Title: The Study of Cardiovascular Tissue Processing in the United Kingdom

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The Study of Cardiovascular Tissue Processing
in the United Kingdom

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THE STUDY OF CARDIOVASCULAR TISSUE PROCESSING
IN THE UNITED KINGDOM

by

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ABSTRACT

The study of United Kingdom cardiovascular tissue banking practice has required research into areas of cardiovascular tissue banking that have previously not been clarified, explored in detail or reported. The pressures which have been the driving forces for change in tissue banking in recent years have been identified and the National shortage of cardiovascular tissue donors in relation to the increasing surgical demand has been quantified for the first time in the UK. An examination of the evolution of cardiovascular tissue banking enabled subsequent identification of the inconsistencies reported and the importance of recording small differences in processing details. A detailed overview of current UK cardiovascular tissue processing methodology was collated which, for the first time, established the differences in current practice. The study subsequently identified tissue bankers concerns. The study has identified areas where there may be a need for control, standardisation and improvement of methodology and has highlighted areas where it seems to be agreed that there is need for optimisation of processing technique. The study has also uncovered areas where there is a lack of understanding amongst tissue bankers regarding cryopreservation principles such as freezing and chilling injury and also confirmed the need for more careful control and measurement of techniques used such as cooling and warming rates which may or may not affect the quality of the tissue processed.

The study confirmed that processing techniques were generally empirically based but despite this, the processed tissue seems to be effective. Further work has been suggested which includes recommendations for methods which could be used to monitoring tissue quality and subsequently for harmonisation of tissue banking procedures.
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1.0 INTRODUCTION

Cryobiology is a fascinating sector of biology in which there is still a vast amount yet to be learnt. The term ‘Cryobiology’ was first coined by the late Sir Alan Parkes who defined it as the study of ‘frosty life’. The first book devoted to the subject entitled ‘Life and Death at Low Temperature’ was written in 1940 (Luyet and Gehenio, 1940). The object of ‘Cryopreservation’ is to minimise damage during low temperature freezing and enable prolonged storage. Scientists working with plants, animals, and medicine have been keen to develop this area of biology in order to capitalise on the tremendous benefits that it could bring to their areas of speciality. There are currently successful applications of cryobiology in the fields of plant and animal biology and conservation, in areas of biomedicine including human gamete and embryo cryopreservation, transfusion medicine and haematology, cell and tissue banking and cryosurgery.

The most significant ‘early’ advances in cryobiology were described more than fifty years ago by the classical biologist Audrey Smith (Smith, 1961). The most critical development in cryobiology is recognised to be that made by the ‘Pioneers’ who reported (Polge, Smith & Parkes 1949) that they found that addition of glycerol prior to freezing increased the proportion of fowl spermatozoa that could survive and be revived after vitrification at - 80°C. This was then followed up by a report (Smith, 1950) which confirmed that glycerol was an appropriate cryoprotectant for red blood cell freezing. Mouse haemopoietic stem cells were later preserved and then used to successfully treat animals exposed to a lethal dose of ionising radiation (Barnes and Loutit, 1955). The cryoprotectant dimethyl sulphoxide (DMSO) was introduced as an improved alternative to glycerol (Lovelock and Bishop, 1959). It more rapidly diffused into cells and this induced less osmotic stress. DMSO is now the most commonly used cryoprotectant.

Scientists have since repeated and reviewed early experimental studies. A wide variety of methods of cryopreservation have now been successfully used which minimise damage during low temperature freezing and storage (Fuller, Lane and Benson, 2004).

Minimising changes in genetic material of the cells concerned are a primary focus and the required patterns of gene expression must be reliably reproduced in recovered material (Watson and Holt, 2001).

1.1. PRINCIPLES OF CRYOBILOGY

In conventional cryopreservation, water which is the major component of all living cells, changes to ice and cellular metabolism ceases. A level of stability is imposed upon the system by ultra-low temperatures
(i.e. <-130°C) and storage is at, or close to, -196°C using liquid nitrogen (or liquid nitrogen vapour immediately above it), as a practical and convenient cryogen.

When cells or cellular systems are exposed to these sub zero temperatures this is accompanied by ice formation and potentially, by freezing damage. Freezing damage will depend upon the amount of free water in the system and the extent it is able to crystallize during freezing. In the initial stages of freezing, ice is formed in the extracellular environment which causes an increased salt concentration. As a result there is an osmotic imbalance intracellularly and water passes to external environment by osmosis. The cell dehydrates which can be damaging or lethal.

Cryopreservation studies focus upon the complex interaction of several variables which must be optimised for each given cell type, suspension or tissue. These variables include cryoprotectant, cooling rate, storage temperature and thawing rate. Failure to optimise these variables will result in injury which may be irreversible and ultimately detrimentally affect cell recovery or performance post re-warming. Understanding the mechanism of chilling ad freezing injury is therefore fundamental.

1.2. CHILLING INJURY

‘Chilling Injury’ can be defined as the damage incurred by living cells during exposure to sub-physiological temperatures (Watson and Morris, 1987). Many types of cells and tissues are damaged when they are chilled (cooled) to temperatures which are close to, or < 0° C without freezing. Chilling injury was first used by Botanists in the 18th century (Sargent, 1764 & 1764) to describe the irreversible plant damage after chilled storage at temperatures above 0° C. However, despite considerable studies to develop an understanding of the biomechanical mechanisms responsible for initiation of Chilling Injury, there is still no general agreement on the cause, nature or primary event triggering this low temperature damage.

In 1934 ‘temperature shock’ was first used (Drobnis 1993) to describe the irreversible damage to mammalian sperm when cooled rapidly (> 2-3 ° C per minute) to below body temperature. Both phenomena are more commonly described by the terms ‘direct chilling injury’ or ‘cold shock’ which are now accepted as the affect observed quickly when temperature is reduced quickly and is dependant on cooling rate.

A second type of chilling injury is termed ‘indirect chilling injury’. This is independent of rate of cooling but usually observed after long periods of exposure at low temperature.

In some cases, it is difficult to classify the effects of cooling as due to exclusively cold shock or indirect chilling injury. Therefore these two categories may well be related and differences in cell response may be quantitative rather than qualitative.
1.2.1. ‘Direct Chilling Injury’ or ‘Cold Shock’

There are several essential features of cold shock:-

- It affects all types of cell provided that they are cooled rapidly enough to a sufficiently low temperature
- Cellular viability is dependant upon the rate of cooling, with more injury occurring at ‘rapid’ rather than ‘slow’ cooling
- It is almost independent of the rate of warming
- It is increased as the period of incubation at the reduced temperature is extended
- Loss of membrane permeability occurs following rapid cooling, but, in some cases, this may be reversed upon re-warming
- The response of any cell type may be modified by adapting the culture conditions before cooling or by the addition of specific compounds

Generally accepted hypotheses suggest that the primary response to direct chilling is the lipid phase changes in cellular membranes which then permit the affected tissue to be further susceptible to development of secondary disorders. The first systematic evidence that cold shock results from lipid phase transitions in cell membranes was shown using Fourier transform infrared spectroscopy to measure the transitions in marine shrimp and pig sperm (Drobnis 1993). It was believed that membrane lipid phase transitions leading to loss of membrane integrity and physiological dysfunction were the affects induced at low temperatures (Lyons, 1973). This theory had to be modified however, to account for observations of tissue with high melting phospholipids that appeared to be ‘chill sensitive’. Young drosophila embryos (<3hrs at 25°C postoviposition) have been shown (Steponkus) to be sensitive to chilling but older embryos (>4hrs at 25°C) seem to be ‘chill resistant’.

It is now more generally agreed that, during low temperature exposure, discreet lipid domains are changed physically and small amounts of gel phase or ordered membrane lipids can induce physiological imbalances.

Cryopreservation of many species of fish sperm has been successfully applied however; there has been no success with fish embryo cryopreservation (Billard 2001). The studies of cryopreservation of fish oocytes (Pearl 2000), illustrated that the chilling sensitivity is related to lipid phase transition. A further systematic study (Isayeva 2004) confirmed that zebra fish oocytes are very sensitive to chilling with survival decreasing with decreasing temperature and increased exposure period.
Liu (2008) has recently shown that partial removal of yolk of zebra fish embryos can mitigate the effects of cold shock injury. These recent developments in understanding the nature of chilling sensitivity should help in the optimisation of cryopreservation protocols.

1.2.2. ‘Indirect Chilling injury’

Indirect Chilling Injury is usually evident following a relatively long exposure at reduced temperatures, and it is independent of the rate of cooling. However, it can manifest rapidly (e.g. in mammalian oocytes and embryos and some early stage insect embryos). This may be as a result of the modification of the structure and activity of both proteins and lipids following the reduction in temperature. Low temperature chilling may affect the structure and function of proteins by decreasing the rate of enzyme activity and the denaturation of proteins.

A reduction in temperature is likely to affect different enzyme reaction rates to a different extent. The effects of alterations in enzyme activity on a series of linked reactions are complex and this may cause uncoupling of metabolic pathways.

The disorder of metabolic and enzymatic processes can be especially detrimental in fast developing embryos such as zebra fish (Liu 2001), and such injury will increase rapidly at lower temperatures because the loss of co-ordination may be increased with decreasing temperature.

Parkin (1988) suggests that loss of membrane integrity may lead to diminished regulatory control of cellular energy generation, membrane semi permeability, and metabolite and ion compartmentation and further influence membrane enzyme activities. The hypothesis suggesting that the primary cause of chilling injury may be due to the redistribution of cellular calcium transducer (Minorsky 1985) is founded upon calcium bearing the secondary messenger role for many cellular functions. The inability to maintain calcium gradients due to loss of membrane semi permeability may increase the ultimate loss of metabolic control. Chilling stress has also been shown to evoke an elaborate membrane repair response via activation of hydrolytic and oxidative enzyme activities, which may lead to increased fluidity at low temperatures. The rate of membrane repair may determine whether the damage is reversible.

Lipid peroxidation may also cause irreversible chilling injury and the effects similar to senescent processes of free radical damage to tissue and ultimately result in rigidification of the membrane.

Chilling injury remains a significant obstacle to successful mammalian oocytes cryopreservation (Woods 2004) despite the introduction of strategies to improve cryopreservation (Ledda 2007). More recently, alterations in the oocyte membrane have been shown to underlie the developmental competence in
mammalian oocytes exposed to the thermal stress of chilling injury and also similarly to that of heat shock (Arav and Roth, 2008).

Until the mechanisms of chilling injury are fully understood, it seems that efforts to ameliorate the associated problems will be unlikely to succeed.

