is. Bax was consistently expressed by myocyte and endothelia in all the hearts studied. The only exception was the L1/R1 biopsy, where bax was present on the endothelia in 3 brain-dead donors only. Bcl-2 neutralises the apoptotic tendencies of bax by formation of homo- and heterodimers (Badrichani, 1999). The reasons for the lack of anti-apoptotic bcl-2 in our patients are speculative. Perhaps cells adopt a survival programme which involves mediators other than bcl-2. A stepwise downregulation of the antioxidant gene bcl-2 may also be taking place in the allograft, as shown by the experimental
model of Grunenfelder and colleagues (Grunenfelder, 2001). It is not clear however to what extent this mechanism is also active in the human heart for two reasons: firstly, it is surprising that bcl-2 is absent even before ischaemia; secondly, caspase-3, which is the cleaving agent of bcl-2, has not been detected in human donor hearts by other investigators (Birks, 2000). In a canine experiment of ischaemia and reperfusion Zhao et al showed a similar imbalance of bax and bcl-2 (Zhao, 2000). The fact that such an imbalance is possible clinically is demonstrated by the overall satisfactory performance of the hearts studied by us. Depre and Taegtmeyer emphasise that a simultaneous perturbation of energy metabolism, contractile function and gene expression is necessary before the cells commit themselves to apoptosis (Depre, 2000). How the survival programme is achieved in the majority of allografts is not known. Interestingly, new evidence is accumulating to suggest that both the endothelium and the myocardium act in concert to preserve function. The ubiquitous CD31 (PECAM-1) molecule, demonstrated on the endothelia in all our cases, along with other cell surface markers such as osteoprotegerin and the 3 integrin seem to be part of an NF-κB-dependent range of pro-survival products (Buckley, 2001). As for the myocytes, their apoptotic stimuli are abundant on both cytokine (e.g. TNF-α) and mitochondrial (e.g. free radicals) pathways. Since programmed cell death is an energy-dependent process, severe energy depletion leads directly to necrosis. The levels of ATP, bax and bcl-2 required to progress to apoptosis are not defined. Our study suggests that a marked imbalance in favour of bax is compatible with allograft survival. Nevertheless, experimental studies showed that upregulation of anti-apoptotic bcl-2 is worth exploring as a therapeutic avenue to reduce endothelial activation (Badrichani, 1999) and enhance allograft function (Grunenfelder, 2001; Murata, 2002). The salutary activity of bcl-2 extends in fact beyond its anti-apoptotic effects through inhibition of NF-κB activation (Badrichani, 1999).

NO is a potent vasodilating autacoid whose role in transplantation is still under debate (Cannon, 1999). Of the three NO synthases, the inducible isoform is cytokine-regulated and not expressed in the normal heart (Lewis, 1996). One study measured mRNA iNOS in 26 donor hearts (including 10 dominos) and found expression in one heart only, from a brain-dead patient (Birks, 2000). That particular heart however did not express TNF-α, which is one of several triggers for iNOS transcription. In contrast, this study showed that all 10 donors evaluated had iNOS staining in the endothelium and the myocytes. This may be explained by the different methods employed, as there can be less than good correlation between mRNA expression and immunostaining (Lewis, 1996). Expression of iNOS by the 3 domino donors is difficult to explain. It may be related to the pre-existing intrathoracic inflammation observed in cystic fibrosis. Postoperative
expression of iNOs is in keeping with other studies which reported increases at different time points in the transplanted heart (Lewis, 1996; Wildhirt, 2001; Vejlstrup, 2002). Together with the elegant experiment of Skarsgard et al. these results suggest that the transplanted heart has a profound NO-mediated vasodilatation, resulting from both eNOs and iNOs, which may further influence allograft performance and subsequent vasculopathy (Skarsgard, 2000).

**Trend over time and RV vs. LV**

The current study showed a gradual increase in the number of cases staining positive for bax and iNOs (Figures 6.2.1 to 6.2.3), with no difference between the two ventricles. It is to our knowledge the first study to report the time trend for expressing these markers in the human allograft. The pattern observed suggests that the maximal inflammatory insult takes place between reperfusion and 1-week postoperatively, although the end result is probably cumulative. These data parallel the observations of other groups who studied apoptosis in a murine model (Scarabelli, 2001). After ischaemia and reperfusion, programmed cell death of the myocytes is preceded by that of the endothelium, the first to be affected being the small coronary vessels. Endothelial apoptosis reached a peak at 60 minutes after reperfusion. Cardiomyocyte apoptosis was initially taking place in a perivascular location and it assumed a more homogenous distribution at 120 minutes post-reperfusion. Taken together, these results indicate a potential role for endothelial-released factors which would act in paracrine fashion to propagate injury to the cardiac parenchyma.