1.3. FREEZING INJURY

Liquid water is essential to the structure and function of living cells, and solidification of water by freezing is often destructive or even lethal to cells.

The stresses to which cells are exposed during freezing mainly result from:

- The mechanical effects of extra cellular ice crystals at cell surfaces especially in tissues with cellular interconnections
- Alterations in the physical properties of solutions outside the cell, including the concentration of solutes which results from the nucleation of a proportion of extracellular water inducing osmotic stresses and toxicity effects.
- Intracellular freezing, if it occurs

When the temperature is lowered to approximately 0 to -5°C, the cells and their surrounding medium remain unfrozen both because of super cooling and the depression of the freezing point by the protective solutes that are frequently present.

Between -5°C and -15°C, ice forms in the external medium, spontaneously or as a result of deliberate 'seeding' of ice. But the cell contents remain unfrozen and super cooled.

The presence of ice in the external medium significantly affects the composition of the unfrozen fraction of the extracellular solution. When extracellular ice forms the solute concentration increases as the temperature decreases and the ice phase grows. This will result in a chemical potential imbalance between the biomaterial and the unfrozen external solution. The super cooled solution in the cells has, by definition, a higher chemical potential than that of water in the partly frozen extracellular solution, and in response to this difference in potential, water will then flow out of the cell and freeze externally.

The subsequent physical events in the cell depend on the cooling rate:

- If cooling is sufficiently slow, the cell is able to lose water by exosmosises and concentrate the intracellular solutes sufficiently to prevent super cooling and maintain chemical potential balance
between intracellular and extracellular water. The cell will dehydrate and intracellular freezing is
prevented.
- If cooling is too slow, cells will experience ‘solution-effects’. i.e. the cells are exposed to the changes
in the hypertonic liquid environment as ice crystallises out of the solution.
- If cooling is very slow, cells will be exposed to extreme hypertonic conditions in the residual liquid
fraction for a sufficiently long period to be damaged before reaching the ‘safe’ ultra-low temperatures
used for storage.
- If the cooling is too rapid, the rate at which the chemical potential of water in the extracellular
solution decreases is much faster than the rate at which water can diffuse out of the cell. The
remaining intracellular water will then freeze. Intracellular ice formation (IIF) is usually lethal to the
cell.

1.3.1. Extracellular freezing Injury

Biological systems will be subjected to a series of stresses arising from the formation of extracellular ice.
There have been several theories suggested to explain the cause of the damage although the exact
mechanisms of cell damage during extracellular freezing are not yet fully understood.

*Increase in concentration of extracellular electrolytes*

Lovelock suggested, on the basis of his work freezing red blood cells (Lovelock, 1953), that freezing
damage could be largely accounted for by the increased salt concentration, particularly electrolytes in the
unfrozen aqueous fraction and this led to an increase in concentration of intracellular electrolytes. There was
evidence that hypertonic salt solutions caused denaturation of lipoproteins, and that this could induce
haemolysis in red blood cells. At higher electrolyte levels, the weak molecular interactions responsible for
maintaining the native protein structure may be modified and this may result in the denaturation of enzyme
activity.

*Reduction in cell volume*

Meryman and his associates however, proposed the ‘minimum cell volume theory’ (Meryman 1968) to
account for cellular damage associated with freezing and thawing. Like Lovelock’s theory, it proposed that
high salt concentration during freezing was important, but Meryman suggested that salt concentration was
indirectly responsible for damage. He suggested that the high extracellular osmolarity caused cells to be
dessicated beyond their limit, or shrunken below their ‘minimum cell volume’, and this would be lethal to the
cells.
**Mechanical effects of ice**

It has been shown that cells may also be damaged by the mechanical interaction between the growing ice phase and cells sequestered between the shape and size of the ice crystals. Takamatsu (1999) observed that human prostrate primary adenoma cancer cells were destroyed by compression between ice crystals.

**Other factors**

Farrant (1977) suggested the hypothesis that the damage or survival of living cells during freezing and thawing is primarily linked to bulk water transport across membranes. Some physical factors such as pressure changes and formation of gas bubbles may also be involved in the destruction of the cells.

1.3.2. Intracellular freezing Injury

It is assumed that formation of ice inside the cell is inevitably lethal. Many studies suggest that intracellular ice formation (IIF) during freezing does correlate with death of cells (Karlsson, 1999 and Koebe 1999) whilst the specific mechanism of the injury and the interactions with ice recrystallization during re-warming are unclear. Mazur (1963) determined a formula for quantitive calculation of the amount of water in a cell and temperature. It involves variables such as cooling rate, surface-volume ratio, membrane permeability to water, and the temperature coefficient of the permeability constant. Numerical solutions to this equation give calculated water contents which permit predictions as to the likelihood of intracellular ice formation.

Apart from the presence of intracellular ice and the size of each crystal, the total amount of ice is another factor that may be relevant to cell survival.

There is strong evidence that some cells survive intracellular freezing providing that thawing is extremely rapid, especially through the range -30°C to 0°C. Some Antarctic species have been reported to have survived intracellular freezing as intact organisms but the underlying mechanisms for this are still relatively unknown. Antarctic nematode Panagrolaimus davidi is able to survive intracellular freezing. The nematode has a variety of mechanisms for its survival in such harsh an environment.

There are three possible mechanisms by which intracellular ice formation is believed to occur:-

1. Homogenous nucleation
2. Heterogenous nucleation
3. Seeding by the extra cellular ice

Homogenous nucleation temperature of a 1um droplet of pure water is -39°C. This increases by 2°C for a ten fold increase in droplet diameter. Solutes depress this by 3.3°C for each unit rise in osmolarity. Rall and colleagues (1983) showed that the range of homogenous nucleation temperature of cells is -38 to -44°C.
Heterogenous nucleation relies on the presence of intracellular nucleating agents. Franks et al (1983) produced evidence for this when he observed IIF at -31°C to -38°C in cells cooled in micro-droplets. Seeding seems to be the predominant mechanism for IIF in many cases as IIF occurs at median temperature of -10 to -20°C in many cell types.

1.4. THAWING INJURY

Although cells can be damaged due to crystallisation of ice during cooling, damage can also occur during warming. Small innocuous intracellular ice crystals in rapidly cooled cells will grow if warming is too slow, a process known as ‘recrystallisation’ and this can be lethal. Rapid thawing generally will improve survival. It has been shown (Acker 2003) that some cells such as Chinese hamster fibroblasts and canine kidney cells which have intracellular ice can survive if thawed very rapidly.

Cells cooled at rates faster than the optimal rate for intracellular ice formation are more sensitive to a decrease in the rate of thawing. Rapid warming results in complete thawing of the suspension before significant dehydration.

However, there are instances where slow thawing is less damaging than rapid thawing. Zhang et al (1989) observed this during studies using embryos of common carp (Cyprinus carpio). They showed that optimum survival required warming at a slow rate (8°C/min) but faster rates killed the embryos.

Ashwood-Smith (1980) and Farrant (1980) suggested that injury caused by rapid thawing was due to the presence of a small amount of intracellular ice which seals the cells and results in osmotically induced damaging water movements during the initial part of thawing.

Vorotilin et al (1991) suggest that, in the rapid heating of a frozen biological system, mechanical stresses can affect the cells and cause additional damage to them due to the limited thermal conductivity of the system. A two-step warming, a combination of slow warming and rapid warming was therefore advanced to improve survival (Campbell 2001).
1.5. CRYOPROTECTANTS

A ‘cryoprotectant’ is any additive which, when provided to cells before freezing, can enable a higher post-thaw recovery than can be obtained in its absence. Cryoprotectants are highly soluble and have a low toxicity.

There are two major categories of cryoprotectants:-

1. Permeating cryoprotectants. E.g. methanol, dimethyl sulphoxide (DMSO), glycerol and propylene glycol (PG).

2. Non-Permeating cryoprotectants. E.g. hydroxyethyl starch, polyvinyl pyrrolidone and various sugars.

Permeating cryoprotectants are low molecular weight and can penetrate the cell membrane. They produce a considerable freezing point depression in addition to that due to any electrolytes present, eventually leading to a ‘ternary eutectic point’ (cryoprotectants-salt-water) at a low temperature. In a freezing system, total solute concentration in the unfrozen phase is uniquely determined by temperature. Therefore, the inclusion of a cryoprotectant to the solution will result in a decrease in concentration of other solutes.

As the increasing salt content of the residual liquid is the main cause of so-called ‘solution effects’, both the reduction of damaging salt enrichment and its shift to lower temperatures are believed to be beneficial effects. This explanation of cryoprotectant properties is also known as ‘osmotic buffering’.

Non permeating cryoprotectants have a high molecular weight and cannot enter cells. The effect of Non permeating cryoprotectants is mainly based upon their ability to dehydrate the cells prior to cooling. This then results in reduced ice crystal formation during freezing. Some high molecular weight (>50,000) cryoprotectants such as polyvinyl pyrrolidone, polyvinyl alcohol and hydroxyethyl starch, will protect cells during freezing and thawing by altering ice crystal formation to an innocuous size and shape.

Toxicity of Cryoprotectants.

Whilst cryoprotective agents can protect living cells from massive distortions of cellular and environmental geometry, a variety of them can be damaging to cells, especially when used in high concentrations. It seems that the basis for detrimental effects of cryoprotectants is not simply osmotic but due to direct ‘bio-chemical’ injury.

Injuries caused such as inactivation or denaturing of specific enzymes, disruption of transmembrane ionic pumps or other related perturbation of cellular structure and function. They are most likely due to the direct
interaction of the cryoprotectant with proteins and biological membranes. Toxicity may increase with increasing temperature.

It is important to know the permeability of the membranes of cells to water and cryoprotectant in order to optimise the protocols for addition and removal of cryoprotective solutes and the cooling rate for cell preservation. It is also important to characterise the fundamental physical mechanisms of solute and water transport across the cell membranes (e.g. channels versus lipid bilayer).

A number of different formulae are available for determining cell membrane permeability parameters. These include a one parameter (solute permeability) model described by Mazur et al (1974), a two parameter model (water and solute permeability) model described by Jacobs (1932-33) and a three parameter (water, solute and a solute solvent interaction term known as ‘sigma’) described by Kedem and Katchalsky (1958). The recent discovery and characterisation of water channels (proteins known as aquaporins) in biological membranes reveals that aquaporins are highly selective for water and do not typically co-transport cryoprotectants.