**Donor organ failure**

In patients with chronic heart failure there is overexpression of bcl-2 but not bax compared to normal hearts (Olivetti, 1997). The work of Olivetti is limited in this conclusion by the small number of patients in each subset of the study and by the use of imperfect controls – 3 out of 11 controls were brain-dead donors. In contrast to this pattern, we found overexpression of bax in human donors and no bcl-2. The inability to correlate expression of the stress markers that we studied with donor organ performance may be related to the small number of observations or to the relatively crude definition of allograft failure used.

The current study reports on the expression of 'stress markers' before the onset of acute or chronic rejection. Others showed that acute rejection is associated in the human allograft with
iNOS expression and apoptosis (Szabolcs, 1998). Inhibition of iNOS in the acutely rejecting murine heart minimised the reduction in left ventricular diastolic filling (Soto, 2000). In separate experiments with iNOS-null recipient mice, other groups showed that iNOS increases allograft survival, possibly by stabilising the Weibel-Palade bodies (Qian, 2001). The rejecting allograft performs poorly for obvious reasons. When iNOS and cardiac performance are analysed in the absence of rejection the results are conflicting.

Evidence was produced for a relationship between iNOS expression and systolic and diastolic dysfunction in the human allograft (Lewis, 1996; Paulus, 1997). All recipients express iNOS mRNA at some stage after transplant, but this is more frequent in the first 6 months (Lewis, 1996). Furthermore, Wildhirt and colleagues examined coronary flow reserve in the first year post-transplant and demonstrated microvascular dysfunction in 30% of recipients in relation to increased iNOS (but not eNOS) mRNA expression (Wildhirt, 2001). The vasoplegia observed in the murine allograft involves eNOS- and iNOS-based NO production (Skarsgard, 2000). This enhanced vasodilatation naturally predisposes to interstitial oedema and ventricular stiffness. Taken together, these findings have important long-term consequences for the transplanted heart, since staining for iNOS persists for longer than 3 years in the absence of rejection (Vejstrup, 2002). Early reperfusion after cardiopulmonary bypass is insufficient to induce iNOS mRNA expression in the ischaemic human myocardium (Nelson, 2002). The inflammatory load of the transplanted heart may therefore be higher than in conventional cardiac surgery, with iNOS induction starting as early as in the donor.

Unused donor hearts

Poorly functioning donor hearts have more intense myocyte staining for bax and iNOS but much less frequent iNOS expression on the endothelium (Figures 6.2.4 and 6.2.5). It appears that in some hearts brain death and the ensuing circulatory abnormalities induce expression of iNOS and bax beyond a threshold which is compatible with satisfactory pump function. A cause and effect relationship is however not demonstrated in the current study. Before concluding that iNOS is deleterious for cardiac contractile function, results from two recent experiments should be noted. Transgenic mice with marked overexpression of iNOS were surprisingly similar to wild type controls in terms of cardiac performance and myocardial energetics (Heger, 2002). Conversely, genetically iNOS deficient mice were very vulnerable to ischaemia and reperfusion, the
mechanism probably being perturbed transcription of NF-κB-dependent inflammatory mediators (Zingarelli, 2002).

Finally, in none of the studies in this dissertation cause of BSD and time from BSD to explantation were analysed in relation to cellular markers. This is for two main reasons. Firstly, the existing literature suggests that BSD can cause substantial cardiac damage irrespective of its aetiology. Secondly, the magnitude and rate of increase in intracerebral pressure and the exact moment when brain stem activity ceases are variables which are difficult to measure clinically.
6.1 Bet-2 expression in a reperfusion biopsy. There is positive staining of activated lymphocytes, demonstrating that the antibody is working.

6.2 Hop-70 in a 1-week biopsy showing intense staining of myocytes.

6.3 Intense iNOS staining of endothelium and myocytes in an intraoperative biopsy.

6.4 Intense iNOS staining of endothelium and myocytes in a 1-week postoperative biopsy.

6.5 Bax staining of a LV donor biopsy. Note positive central vessel and granular staining of surrounding myocardium.

6.6 RV end-ischaemia biopsy showing intense Bax staining of microvasculature and surrounding myocardium.
In the current study we have shown that adenine nucleotide metabolism is temporally similar in both ventricles across transplantation. Brain-dead donors do not have an impairment of energetic metabolism in gross quantitative terms. Contractile function appears to be preserved over a wide range of HEP contents. Prior to implantation there is almost a dissociation between biochemical and contractile function in the human donor heart, with little correlation between contractile indices and HEP values. However, failing organs sustain the biggest injury at reperfusion and generally have difficulty in replenishing their HEP stores. The endothelium is probably involved in the dysfunction seen. Cardiac transplantation (including domino) is associated with marked endothelial changes, with no difference once again between the two ventricles. Endothelial activation is present as early as after brain death and reaches a peak at reperfusion. Activation persists in the postoperative period even in the absence of rejection. The most remarkable changes were seen for P-sel (type 1 activation), VCAM-1 (type 2 activation) and thrombomodulin. Upregulation of the Bax and iNOS pathways is present in the endothelium from brain-death onwards, with the myocardium being also affected in time-dependent fashion. This however is compatible with normal allograft function. Heat shock protein is absent from the endothelium in the acute phases of transplantation but is uniformly present in myocytes at 1-week postoperatively. Bcl-2 seems to have no role to play in the acute or early postoperative phase. None of the biochemical or endothelial markers studied was sufficiently predictive of donor organ failure, defined as the need for targeted therapeutic measures.

Limitations of the study

Our conclusions are limited by a number of factors. In terms of patient enrollment, we were unable to obtain a simultaneous biopsy and conductance catheter evaluation in many cases. This was often due to the time constraints of the transplant procedure or to lack of consent from the donor family or from the recipient. When consent for research was granted, we were always guided by the transplant clinicians, who judged the appropriate level of invasive studies in any given case. The biopsy technique itself is theoretically prone to sampling error. By biopsying the
same area it is possible in some cases to sample tissue which suffered microtrauma from the previous biopsy. Standard histology performed on all biopsies showed that this was not the case, as the architecture of the tissue was intact. Furthermore, the symmetrical trend observed in both ventricles suggests a true biological variation and almost acts as an internal control of our experiment. In terms of evaluating a dynamic and intricate process, serial histology offers little more than snapshots. We aimed to find a compromise between minimal sampling over time and getting the maximum amount of information. Due to the limited amount of tissue available, we were therefore able to report results only in non-muscularised vessels, and nucleic acids or the corresponding proteins were not measured in tissue lysates. Leukocyte infiltration of the tissue was not quantified either. The last intraoperative biopsy was after 10 minutes of reperfusion, which is probably before many markers reach their peak expression (Ichikawa, 1997). Since rejection was such a rare event in our series we could not correlate peritransplant endothelial activation with this outcome. Our main focus for the biochemical component of the study was on the relationship between high energy phosphates and functional recovery, and so the evaluation did not include catabolic products (e.g. lactic acid, inosine) or serum markers of cellular injury (troponins). Finally, the biochemical comparison between hearts from live and brain-dead patients included 3 patients with LV hypertrophy from aortic stenosis – this methodological flaw was expanded upon in chapter 4.