The Boyle Van’t Hoff relationship will facilitate determination of the minimum shrinkage volume which is known as the ‘Osmotically inactive water volume’ (OIV). A Boyle Van’t Hoff plot of relative cell volume versus 1/relative osmolality does not cross x axis at zero since there is a ‘non osmotic volume’ which consists of cell solutes and some water which is bound and cannot move by osmosis. This needs to be determined to optimise conditions for addition and removal of cryoprotectants. The higher the OIV, the lower the permeability.
1.6. APPROACHES USED IN CRYOPRESERVATION

There are two approaches used for cryopreservation:

1. Controlled slow cooling
2. Vitrification

Cells are at greatest risk of injury during cooling and thawing but risk is low during storage below -130°C. Other dangers exist such as contamination during storage and that of genetic drift. Cryopreservation is a method by which cells can be stored in suspended animation, essentially biological time is stopped (e.g. this can be observed with mammalian sperm).

It is possible to measure the internal energy in a system $T^\circ$. In fluids it allows molecular motion which is vital to living cells. As $T^\circ$ decreases, motion decreases down to the glass transition temperature at -130°C. Below -130°C liquid water does not exist and diffusion is insignificant. The challenge of cryopreservation is to exploit this phenomenon of storage at low $T^\circ$.

1.6.1. Controlled Slow Cooling

Controlled slow cooling is characterised by the addition of molar concentrations of penetrating cryoprotectants such as dimethyl sulphoxide (DMSO), glycerol or other cryoprotectants to the cell suspension. It is also conventional to use controlled rate freezing to lower the cell suspension to the storage temperature. The basic steps required to preserve embryos using this approach are:

- Collect and assess cell quality
- Equilibrate cells in a solution containing molar concentrations of a cryoprotective solute
- Freeze cell suspension using controlled cooling to temperatures below -130°C
- Low temperature storage at – 196°C
- Warm and thaw cell suspension using controlled conditions
- Remove cryoprotectant solute from the cell suspension
- Return cells to normal physiological conditions

Factors commonly considered in embryo cryopreservation include: cryoprotectant concentration, equilibration time, embryo development stage, seeding temperature, cooling rate, holding period, thawing methods and post-thaw fertility of frozen cells.
In order to use this methodology, the cell suspension with cryoprotectant needs to be transferred into
cryovials or straws which have been designed to withstand the cooling and thawing temperature changes
and the long term storage at cryogenic temperatures. A controlled rate freezer with liquid nitrogen supply and
ultra low temperature electrical or nitrogen storage freezers are also essential.

1.6.2. Vitrification

Vitrification was first successfully used for the cryopreservation of mouse embryos in 1985 by Rall and Fahy.
Since then preservation of tissues, blood cells, plant somatic embryos Asparagus officinalis, Drosophila
melanogaster embryos and oocytes and embryos of many mammalian species. Many mammalian systems
have been successfully cryopreserved by controlled freezing methods but, in the case of Drosophila
melanogaster, vitrification has succeeded where controlled freezing has, so far, failed.

Vitrification is the solidification of a liquid brought about by an extreme elevation in viscosity during cooling,
rather than by crystallisation. During vitrification the solution is said to become a glass. Biological time is
effectively ceased since the molecular motions are significantly arrested, but without any of the 'normal'
changes brought about by freezing. Cells capable of being vitrified need no longer satisfy classical
constraints of optimal cooling and warming rates, but instead can avoid both 'solution effects' injury and the
dangers of intracellular freezing.

Successful vitrification requires optimisation of:-

- The concentration and composition of the vitrification solution
- The procedure used to equilibrate cells in the solution – *it is common to use step wise addition of*
vitrification solution with change in temperature to *minimise damage*.
- The cooling and warming conditions
- The procedure used to dilute cells from the vitrification solution – *to avoid injury related to toxicity*
  and osmotic shock

Despite differences in the method used to produce osmotic dehydration, the basic steps of vitrification are
similar to those of controlled freezing:-

1. collect and assess embryo quality
2. equilibrate and dehydrate embryos in a concentrated, but low-toxic, solution of
cryoprotectants (the vitrification solution).
3. vitrify embryo suspension by cooling to temperatures below -130°C
4. store at low temperatures (-196°C)
5. warm and soften the glassy solution to obtain the liquid state and immediately remove cryoprotective solutes from embryo suspension – *this is very rapid to reduce risk of internal ice crystal formation*

6. return embryo to normal physiological conditions

The equipment required for vitrification include the cryovials, straws, metal grids, plates and tubes. Liquid nitrogen, nitrogen slush or liquid helium may also be required.

Most of the differences between vitrification and controlled slow freezing are related to the need for optimisation of a vitrification solution consisting of one or several cryoprotectants. It is also very important to establish optimal conditions for equilibration in the vitrification solution and dilution from the solution – *it may be suitable to choose low concentration with high temperature or high concentration with low temperature or choose room temperature.*

The use of vitrification solutions with low toxicity seems to be essential – *but these solutions may be very concentrated.*

The greatest challenge in developing a successful vitrification protocol is to formulate a vitrification solution that satisfies two requirements:

1. It must be sufficiently concentrated to avoid crystallisation during cooling and it must be able to vitrify into a glassy solid – *i.e. physiochemical properties are important*

2. It is also important to match the choice of cryoprotectants with the intrinsic permeability and toxicity properties of the cells to be cryopreserved. Ideally at least one of the cryoprotectants in the vitrification solution must be able to permeate the cytoplasm, but the overall composition must not produce excessive osmotic stress or chemical toxicity.

**Cryoprotectant Interactions.**

Successful vitrification requires the use of highly concentrated mixed solutions of cryoprotectants. Different cryoprotectors may interact with one another because of different mechanisms of protection or because they protect by the same mechanism but via different routes.

Fahy and Hirsh (1982) proposed that high concentrations of permeating cryoprotectants could be used to achieve vitrification at cooling rates low enough to be practicable for organs. However, using a cryoprotectant in such a way is unconventional; normally it is used as a mitigator of slow freezing injury. They chose instead, to select these cryoprotectants because of their high solubility in water and relatively low toxicity to cells at multi-molar concentrations. Adding solutes enables the colligative depression of both the
equilibrium freezing and homogenous nucleation temperatures. It also results in an increase in the viscosity of the solution at any subzero temperature.

It was found that solutions containing both glycerol and propane-1, 2-diol proved to be less toxic for rabbit kidney than those containing one of these components alone. This demonstrates that a combination of two agents of low toxicity can significantly lower the cryoprotectant induced toxicity.

**Stability of amorphous state of vitrification medium**

An approach to understanding the action of low molecular weight penetrating cryoprotectant agents is to consider the stability of the amorphous (or glassy) state. The amorphous state may be defined by the critical warming rate at which the difference between the devitrification and melting temperature vanishes. Under such circumstances, ice formation and damaging salt enrichment are completely avoided during both cooling and subsequent warming. It is also evident that the more stable the amorphous state is, or the lower the critical warming rate, the better the cryoprotective action.
1.7. CRYOPRESERVATION OF TISSUES

1.7.1. Introduction

Due to the advances in surgical treatment, there is an increasing demand for mammalian tissue for transplantation for therapeutic use for patients suffering with disease, infection or congenital deformities. Tissue procurement is often performed at sites distant from where there is a surgical demand. To minimise risk of transmission of infection to recipients, this tissue has to be sent for microbiology testing, virology screening and the donor records reviewed. The logistics involved therefore demand some form of preservation method to maintain the quality of tissue until transplantation. The most common method of tissue preservation has been chilling in a refrigerator, or cryopreservation followed by storage at <-70 to -196°C. The advantage of hypothermic, but non-frozen storage is that this tissue is ensured that it is free from the effects of ice damage but however, it can only be safely maintained at such temperatures for a relatively short period. This is typical of refrigerated skin and cornea storage methodologies (May, 1985 and Taylor 1986). The advantage of cryopreservation is that it may offer long term storage (more than 5 years) but not, however, if there is a lack of understanding of the mechanisms of freezing damage (‘cryoinjury’) in order to minimise ice formation during the cooling and warming processes. Cryopreservation, generally is more advantageous over refrigerated storage, provided that effective methodologies are optimised for each specific tissue.

1.7.2. Difficulties with tissue cryopreservation

It is not easy to generalise the inherent difficulties in the cryopreservation of tissues since they are often specific to the tissue and the range of tissues is vast. However the most important difficulty relates to the tissues variation in their mass and geometry which directly affects the control of cooling, warming, and cryoprotectant movement through the tissue. In thick tissues, or tissues with a mass that prevents rapid equilibrium and homogeneity of temperature throughout the tissue, then prolonged super-cooling and extended exothermic temperature plateaus during cooling and endothermic plateaus during warming will be observed which are damaging. Significant temperature gradients during the cooling and warming of thinner tissues such as skin or heart valves are not normally observed between the surface and interior of these tissues.

Thin tissues can thus be satisfactorily cryopreserved from cooling or warming of the outside of the tissue. The next major issue is that most tissues incorporate a mixture of cell types. This affects the design of the optimised cryopreservation methodology since it would have to take into account the different sensitivities to freezing damage of each cell type to ensure that some cell types did not suffer cryo-injury at the expense of...
others. This has been observed in the case of the epithelial and fibroblastic cells of skin. There is difficulty relating to the density of cells within a tissue. If cells are packed densely, for example similar to that found in organs (which may be approximately 80%), then the survival after cryopreservation may be reduced significantly (Jacobsen, 1990). This is thought to be due to the inability of the cryoprotectant to access the cells, the ability of water to move across the cell membrane and the effect of the formation of ice (Pegg, 1979). The general structure of the tissue itself also affects the tissue cryopreservation particularly if it’s different cell types are arranged in layers (such as in skin or the Islets of Langerhans in pancreatic tissue). Fractures in the planes between the layers may be observed during freezing of such tissues. After thawing or even transplantation, separations within the dermal and epidermal layers of skin have been observed (Heck, 1985). Finally, there may be additional difficulty in some tissues if maintenance of the functioning of vascular components is required post transplant. Tissues that depend upon simple diffusion for the supply of nutrients, and thus do not need a functioning vascular system, will be less difficult to cryopreserve.

Tissue whose performance depends upon maintenance of a viable vascular system will be inherently difficult to cryopreserve because vascular systems incorporate endothelial cells which are very sensitive to any form of injury. Procurement and Cryopreservation processes will inevitably damage the vascular system of most of these tissues. The endothelial cell viability of donated kidneys is damaged by cessation of circulation whilst perfused in a cooled bloodless solution prior to transplantation (Pegg, 1973).

1.7.3. Optimisation of tissue cryopreservation variables

There are at least ten variables which ought to be taken into account when designing a cryopreservation protocol for a tissue:-

i. **Tissue size and geometry**

This can affect the thermal homogeneity within the tissue and thus affect the ability to control the temperature throughout the tissue, risking the harmful effects of exothermic temperature plateaus. Control of cooling and warming rates is inevitably reduced.

ii. **Tissue transport fluid & cryoprotectant media**

To minimise tissue damage post procurement due to anoxia or hypoxia, tissue is immersed prior to transplantation in a suitable fluid which has been chosen to balance nutritional needs of the cells in order to provide adequate energy for the ion pumps of the cells. This fluid must also balance the osmotic requirements of the cells to prevent dialysis of required intracellular solutes. Similarly, the cryoprotectant is delivered to the tissue in a base media which may be identical to the transport media.
The cryoprotectant must be of type and of a concentration which is only of low toxicity to the tissue. It must only be exposed for sufficient time and appropriate temperature to enable adequate uptake within the tissue. Cryoprotectants may be toxic to tissues if exposure period is too long. For skin preservation, this typically may involve a 15 minute exposure at 4°C (Bravo, 2000). Using a two step addition of the cryoprotectant with an appropriate equilibration time between the two stages may minimise risk of osmotic stress on the cell membranes such as in the cryopreservation of human ovarian tissue (Gook, 2000).

iii. Cooling Rate
Prefreeze cooling or ‘chilling’ from donors body temperature (37°C) to refrigerator temperature is usually achieved in one step typically from procurement to ice cooled transport fluid and then transferred to refrigerator, or if two step, then method may involve a room temperature stage in between. During this chilling period, the cell membranes and rates of biochemical reactions in the cell will be affected and the cell may be unable to provide sufficient energy levels to maintain isotonicity. It is important therefore, that the transport fluid is isotonic for as many of the tissues constituent cells as possible to mediate this effect. The cell membranes will shrink during pre freeze cooling, and cooling below freezing point, thus sufficient time must be allowed for this shrinkage to occur in the idealised cooling program. Generally a slow cooling rate such as -1 to -5°C per minute is sufficient (Ashwood-Smith, 1980).

iv. The ‘state’ of the water
The ‘state’ of the water varies depending upon the final storage temperature and the duration of the storage is also important. No water movement and recrystallisation of ice will occur below the ‘glass transition temperature’ (which is at least -139°C) which otherwise could have induced mechanical damage to the tissue. Also, rates of chemical reactions and biophysical processes are sufficiently slow below this temperature to minimise cell damage (Grout, 1987).

v. Warming Rate
In order to minimise risk of potentially harmful temperature plateaus during warming, the warming must be controlled sufficiently so that homogenous warming is achieved throughout the tissue. This is controlled by using very rapid rates of warming which are achieved by taking the tissue directly from its low storage temperature and placing it directly into a water bath at body temperature. Warming rates of more than 100°C per minute are typical (May, 1985). However, it has been shown that, in some tissues, if rates of warming are faster than the glass transition temperature, then this can induce fracture formation. The inclusion of a first slower warming rate of <50°C has been shown to prevent cracking in these tissues such as carotid arteries (Pegg, 1996).
vi. Thawing media and removal of cryoprotectant
The media used to thaw and dilute out the cryoprotectant should also balance the tissues nutritional and osmotic needs. The cryoprotectant should be dialysed out at a rate which minimises osmotic damage and this is often achieved by two or more serial dilution steps.

vii. Viability
Preservation of an adequate structure and function may depend upon the viability of cells in a tissue. If this is the case, a critical viable cell function in the tissues post transplant performance must be defined and identified. One or more of these critical functions must ideally be assessed to determine true tissue viability (Southard, 1989 and Pegg, 1989).

1.7.4. Successfully Cryopreserved Human Tissues
There are many tissues that may be successfully cryopreserved which include those tissues whose role post transplantation is structural such as bone, skin, dura mater, fascia lata, veins and arteries and heart valves. This group of tissues generally has similarities such as, relatively high collagen content. They have relatively low cell density, few cell types and simple architecture with few layers and have relatively active pentose phosphate pathways. These characteristics will aid cryopreservation protocol optimisation. In some cases it is not certain whether cell viability is maintained post cryopreservation because, in some structural tissues, their post transplant performance is not dependant upon viability but instead, relates more to in growth and re-colonisation of the transplanted tissue by the recipients' cells.

The other group of tissues that can be cryopreserved are those with a biosynthetic function such as embryos and foetal tissues, pancreatic tissue, parathyroid and thyroid tissue. This group do not generally possess the beneficial characteristics of the structural tissues, but instead are of small size and thus permit easy permeability and temperature homogeneity. Some of these tissues are glandular in function and only small portions of the tissue are cryopreserved (Wagner, 1998). It seems that this group of tissues may require cellular viability and function in order to perform their biosynthetic function post transplantation.

1.7.5. General Cryopreservation methodology
There are common features of the methodology used for cryopreservation of tissues. Generally, many are placed in phosphate buffered saline after procurement, they are then transferred to a tissue culture nutrient media and permeating cryoprotectant added in one or two steps until the tissue is equilibriated at a relatively high cryoprotectant concentration (most often >0.5M glycerol or dimethyl sulphoxide). The tissue is then slowly cooled (between -1 and -5°C) and is the transferred to storage in liquid nitrogen (-196°C) or vapour
phase (<-140°C). It is most often transported to the surgical centre and warmed rapidly immediately prior to use in a single step (>100°C per minute) by removing from liquid nitrogen and directly immersing in a water bath at body temperature or using an added preliminary, less rapid step by holding in dry ice before immersing in a water bath thus achieving a warming rate of 50°C. Cryoprotectant is removed by one or more steps of serial dilution in phosphate buffered saline or tissue culture media. Viability of the tissue graft may be measured by the post transplant acceptance in the recipient patient or by specific biochemical markers. General methods however, although they may be suitable for some tissues, they are only partially effective in the cryopreservation of other tissues.

1.7.6. New developments in tissue cryopreservation

New techniques of ultra rapid cooling (to some where near -300°C per minute) which convert the water by vitrification into a glassy state, minimise the risk of formation of damaging ice crystals because they have not had time to grow. This has to be accompanied by equally rapid warming to prevent ice crystals forming during warming and has been applied to methods to cryopreserved tissue required for in vitro fertilisation (Baudot, 2007). High concentrations, and/or combinations of cryoprotectants are being developed (Fahy, 2004) for use to vitrify tissues at higher temperatures but careful choice of the cryoprotectants composition and concentration for individual tissues is necessary to avoid tissue damage due to cytotoxic effects. Vitrification methodology using the liquidus tracking method has been developed utilising increasing cryoprotection concentration in several stages during cooling to lower the freezing point and subsequently warming and decreasing concentration in the same stages. It has been recently shown to be a potentially suitable method for preservation of cartilage tissue (Wang, 2007) compared to conventional cryopreservation or conventional vitrification. Viability assessment by glucosaminoglycan (GAG) synthesis comparison showed liquidus tracking to enable 95% recovery compared to 5% for conventional cryopreservation or 20 % for vitrification methodologies.

The surgical requirement for human tissues for therapeutic use is the driving force behind the design and development of new methods of tissue preservation and for the improvement of existing techniques. There is no doubt that, in the future, the number of tissues which may be successfully cryopreserved will increase as a result.
1.8. PERSONAL HISTORY & EXPERIENCE

The author has worked in the field of cardiovascular tissue banking since leaving university in 1984. She first worked in the Homograft department at the National Heart Hospital in London for pioneers Mr Donald Ross FRCS, Sir Magdi Yacoub and Mr Marc de Leval. These leading consultants were all advocates of human heart valve transplantation. Donald Ross performed the first human valve transplant in 1962 and went on to introduce many new types of homograft surgery for both adults and children and he also performed the first heart transplant in the UK at the National Heart Hospital. Magdi Yacoub extensively used unpreserved homovital transplantation of human heart valves procured from heart transplant recipients and enthusiastically went on to perform the biggest series of homovital valve transplants to date. Magdi Yacoub went on to perform an equally notable series of heart transplants at the National Heart Hospital and the Harefield hospitals in London. Mr Marc de Leval focussed upon introduction of new methods of paediatric heart valve surgery using human homografts which he advocated extensively throughout his career which was based in London’s Great Ormond Street Hospital.

Throughout her working career in London which was based at the National Heart, but involved also working at Great Ormond Street, Brompton and Harefield Hospitals and also the Harley Street Clinic between 1984 – 1990. The author worked as a technician and was involved in routine tissue processing and tissue allocation. Whilst working closely with these surgical pioneers, she also worked in the operating theatre with the surgeons in order to pursue a variety of research and development projects and she took on an additional role to her full time post, working as a research assistant for Mr Ross.

The author was asked to observe surgical techniques in the operating theatre in order to produce drawings of the operation techniques for surgical training, publication and operative technique development (for example in articles by Ross, 1987 and in Dhalla & Yacoub, 1986). The author has worked with the surgeons abroad introducing and demonstrating new surgical techniques and human tissue valve surgical preparation such as Mitral top hat Ross procedures (Kabbani 1999). The author was trained to mount human valves onto frames similar to those used for porcine tissue prosthesis such as development of the xenograft mounted tissue bank in Brazil in 1987. The author was asked to set up human heart valve banks in various parts of the world including Brazil (da Costa, 2001), Syria (Kabbani, 2000) Pakistan and Thailand. The author was also involved in the extensive data collection and analysis of human heart valve transplant recipient follow up studies for Mr DN Ross, many of which were presented and published, not all including acknowledgement of this author (Gerosa1994 & 1991; Vesley 2000; Bodnar 1990; Ross 1995, 1997; Matsuki, 1988, Glazier 1991).
Retrospective studies included those focussed on operative techniques and long term recipient outcome (Matsuki, 1988, Gerosa, 1989 and Glazier, 1991) and others on comparisons of preservation and disinfection techniques and also on different types and methods of valve transplantation (Bodnar, 1990, Ross, 1997 and Kay, 1986).

In 1990, the author was asked to move to set up a new human heart valve bank in Oxford and, in 1991, this bank was opened. The valves processed by the Oxford bank are used for paediatric and adult patients in Oxford and a significant quantity are supplied to other UK hospitals, particularly to the Guys and St Thomas Hospital and the Great Ormond Street hospital in London. More than 3000 valve have since been processed by the Oxford bank and currently it procures approximately 200 human donor valves per year and as such, is believed to be the second largest bank in Europe. UK and European tissue banking quality and safety legislation has been recently introduced and licensing/accreditation of the bank has been achieved. A recent Human Tissue Authority phase 2 inspection reported that it is currently the model of good practice in the UK.