**Strengths of the study**

The project linked clinical aspects, such as donor organ failure, to recent experimental findings suggesting protective or deleterious roles for various tissue markers. Both ventricles were serially sampled during the transplant operation, starting as early as in the brain-dead donor, and the follow up extended up to 3 months. The load-independent assessment was focused on the right ventricle, reflecting its more vulnerable position in the postoperative period. The Papworth protocol of donor management ensured that the evaluation was done in optimised donors, with presumably good coronary perfusion pressure. We feel that the vasoplegic effect of brain death (Szabo, 2001) has been well counteracted, at least at the time of our assessment. All donors assessed by the Papworth team were managed according to our protocols and the donor hearts retrieved by other teams were accepted based on established criteria (Stoica, 2002). To what extent varying loading conditions after brain death render a donor heart unusable is still to be established.
The current study provides a more accurate description of the donor heart physiology but is unable to offer a predictor of donor organ failure. It is interesting to note however other recent evidence which indirectly supports our findings. When the time from the incident leading to brain death to the time of donor aortic cross clamping was analysed, it was found that recipients in which this interval is less than 72 hours have a survival advantage (Cantin, 2002). It is logical therefore for the retrieval team to arrive at the donor hospital as early as possible and to counteract the donor's physiological decay as best as they can. Although donor organ failure and allograft vasculopathy have generally been analysed as separate outcomes, they may be two separate ends in a single spectrum of injury. Echocardiographic fractional shortening in the LV during the first week is inversely correlated with narrowing in the epicardial vessels one year after transplantation (Bolad, 2002). These authors also conclude that better protection against ischaemia-reperfusion injury may reduce the incidence of chronic rejection. How can this be achieved in coming years?

**Future directions**

In the last decades cardiovascular physiology has been somewhat a victim of its own success. Myocytes represent only one third of the heart cells, and the paradigm that cardiomyocyte preservation automatically leads to good pump function and good outcomes is about to change. It is now clear that the acute injury propagates beyond the endothelium to the smooth muscle cell and the matrix, an area that has received virtually no attention so far in preservation strategies. The in vitro stretch experiments are unable to provide the complex environment in which myocytes and fibroblasts interact in autocrine, paracrine and direct fashion (MacKenna, 2000).

It has gradually become apparent that different pathways of reperfusion injury - complement (Collard, 1998), cytokine (Azzawi, 1999), lymphocyte (Land, 1999), neutrophil (Jordan, 1999), or reactive oxygen species-mediated (Kunsch, 1999) - meet at closely linked subcellular levels. Simplicity in any of these models remains out of sight, especially when some cytokines or cytoprotective pathways were shown to have a dual role. For example, the effect of tumor necrosis factor on post-ischemic cardiomyocytes is concentration-dependent and ranges from adaptive reduction in contractility to deleterious inflammation (Sack, 2000). Likewise, induction of heat shock proteins may have dual consequences, as the highly immunogenic and crossreactive hsp60 is released into the circulation following cardiomyocyte ischemia (Schett, 1999). More
clinical studies into peritransplant cytokine biology could pave the way for pharmacological modulation.

Myocardial protection intraoperatively and immunosuppression after the operation have, alone, been unable to generate improved long-term outcomes for cardiac transplantation. The endothelium may hold a pivotal position in the injury sequence to the allograft holds and should be one of the main targets for intervention (Table 7.1). The next decade is therefore faced with a twofold challenge. First of all, elucidation of the mechanisms involved in the convergent pathways should permit better cytoprotective interventions. Secondly, understanding the contribution of each of these mechanisms at the stages between brain death and reperfusion will enable the development of comprehensive strategies to preserve whole organ function.

Table 7.1 Targets, agents and strategies to reduce endothelial injury in cardiac transplantation.

<table>
<thead>
<tr>
<th>Targets</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Cytokines</td>
<td>(Jordan, 1999; Wang, 1998)</td>
</tr>
<tr>
<td>- Complement</td>
<td>(Klima, 2000; Baldwin, 2000)</td>
</tr>
<tr>
<td>- CAMs</td>
<td>(Lefer, 1999; Jordan, 1999; Pinsky, 1996; Wang, 1998; Jaakkola, 2000)</td>
</tr>
<tr>
<td>- TNF-α</td>
<td>(Meldrum, 1997; Birks, 2000)</td>
</tr>
<tr>
<td>- MHC molecules</td>
<td>(Rose, 1998)</td>
</tr>
<tr>
<td>- Coagulation signalling (e.g. PARs)</td>
<td>(Poullis, 2000; Napoli, 2000)</td>
</tr>
<tr>
<td>- Adenosine signalling</td>
<td>(Meldrum, 1997)</td>
</tr>
<tr>
<td>- NO synthesis</td>
<td>(Wildhirt, 2000; Cannon, 1999)</td>
</tr>
<tr>
<td>- Oxidative cascades</td>
<td>(Land, 1999; Kunsch, 1999)</td>
</tr>
<tr>
<td>- Transcription factors (AP-1, NF-κB)</td>
<td>(Boyle, 1999; Feeley, 2000; Feeley, 1999)</td>
</tr>
<tr>
<td>- Fas-FasL system</td>
<td>(Martinez, 1999, Thomas, 2000)</td>
</tr>
<tr>
<td>- Anti-apoptotic genes (bcl-2 family)</td>
<td>(Badrichani, 1999; Grunenfelder, 2001)</td>
</tr>
<tr>
<td>- Cytoprotective genes (Hsp70, HO-1)</td>
<td>(Hayashi, 1999; Hiratsuka, 1999)</td>
</tr>
</tbody>
</table>
Methods