The author was a founder member of the only professional tissue banking body known as ‘The British Association for Tissue Banking’ (BATB) and is also a longstanding member of the European and American Association of Tissue Banks (EATB and AATB respectively). Currently the author is qualified by the AATB as a ‘Certified Tissue Banking Specialist’ and was a founder graduate of the Barcelona University Tissue Banking Diploma. The author regularly presents scientific papers concerning human heart valves at the BATB and EATB Annual Scientific meetings (Davies 2007 and Womack, 2007). The author is currently and has been an Executive member of the BATB for more than 5 years and is responsible for the association website, chair of the Public relations committee and past chair and secretary of the Cardiovascular special interest group. The author, together with AATB and EATB representatives, was asked to host the November 2008 first ever Cardiovascular workshop day held jointly by the AATB, BATB and EATB.

During the time spent in the London and Oxford hospitals, the author was involved in the processing and preservation techniques of antibiotic disinfection and refrigerated valve storage and the introduction of the use of liquid nitrogen vapour freezing as an alternative to refrigerated valves (Davies 1997). The author was involved in setting up BATB and EATB cardiovascular tissue banking guidelines (Parker, 2000) which led to the need to prolong storage whilst additional tests and information had to be gathered. Later, the author helped to set up the controlled rate freezing program and develop the use of dimethyl sulphoxide cryoprotectant and subsequently withdraw the use of glycerol. Controlled slow cooling with DMSO was always used in Oxford bank. Unique surgical procedures were developed using homografts which included the first mitral homografts prepared in the UK and used for Tricuspid repair (Katsumata, 1997), the first
carotid conduits were procured and prepared and used as shunts (Katsumata, 1998, Handa, 2001 and Evans, 2007) and unique aortic root surgery modifications were introduced (Westaby, 1992) Post operative data is still collected currently and trends analysed (unpublished proceedings of National Institute of Biological Standards and control and in press).

The author has been an advocate of tissue banks developing their own quality management systems (based upon ISO 9000: 2001) and was invited to join the working group of the European Commission organised by Bio Implant Services (de By, 1991). Staff training and ongoing performance assessment review have also been central to the changes made in Oxford in the ultimate goal to improve quality and safety of the human tissue supplied for transplant surgery.

Whilst in London, due to the shortage of human valves available, the author was involved in the initial design of an original new type of xenograft which very closely resembled the homograft (stentless but Dacron cloth covered and low pressure fixed in glutaraldehyde). This led to the commercial valve manufacturer ‘Edwards’ developing what is now known as the ‘Prima’ valve and, with D Ross, the author produced the first prototypes and design features. The author has also been a founding member of commercial company (Cambridge Biomedical) producing porcine stentless xenografts. Initial design and development was jointly by the author and DN Ross. The author prepared and wrote the initial drafts of two patents (US Patent 5,352,240, 1994 and US Patent 5,089,015, 1992) which were eventually bought (along with the company) by the US cryoprocessing allograft company ‘Cryolife’. The author has also been involved with a group currently developing a decellularised homograft tissue valve (Della Rocca, 2000 and Weind, 2002). In Oxford, the author has been involved with several clinical trials of stentless xenografts including the Prima 2 xenograft freestyle xenograft and more recently, the 3F stentless xenograft. The author prepared human valves for long term fatigue testing (sewing them onto stents) or the Vesalio tissue engineering project and also prepared valves for video imaging comparison of Medtronic stentless xenografts versus homografts.

The author has also been involved in a myocardial stem cell research project and a Mitral Isthmus Anatomy Study (Ref: 09/H0604/22) which involves MRI scanning for making detailed measurements of donor hearts.
2.0. AIMS OF THE STUDY

There are currently several tissue banks in the UK which process donated human cardiovascular tissue for transplantation. Current National and European requirements do not insist that the processing methods used in the UK are published or standardised. The UK processing techniques appear to be different and this may potentially affect tissue quality. The ambiguity may be of concern to those requiring to ‘import’or ‘export’ within, or outside the UK. The aims of this study has been designed to help alleviate these concerns and has been designed with several objections:-

1. To establish the current National status and issues affecting cardiovascular tissue banking throughout the UK.

2. To investigate the historical background regarding the introduction and application of cardiovascular tissue transplantation and the evolution of cardiovascular tissue processing techniques and the emergence of tissue banks.

3. To collate and review the unpublished processing methodology employed by each of the currently active UK cardiovascular tissue banks and thus to establish the details of current UK cardiovascular tissue processing practice and highlight areas where there are differences in practice. The details of the cryopreservation related processes will then be scrutinised including an investigation of the validation methodology and that of any tissue quality assessment techniques routinely used.

4. To identify if there are any issues which currently concern UK cardiovascular tissue bankers, and to then, following review of published data from non-UK banks, discuss the likelihood that these concerns may be detrimental to tissue quality.

5. Finally, to suggest options for further study of any of the unresolved issues which appear to affect tissue quality. Ultimately, in doing so it is hoped that it will be possible to begin and encourage optimisation of cardiovascular tissue cryopreservation processes and standardise and improve cardiovascular tissue banking methodology.
2.1. MATERIALS AND METHODS

2.1.1. Establish National Status & Issues concerning UK Cardiovascular tissue banks

There has been a great deal of change concerning National guidelines and requirements for UK tissue bankers in the last decade. It appears that this rapidly changing legisatory environment has affected the current state of cardiovascular tissue banking in the UK. By means of a review of current requirements and guidelines using literature searches and interviews with tissue banking professionals, the issues concerned will be established along with the pressures this places upon tissue banking staff. The study method included informal interviews with BATB executive members and former past presidents and data collection of the orders and supply of cardiovascular tissue within bank 1 (see spreadsheet in Appendix 1).

2.1.2. To Investigate the introduction & application of cardiovascular tissue for transplantation & the evolution of processing methodology and emergence of tissue banks

In order to document the historical background regarding the introduction and application of cardiovascular tissue transplantation, a thorough detailed review of published and unpublished material was performed. Personal interviews were set up with pioneering surgeons from the past and present day, eminent researchers and also with tissue banking staff who has worked in the industry for a substantial time. The evolution of cardiovascular tissue processing techniques was also established by similar means, and enabled the author to identify and then critically review the failures and successes of the evolving techniques used and to highlight the contradictory underpinning assumptions made by the homograft bankers at the time which steered their homograft processing methodology changes. Additional unpublished details of the London theatre surgery and homograft department records were, for the first time reviewed and the detailed documents mapping homograft processing changes were accessed. This helped to clarify the uncertainty in contradicting literature such as whether the first homograft used was freeze dried or antibiotic media preserved and confirm when the different preservation methods were started and ended in London and how actually they were performed.

i. The study group

The study group was focussed upon the main protagonists of homograft use in London, New Zealand and Australia, but this was extended this to include international published data reported by other surgeons and tissue banks. Surgeons and tissue bankers currently working using homografts were also approached to capture present day experience and issues.
The author arranged interviews with a number of pioneering surgeons which included, Donald Ross and Magdi Yacoub (London), Bryan Barratt-Boytes (New Zealand) Marc o’Brien (Australia) and Steve Westaby and Ravi Pillai (Oxford), with researchers Endre Bodnar and Jane Sommerville (London), Gino Gerosa (Italy) and Ivan Vesley (Canada). Tissue bankers working in the field of tissue banking for > 20 years were also interviewed (William Wain and Robert Parker) who both were managers of the London bank. Present day pioneering surgeons were also interviewed and these included some of the previous group (since they are still operating today) and also Davis Anderson and Chris Blauth, Ashok Cherian from London, Jave Khan now in Pakistan, Alphonso Robles, and now in Chile and Sami Kabbani now in Syria.

2.1.3 To establish current UK cardiovascular tissue processing practice

The unpublished processing methodology employed by each of the currently active UK cardiovascular tissue banks was collated and reviewed to establish the details of current UK cardiovascular tissue processing practice and highlight areas where there are differences in practice. Additional details concerning the cryopreservation related processes were then scrutinised including an investigation of the validation methodology and that of any tissue quality assessment techniques routinely used.

i. Selection of the study group & information source

In the study period, 2007/08, six tissue banks in the UK were actively involved in processing cardiovascular tissue for therapeutic use. Some of these banks were multi tissue banks (National Blood Service in Liverpool and Scottish National Blood Service in Edinburgh) and others were independent banks based on a hospital site where there is a cardiothoracic surgery service (Oxford bank at the John Radcliffe Hospital, Birmingham bank at the Children’s Hospital, Royal Brompton Hospital bank in London). The remaining UK bank is based in an eye hospital adjacent to an eye bank and a nearby hospital which delivers a cardiothoracic service. In order to ensure compliance with European and UK requirements, each UK tissue bank has developed a unique set of operating procedures which standardise the entire series of complex and interrelated activities from tissue procurement to the implantation of the graft. These procedures are reviewed upon inspection by HTA but do not yet have to be published. Therefore, in order to investigate the details of all of the staged processes used in each establishment, it was found necessary to ask each bank to volunteer this data for study purposes.

The author discussed the survey within the professional UK tissue banking forum – the British Association of Tissue Banking, which has a Cardiovascular Special Interest Group (BATB CV SIG). All UK tissue banking establishments who were members of the CV SIG agreed to co-operate.
As previously explained, each UK bank processing cardiovascular tissue, varies in its origin, size, management and organisational structure. However, the majority of the personnel in these banks are BATB members, most of whom regularly attend cardiovascular Special Interest group (CV SIG) meetings. Historically, these SIG’s were set up by BATB to develop UK guidelines in the absence of any National regulation. More recently this has become the only network for SIG members to interpret, consult and discuss the implementation of the HTA and EUTCD requirements. The questionnaires were targeted at the managers or lead production technicians of each bank, all of whom have had more than 5 years experience in their HTA licensed cardiovascular tissue bank. Each of them had been involved in the implementation of the new National and European requirements and has undergone successful HTA inspection to gain the mandatory license in order to store human tissue for transplantation.

ii. Information Collection Questionnaire

The format of the questionnaires initially, was a data table which had been designed with questions prompting descriptions about each bank’s processing stages (see Appendix I). The questions were set out in one column. The bank number 1 (the authors Oxford bank) was completed as an example to follow in the adjacent column and an empty column was left for the participating bank to complete. This table was distributed to the production lead in each UK bank.

Unfortunately, despite agreeing to do so, these questionnaires were not returned or were incomplete; consequently telephone interviews with the production lead from each bank were then performed. This was then followed up by the author returning the questionnaires for checking and yet more interviews to facilitate additional details as new and unexpected variables were identified. For example, during the initial questionnaire phase, the number, type and stage at which microbiology samples were retrieved were found to be quite different. The answers to some of the questions were found to be too detailed and so standard operating protocols and instruction leaflets were collected by the author where relevant and relevant information extracted and added to the survey worksheets by the author. Questions from survey participants and other interested BATB members were also included and survey extended as the investigation progressed.

Further questions with processing managers were required to clarify the details of the unique processes used and to determine what, if any, validation and quality assessment methods were being employed. Data was tabulated in order to compare and contrast processing techniques. Information volunteered concerning freezing programs, re-warming and dilution instructions were graphically represented.
2.1.4. To identify current concerns in UK Cardiovascular Tissue Banking

i. Establishing concerns

The BATB CV SIG members were asked to voice their concerns anonymously about cardiovascular processing issues which seem unresolved. They were asked to complete a third questionnaire to illustrate where they perceive to be the areas which require further knowledge and understanding and/or experimental study. Data was summarised in an excel spreadsheet. Surgeons’ views were also included as well. This was achieved by collating and analysing surgeon satisfaction surveys and postoperative recipient outcome forms. Leading tissue banks in countries outside the UK were asked to join the review. The Dublin bank (based in the Irish National Blood Service) joined the UK study as they were also active members of the CV SIG. This bank is within the scope of the European tissue banking requirements (EUTCD) but did not have to comply with HTA Directions and HT Act. It is inspected by the Irish Medicines Board. Several European banks, two United States banks, two Canadian banks and one Australian bank were asked and have agreed to join the study and, where relevant, their processes have been compared and contrasted. Information from other banks is due to be added as described in the ‘Future Work’ section of this study.

ii. Literature review

A literature search was performed which focussed on cryopreservation techniques used by non-UK banks and also those designed for non cardiovascular tissue. Following this review of published data, a discussion was possible regarding the likelihood that these concerns may be detrimental to tissue quality. A variety of electronic databases were used via NHS Athens and Oxford University library to perform the necessary literature searches such as Medline, Biosis, Scopus, Web of Science and also, to a limited extent, Science Direct and Google Scholar. Choosing the topic keywords to be used was however, not so straightforward since there are many terms which may be used to describe cardiovascular tissue used for transplantation and also for the processing and associated testing and validation procedures. For example Key words used included terms for the valve tissue themselves such as Homografts, Allografts, Human heart valves, Cardiovascular valves, cardiovascular tissue, Homovitals, Autografts or valved conduits. Historically cardiovascular tissue used for transplantation was called ‘Cardiac Homografts’ but, as the heart transplantation program developed alongside, soon the term ‘Allograft’ was also used. Many British surgeons continue to use the term Homograft whilst virtually all US surgeons refer to Allografts. Other professionals use ‘human’ or ‘donor’ ‘heart valves’ or ‘cardiovascular’ or ‘cardiac’ tissue, valves or conduits. ‘Tissue’ can be, to a surgeon, a term connected with human or biologically derived substance (e.g. tissue prostheses are
often porcine or bovine tissue sewn on to plastic/metal frames or Dacron cloth covered). ‘Tissue’ to a tissue banker is of that of human origin and could also refer to bone, skin or cornea.

There are also different terms for the various types of cardiovascular tissue which are dependant on their procurement/preservation/use. For example human valves taken from heart transplant recipients and then implanted the following day are often termed ‘Homovital’ valves. ‘Autografts’ is one of the terms used for the aortic valve procured from the patient undergoing the ‘Ross Procedure’ but to non cardiac surgeons it may be attributed to any section of the body that is moved from one site to another. Articles describing and studying outcomes of transplantation were also complex to search for and interpret since human valves are transplanted into various positions in the recipient heart using a great variety of techniques. Surgical procedures are named after the type of surgery or by the name of the surgeon who first performed the surgery and, initially, these are not given the surgeons name until a successful cohort of operations have been completed. ‘The Ross Procedure’ was initially termed ‘The Autograft’ or ‘a switch operation’. Surgical outcome studies comparing mechanical and biological valves often use the term ‘tissue’ to mean porcine of bovine valves but sometimes they do include human tissue. Also, the aortic and mitral mechanical and biological available will be inserted into the aortic and mitral position in adults only where as human aortic and pulmonary valves and patches are normally inserted into the aortic and pulmonary position of adults and children and hence long term performance data has to be sourced and compared cautiously.

Also, whilst reading the articles, some of which were translations, the test procedure terms used had to be cautiously interpreted. ‘Viability’ is one of those terms used by many researchers to mean, or measure, quite different properties or attributes and yet they have been successfully published using the same idiom.

In all electronic searches performed, Boolean logical operations were used such as ‘and’, ‘or’ and occasionally ‘not’ and these were carefully used in combination with keywords to condense the searches. As the author was aware of key pioneers and prominent researchers in the field, searches for known authors were also performed, using appropriate ‘wild card’ symbols as required since it was soon discovered that several of these authors varied their given names and initials.

Some of the articles referred to in the older papers bibliography, and some articles which were never published in journals were manually sourced (e.g. validation studies, and MSc study theses).

In the opinion of the author, it also appeared that the amount of new publications concerning cardiovascular tissue processing studies and implantation techniques and recipient outcome had diminished considerably over the last decade. However, a rapidly growing number of articles describing developments in the field of tissue engineering of cardiovascular heart valves or alternatives were observed.
3.0 RESULTS

3.1 Current Status of Cardiovascular Tissue Cryopreservation

The UK has historically been host to some of the most influential pioneers in the field of tissue transplantation and in the development of tissue banking, especially with regard to cardiovascular tissue. Cardiovascular tissue banks have existed in the UK since the first homograft was transplanted in 1962 (Ross, 1962), in fact the first bank ever to be established in the world was in Guys Hospital in London in 1962. It subsequently relocated to the National Heart Hospital site and is still open now based at the Royal Brompton Hospital in London. Other banks were subsequently opened, most of which were associated with a surgical centre that exhibited a clinical need for this tissue.

Over the last decade however, there has been considerable change imposed upon tissue banks and the number of cardiovascular banks has reduced. The closures (at Harefield, Southampton, Cambridge, Wakefield and Wrexham) reflect the challenges of responding to new and changing guidance and more recently to the new and complex legislative requirements and mandatory inspection and licensing program. Financial pressures placed upon tissue banks have been considerable and affected upgrading of facilities and procedures to ensure compliance with such guidance and legislation and staffing and resources have suffered and, as a consequence, only 6 UK banks remain (see figure 1.). A review of the results of the investigation to determine the current status and issues involved in UK cardiovascular tissue banking is discussed below.

3.1.1 UK and European Legislative Requirements

In order to assure quality and safety standards are put in place throughout the European Union the European Commission has established a regulatory framework. ‘The European Union Tissue and Cells Directive’ (EUTCD) creates a common framework that ensures high standards in the procurement, testing, processing, storage, distribution and import/export of tissues and cells across the EU community. The EUTCD Parent Directive (2004/23/EC) sets out the framework of the Directive and the two daughter directives (2006/17/EC and 2006/86/EC) provide technical details. The Directive was transposed into UK law via the Human Tissue (Quality and Safety) Regulations 2007. The Human Tissue Authority (HTA) is the designated competent authority for the implementation of this Directive in the UK. Prior to the transposition of the EUTCD into UK law, the EUTCD has been implemented via the updated Human Tissue Act 2004 (HT Act). The HT Act supersedes the previous ACT (1961) and was initiated in response to the organ retention scandals at Alderhey, Liverpool and other hospitals, to patient (donor’s and recipients) rights and also to
Fig. 1 Map illustrating location of UK & Ireland Tissue Banks currently processing Cardiovascular Tissue. Marker denotes the number of donors processed by each bank (Brompton bank in London processes 250 donor hearts, Oxford processes 180 hearts and all other banks process < 50 hearts).
increasing cultural and ethnic diversity. The new HT Act clarifies a notoriously uncertain area of law but, in
the opinion of some legal critics (McHale, J., 2005; Gillett, G., 2007) may be considered as a fundamentally
flawed Act and a missed opportunity. To address the criticism, the HTA issued a series of Directions
(Directions 001/2006, 002/2007 and 004/2007) which summarise the requirements of the Directives and
Regulations, The EUTCD Directives were transposed into UK legislation 5th July 2007. The Council of
Europe also sets standards for tissue banks, the most recent is ‘The Council of Europe Guide to Safety and
Quality Assurance for Organs, Tissue and Cells’ (3rd Edition). Any tissue bank storing human tissue or cells
for transplantation should have been licensed for this activity from 7th April 2006 and will be inspected by the
HTA on a two yearly basis. The Dublin bank does not have to comply with the UK’s National organisation
responsible for the enforcement of the EUTCD nor does it need to comply with the HT Act 2004 (the Act only
applies within the United Kingdom), instead the Dublin bank has to comply with the Irish Medicines Board
(the equivalent of the UK Medicines and Healthcare Regulations Agency) who have been designated as
Ireland’s responsible authority. Donor selection procedures are carefully controlled in tissue banks and the
most commonly used guidelines in the UK are the ‘Tissue Donor Selection Guidelines for Deceased Donors’
issued by the joint UK Blood Transfusion Services and the National Institute of Biological Standards and
Control Professional Advisory Committee (JACIE). There is also one chapter in the ‘Guidelines for the Blood
Transfusion Service in the UK’ (current version is the 7th Edition) which is devoted to tissue banking. The
National Blood Service (NBS) and the Scottish National Blood Service (SNBTS) obviously are expected to
comply with these guidelines. However, some UK banks will consult the NBS guidelines initially and then
use their own advisory team to make judgement on whether to accept a donor or not (bank 1). Belgium and
US have National donor selection standards. Guidance on the microbial issues of donor selection and
guidance regarding the testing and final, post-processing acceptance/rejection of donor tissue is provided in
the UK by the Department of Health’s Advisory Committee on the Microbial Safety of Blood, Tissues and
Organs (MSBTO), latest published guidelines for the UK (dated 2004).