- **Antioxidants** (Feeley, 1999; Land, 1999; Kunsch, 1999)
- **Complement inhibition** (Baldwin, 2000; Klima, 2000)
- **Protease inhibitors (e.g. aprotinin)** (Gott, 1998; Poullis, 2000)
- **Heparin-coated circuits** (Gott, 1998; Wan, 1999)
- **Preconditioning** (Meldrum, 1997)
- **Heat shock** (Hiratsuka, 1999)
- **Cytokine and CAM removal (ultrafiltration)** (Grünenfelder, 2000)
- **Leukocyte depletion (filters)** (Gott, 1998; Kirklín, 1999)
- **Monoclonal antibodies** (Pinsky, 1996; Poston, 1997; Ardehali, 2000; Kung, 2000)
- **Transcription inhibitors** (Feeley, 1999; Feeley, 2000; Boyle, 1999)
- **Blockade of T cell activation** (Takada, 1999; Land, 1999)
- **Gene therapy** (Feeley, 2000; Hiratsuka, 1999; Ardehali, 2000)
  - blockage
  - induction
  - transfection

Strategies

- What, how much and when?
- Immunosuppressives, anti-inflammatories or both?
- Start in the brain-dead donor? (Land, 1999)
- What cardioplegia, what adjuvants? (Luciani, 1999; Carrier, 1996)
- What storage solution, what adjuvants? (Kirklín, 1999; Jahania, 1999; Stoica, 2001)
- Adjustment of treatment to genotype? (Hutchinson, 1998; Densem, 2000)
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APPENDIX 1

STATEMENT OF ORIGINALITY

The work embodied in this thesis is part of a multidisciplinary project in clinical transplantation. The original idea to characterise the transplanted heart in terms of biochemical, endothelial and haemodynamic function belongs to the principal investigator, Mr S. R. Large (Dept. of Cardiothoracic Surgery and Transplantation, Papworth Hospital). The other main collaborators were Dr. M. Goddard (Dept. of Pathology, Papworth Hospital), Dr. T. Kealey (University Dept. of Clinical Biochemistry, Cambridge) and Prof. A. N. Redington (Cardiothoracic Department, Great Ormond Street Hospital, London). This clinical project was based at Papworth Hospital. Ethics approval was obtained according to strict protocols from the Huntingdon Local Research Ethics Committee (protocol 99/362).

The author personally organised and performed the following:

- Study design for measurement of energy metabolism and endothelial activation
- Ethical protocols
- Research grant application
- Tissue collection from the operating surgeons at the donor and the recipient operations
- Complete biochemical analysis, including animal experiments and assay validation
- Clinical data acquisition and analysis design

In addition, the author participated in the validation of the monoclonal antibody techniques for immunohistology and in histology slide interpretation. The endothelial staining for all the tissue was coordinated by Dr C. Atkinson and performed in semi-automated fashion in the Dept. of Pathology at Papworth Hospital. Dr Atkinson was also the main slide interpreter, and Dr. M. Goddard ensured reproducibility by random verification. (All histology slides were read in blinded fashion in relation to the clinical data.) Drs D. K. Satchithananda and P. A. White acquired and interpreted the conductance catheter data. Mrs. S. Charman and Dr. L. Sharples from the MRC Biostatistics Units, Cambridge, provided assistance with repeated-measurements ANOVA and correction for repeated statistical testing.
APPENDIX

APPENDIX 2
ABSTRACTS AND PUBLICATIONS RESULTING IN RELATION TO THE THESIS

Abstracts


endothelial activation and contractile function. European Association of Cardiothoracic Surgery, Monte Carlo 2002 (shortlisted for Young Investigator’s Award).


**Papers**