3.1.2 National Shortage of Organs & Tissue
There is currently a National shortage of donor organs, cardiovascular and skin tissue (London News, 2009).
The statistics produced by the National organisation which monitors organ transplant in the UK (UK
Transplant) publish data showing numbers of organ donors and numbers of patients who are on the organ
donor transplant waiting list. Historically, the numbers waiting for transplants increases each year but the
number of donors never meets the demand and sadly many patients die whilst waiting for a transplant.
According to published UK Transplant data (uktransplant.org.uk/ukt/statistics) there were 7655 patients on
the active organ waiting list and a further 2,092 on the temporary list in 2007-08. The increase in patients placed on the waiting went up by 10% but the increase in donors was only 2%. The organ donor register is a main method by which the general public may register their willingness to be or to refuse to be a donor after their death. Unfortunately, in March 2008, there were only 15,141,000 people registered which is disappointing proportion of the UK population (approximately 60 million). Only 30% of organ donors in 2007/08 were on this register. Furthermore the donor register does not mention any other tissue other than corneas. This means the public cannot specifically request to be a cardiovascular tissue donor and it also means that there are no statistical data to determine the potential number of cardiovascular tissue donors.

UK Transplant collect data concerning the number of corneas donated and transplanted (4115 and 2490 respectively) and this illustrates the wastage of donor corneas which is predominantly due to the lack of long terms preservation method (they can only be stored for approximately 4 weeks). There are no National statistics which relate to the numbers of patients waiting each year for cardiovascular tissue transplants and this is due to lack of any Government funding (UK Transplant is funded by the Government but this funding is specifically for organs and corneas data collection only).

3.1.3 National Shortage of Cardiovascular Tissue

Although UK tissue bankers believe there to be a shortage of cardiovascular tissue, this has never been quantified. However, the Human Tissue Authority has recently issued a requirement for all licensed UK tissue banks to supply annual statistics to include the amount of donated tissue procured, processed and supplied. This is the first time HTA have issued a spreadsheet for data collection and data terms are still, as yet, undefined. This data collection is still very crude since it does not yet take into account the variables of tissue processed in one year but not used until the following year (see Figure 2). The extract shown in Figure 1. illustrates that 284 units of tissue (heart valves, conduits or patches) were procured and processed by the Oxford bank in 2008. It shows that only 124 units were authorised for clinical use in 2008, but these may have been from 2007 donors. The number of units of tissue discarded was 67 but, although this may seem relatively large, it will include all tissue which is rejected due to damage at procurement and dissection, and those identified during quality assessment as abnormal or diseased and also those which fail microbial or viral testing or patient history review. Alternative sources of data are BATB members’ own collective statistical surveys of supplied tissue which indicate the approximate number of tissues banked. The UK map shown in Figure 1. illustrates that the London bank processes the most donor hearts (approximately 250 per year), Oxford processes 180 hearts and other banks process <50 donated hearts per year. The HTA annual report will be refined and should then offer clear definitions of terminology. Provided it then takes into
account the time lag between procurement and donor tissue authorisation, it may then form the basis of accurate tissue processing data collection.

3. Quantities - Heart Valves

<table>
<thead>
<tr>
<th>Quantities of tissue and/or cells</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Tissue (select from the drop-menu)</td>
<td>Heart Valves</td>
</tr>
</tbody>
</table>

| How many units procured? | 284 |
| How many units tested? | 284 |
| How many units processed? | 239 |
| How many units stored? | 239 |
| How many units released for treatment? | 124 |
| How many units accepted into the TE (from other TEs within the EEA)? | 0 |
| How many units distributed from the TE (to other TEs within the EEA)? | 163 |
| How many units imported (from outside the EEA)? | 0 |
| How many units exported (to outside the EEA)? | 0 |
| How many units disposed of? | 67 |
| How many units were not fully traceable from donor to recipient? | 0 |

Fig. 2. Table extracted from the annual data report completed by the Oxford heart valve bank in response to the mandatory requirement for annual data issued by the Human Tissue Authority. ‘Units’ includes homograft valves, conduits and patches. ‘TE’ refers to Tissue Establishment which is the recognised HTA and EU term for a tissue bank. ‘Units released for treatment’ refers to tissue which has its donor file, results and records authorised for clinical use. ‘Units distributed from the TE’ refers to tissue supplied for transplantation.

The Oxford bank is the only UK group which monitors the numbers of cardiovascular tissue orders against the numbers of valves that are able to be supplied in response to the order (see Figure 3a. below). Data so far collected has, for this study, been analysed and quantifies for the first time in the UK, that there is a definite shortage of cardiovascular tissue in the UK, In 2008, despite 286 requests for tissue, the Oxford bank was only able to supply 164 tissues for transplantation i.e. only 57% of demand.
Fig. 3 a. The numbers of requests for cardiovascular tissue received by the Oxford Bank in 2008 compared to the number of tissues actually supplied, showing that demand is greater than that able to be supplied.

Figure 3 .b. The numbers of requests for different types of cardiovascular tissue received by the Oxford Bank compared to the number of each of the tissues actually supplied, illustrating differences in demand and supply of the different types of tissue.
This data has been analysed in more detail, in order to determine the relative rates of shortage for the different types of cardiovascular tissue required for transplantation. Figure 3b. Shows the differences in demand and supply of these different cardiovascular tissues. The data shows for the first time, that the most common demand in 2008 was for pulmonary valves 131 requests for which 55% were met (73 valves). There were 96 requests for Aortic valves but only 42 (44%) of the aortic demand was met by supply of appropriate valves. There were less difficulties in meeting the demand for conduits (85% supplied) and for patches (75% supplied) and this is due to the fact that there are very few specific requirements for conduits and patches (one size could meet most demand). The demand for carotids seem to have been met, however, this is misleading because, they are currently only used by one surgeon in Oxford for a new transplant technique (Evans, 2007). If this transplant becomes more popular, it may be more difficult to meet the demand.

The BATB cardiovascular tissue banks, keep a register which lists the details of all available tissue. Hence, when Oxford cannot supply tissue as indicated above, the staff at the Oxford bank will check the register of available tissue and pass the requesting surgeon in to another bank that may have a valve instead. This works well, but there often is no other valve available in the UK. If this is the case, then European and American availability is searched using ‘Cryolife’ and Bioimplant services’ tissue availability registers. One of the main reasons why there is a shortage of supply is that promotion of tissue donation is not funded by the Government (which is illustrated by the lack of its mention in the Organ Donor Register) and can only be promoted locally by each tissue bank so hence there are a large number of donors which are never considered for donation. The Oxford bank has recently performed an audit of deaths in its own NHS Trust and found there to be a vast number of potential donors which are never approached for tissue donation (see Figure 4.). This audit was performed using Oxford death data and showed that there out of the total deaths between April 2006 and March 2007 (2392), were a vast number of potential heart valve donors (172). The suitability of each one of the ‘potential donors’ group was determined by review of the details of the patient death record. Age, cause of death and past history were factors which were examined. The data is now being used to promote tissue donation in Oxford and to attempt to secure funding upon the basis of surgical demand for such potential donor tissue.
Figure 4. Results of a death audit performed in the Oxford Radcliffe Hospitals NHS Trust to determine the number of potential cardiovascular tissue donors in 2006/07. The total number of deaths (2392) was plotted against the potential cardiovascular tissue donors (172) and the numbers of actual heart valves donated in that year.

Another reason for the lack of availability is that there are also more surgical centres in the UK than there are tissue banks (see figure 5). More than 20 surgical centres routinely use cardiovascular tissue in the UK. This is because human cardiovascular tissue is required by every cardiac unit performing surgery on paediatric patients, adults with congenital heart defects and adults with infective endocarditis, since they prefer to use cardiovascular tissue for such surgery as it is considered in the UK to be far superior to that of the alternative mechanical or biological prosthesis. Also, when a request is made for tissue for transplant into a child and the donor tissue has to be from a child donor, then the demand is very high due to the lack of child donor tissue. This shortage has forced surgeons to use the less appropriate mechanical valves or xenografts (Kieboom, 2006 and Boethig, 2004) because their patients are too sick to wait for a homograft to become available. This shortage of homograft valves for paediatric patients is one of the main driving forces behind the search for tissue engineered alternatives (Leyh, 2006).
Figure 5. Map of UK & Ireland Surgical Centres which routinely transplant Cardiovascular Tissue. The circular markers represent one hospital in all but London where there are >6 hospitals using this tissue.
3.1.4 Delay in release of donor tissue for transplantation

Donor Tissue which is retrieved, processed and stored prior to transplantation is currently strictly controlled within the European Union to minimise the potential risk of transmission of disease from the donor tissue to a recipient. It is necessary to store the tissue in quarantine for at least 3 months until all recommended quality and safety checks are finalised. Typically, donated cardiovascular tissue is released within 6 months but bone tissue may often be released more than 12 months after donation (source: BATB members). This means that currently patients may die or suffer whilst on the transplant waiting list due to shortages of available tissue. Prolonged storage necessitates cryopreservation of the tissue to avoid its deterioration whilst the tissue is in quarantine. Most tissues, with the exception of corneas, are routinely stored in a frozen state. These tissues are stored in conventional freezers at <-20°C, electric -80°C freezers or in electric freezers at <-140°C or in liquid nitrogen vapour phase at <-160°C. Without the ability to safely store quarantine tissue for extended periods, the majority of UK donated tissue would have to be discarded prior to use since the authorisation procedure is now detailed and time consuming. Lack of satisfactory long term preservation method is the main cause for the high discard rate of donated corneas (Camposampiero, 2003). Cryopreservation therefore plays a vital role in cardiovascular tissue banking in the current climate, since there is a great demand for high quality safe tissue.

3.1.5 National Tissue Banking Association

Each UK Tissue Bank is required to comply with all relevant European Union Directives and Human Tissue Authority Directions, codes and licensing requirements as well as other guidance. It is hence a rapidly changing and demanding profession. Many Tissue Bankers belong to an organisation ‘The British Association for Tissue Banking’ (BATB), founded in 1985. The aims of this organisation support tissue banker’s needs at such a time. The BATB holds scientific meetings, workshops and special interest group meetings which provide a forum for networking, benchmarking and improving tissue banking within the UK and Internationally. The BATB is asked to consult on UK and EC legislation and will also volunteer suggestions and comments from tissue bankers whenever appropriate. BATB also encourages communication with the sister organisations ‘The European Association of Tissue Banks’ (EATB) and ‘The American Association of Tissue Banks’ (AATB). The EATB was founded in 1985 and published general standards, cardiovascular standards (Parker et al 2000) and instigated the set up of an independent Bioethics group (anonymous, 2001). The AATB was founded in 1976 and, in the same year, issued the first tissue banking guidelines and published tissue banking standards (Joyce, M., 2000) before there were
governmental controls (FDA; Khoie et al 2008). They also built up a voluntary program of inspection and Accreditation and set up a certification course for personnel who work in tissue banks. In 2008 there will be a joint meeting of the BATB and EATB and this will be preceded by a cardiovascular tissue study day in conjunction with AATB.

3.1.6 European Cardiovascular Tissue Working groups

General standards of cardiovascular tissue processing were developed by a joint working group of the British and European Associations of Tissue Banks (Parker 2000). The author was a member of this group. Several attempts have since been made to start to ‘harmonise’ cardiovascular tissue banking across the EU. The EUTCD provides some guidance about quality and safety issues but not about the actual methods of tissue processing. Processing methods used within the EU vary and there has been a drive to encourage banks to agree upon some common processing techniques. The main focus of working groups was to harmonise techniques such as sizing, grading, cryopreservation and thawing. The banks were encouraged to distribute their tissue information sheets and thawing instructions that they issue to surgeons. However, these groups did not complete this task although they were keen to do so.

Seemingly one of the main reasons for failure of these harmonisation groups was that they had no collated details of where their processing techniques actually varied. Therefore, this study fulfils a UK need to establish processing variables within the UK. The preliminary results of this study have already been used to encourage other European banks and US, Canadian and Australian banks to participate. This study is to be expanded in the future to include these other banks methods and experience.

3.1.7 Requirement for Quality Assurance & Validation of special processes

In order to full meet the validation requirements of the EUTCD, each technique used for the preservation, processing and storage of the tissue must be validated. The EUTCD states that “this validation may be based on studies performed by the establishment, or data from published studies or, for well established procedures, by retrospective evaluation of the clinical results for tissues”. It appears that cardiovascular tissue bankers have not published or presented detailed validation of some stages of their own banks tissue processing. The EUTCD requires that validation records should be available for inspection but does not insist that they be openly published. It is likely that the areas where the tissue processing varies could well be those where there is little detailed validation. Some of these processes may be performed adequately in a standard and consistent manner but yet remains based on crude validation work carried out many years previously and thus is historically based rather than based upon a scientific evidence base. Unlike the
current methods used for vascular grafts, the clinical cardiovascular tissue bankers have been criticised on
the basis that the current methods used do not rest on a substantial body of experimental data but instead,
have been derived generally from protocols used for the preservation of single-cell suspensions (Wusterman,
M., 2004).

One of the significant challenges of licensed tissue banks is therefore to ‘re-validate’ their special processes
using up to date evidence based information, and to also recognise where this is unavailable and, ultimately
to commission experimental studies to fulfil the needs.

3.1.8 Tissue Bank staff Professional Registration & Career framework

The BATB has recently linked with the Voluntary Registration Council (VRC) in order to begin the procedure
toward professional registration of tissue banking staff. The VRC currently is helping tissue banking
profession (via the BATB) with the detailed process leading up to acceptance with the regulatory body known
as the Health Professions Council (HPC). HPC stipulate that more than 25% have to be on a voluntary
register (VRC) and that they must have a professional body which represents more than 25% of the
profession before they will accept tissue banking as an ‘aspirant group’. This work is underway and tissue
banking is soon to be accepted as a ‘profession’. There is also one Department of Health working group
developing a career pathway (‘Skills for Health’ working group) and another team working out National
Occupational Standards for the variety of tissue banking roles and also another Department of Health team
(Workforce Review Team) working with tissue bankers to assess the amount and skill mix of tissue banking
staff needed for the future 10 years.

However, there is still no formal training program within the UK, which addresses the academic, vocational
and professional needs of the tissue banking profession. This is major hurdle in acceptance of the profession
by HPC and it is also now a requirement of The EC. The technical details of the EUTCD does not give
specific details guidance about training of staff nor does it detail the processes which should be used for
tissue processing. Tissue banks have to train all new staff and it is therefore up to individual banks to
determine how they will deliver this training. Many incoming staff are graduates with a biological sciences or
relevant degree, but undergraduate level staff are also required for the routine tissue processing. Tissue
bank staff training is therefore initially based upon their individual banks processing techniques and, since
these techniques vary, this training is not necessarily transferable should staff leave and join a different
tissue banking organisation.

European and International training packages offer some general basic tissue banking training (University of
Barcelona Tissue Banking Certificate, International Atomic Energy Authority Tissue Banking course). Efforts
have also recently been made in the UK to rectify this lack of formal training which is a requirement for EUTCD. A UK Training program has been launched in December 2007, ‘the BATB/BBTS Specialist Certificate for Cell and Tissue Transplantation Science, and, although this is set at post-graduate level, it is intended to be a first step toward ‘National’ training of tissue bank staff (Gillan, H., 2007). The course is ‘self-taught’ and will only be accessible to tissue banking staff since the candidates will be required to use knowledge and skills gained whilst working in the profession. The syllabus covers all aspects of tissue banking which is briefly detailed in a textbook, which also includes further reading lists and examples of questions and answers. The BATB will hold seminars and study days to support the course and there will be an exam held annually.

However, this course is still flawed by the fact the technical processing content is, so far, based only upon one tissue banks processing techniques and not that of the whole UK experience. The processing detail in the training booklet where there is little detailed validation (such as cryoprotection, re-warming and dilution) may also be of questionable quality. However, these errors will be rectified as the course develops. This remains undoubtedly however, an exciting new opportunity for tissue banking staff and a major advancement towards recognition and acceptance of the ‘tissue banking profession’.
3.2. DEVELOPMENTS IN CARDIOVASCULAR TISSUE PROCESSING

In order to appreciate why there are different processing methods currently in use in the UK, the historical background of the use of homografts, the processing methods previously used and the basis for the differences in practice must first be understood. This is not evident from purely a review of reported published articles, and so the data collected from surgeons’ interviews were particularly helpful for clarification of these events.

3.2.1 Introduction

Cryopreservation of cardiovascular tissue is implemented to preserve the function of any living cells within an intact extracellular matrix. Tissue cryopreservation is a considerably more complex situation than that of single-cell suspensions as previously explained. Cardiovascular tissue is clinically useful only if, following cryopreservation and re-warming, it has maintained its ability to provide mechanical support by virtue of adequate retention of its intact extracellular structure. It seems apparent that all tissue banks preparing cardiovascular tissue for therapeutic use (which may also be termed ‘cardiovascular tissue banks’ in this study) operate independently using their own uniquely derived tissue processing protocols. These tissue banking protocols have not previously been established nor compared or benchmarked.

Tissue banking has facilitated the increased availability of tissue and this has enabled a sizeable number of patients to undergo some form of therapeutic treatment based upon the use of cardiovascular tissues. Processing protocols have varied greatly since the first cardiovascular tissue aortic homograft valve was implanted in 1962 (Ross, 1962). The differences between processing which exist may result from their design having been empirically derived and this may influence tissue quality and function. There are few details published regarding routine tissue banking Quality Assessment and Validation protocols. Ultimately however, tissue quality and/or durability may be best measured by Postoperative Performance Assessment. Published long term postoperative database software and studies are most often focussed upon surgical techniques and have little detail regarding the processing variables and resulting risk factors. Regardless of this, reported tissue performance results have been very mixed - the poorer results leading to abandonment of some processing techniques such as irradiation sterilisation. There are differences of belief in the need for ‘viable’ cells in the processed tissue and this underpins the tissue processing design. There remains no harmonisation between protocols used and as a result there may be differences in the quality of the tissue ultimately processed and transplanted.
3.2.2 Terminology

Although the British convention amongst surgeons is to use the term ‘homografts’, it is gradually being superseded by the term ‘allograft’. The former term describes tissue where the donor and recipient are from the same species whilst the latter term more specifically denotes that the donor and recipient are genetically different. An ‘Autograft’ is a term used to describe cardiovascular tissue removed from one location and transplanted into another in the same person. This is a term used to describe the pulmonary valve which is moved in a unique surgical procedure from a patients own pulmonary position into its aortic (or mitral position). This technique is named after Donald Ross who first developed the procedure (Ross, 1967).

The term ‘Homovital’ when associated with homografts or allografts describes a valve removed from a heart transplant recipient under sterile conditions which is minimally disinfected but transplanted within 48hrs of procurement into a patient requiring valve replacement or reconstructive surgery. All terms will appear at intervals throughout this text and in referenced bibliography. Use of the term homografts and allografts may be interchangeable, but the most accepted terms used internationally now are allograft (instead of homograft) and autograft (meaning auto transplanted pulmonary valve in Ross procedure).

3.2.3 Historical Background

The first successful and ground breaking human heart valve replacement was that of a mitral valve using a mechanical or prosthetic designed valve with consisting of a caged ball with dacron sewing ring configuration known as the ‘Starr Edwards’ valve (Starr, 1961). Within a short time, an aortic Starr valve was also implanted on its own and in combination with a mitral valve (Starr, 1961). However, there was soon significant evidence of problems relating to their inherent thrombogenicity and unnatural harshness on blood cells inducing valve failure leading to sudden patient death. Other mechanical valves such as tilting disc and bileaflet valves were subsequently used but experience highlighted similar problems to that with Ball valves. Porcine and later bovine heart valves mounted on cloth covered frames or ‘stents’ (known as xenograft or bioprosthetic valves) were used widely from 1969. However, to prevent rejection after implantation in humans, they had to be sterilised (or ‘fixed’) in harsh chemicals such as formalin and then glutaraldehyde, and this led to collagen disruption and early failure most often culminating in leaflet tears and calcification.

Although Xenograft valves are moderately stenotic with the stent reducing the effective orifice area, they do have markedly reduced thromboembolic complications compared to mechanical valves but their main limitation is that their durability particularly in paediatrics (Walther, 2004 and Dittrich, 2000). The search therefore remained to find the ideal valve substitute and interest focussed on the anatomically, physiologically and functionally more natural human tissue alternatives.
Laboratory investigations by Lam led to implantation of an allograft aortic valve into the descending aorta of a dog (Lam, 1952). This was then followed by implantation of an aortic valve into a patient with native aortic insufficiency (Murray, 1956). Duran and Gunning (1962) developed the technique of implantation using a running single suture line which was then used by Ross for the first allograft subcoronary transplant which took place in Guys hospital, London in 1962 (Ross, 1962). The next major landmark in the use of aortic homografts was the use of an aortic homograft for right ventricular outflow tract reconstruction in a paediatric patient with Pulmonary Atresia (Ross, 1966). Homografts were used soon afterwards to repair more complex abnormalities such as ‘truncus arteriosus’ (McGoon, 1968) and ‘transposition of the great arteries’ (Rastelli, 1969). The first Ross procedure was performed in 1967 as previously explained and several modifications subsequently successfully developed such as using autograft as a root replacement (Gerosa, 1994). The most courageous development was that of using the patients’ normal pulmonary valve, after sewing it onto a frame, to replace abnormal mitral valves which were first performed in 1968. Other modifications included implanting the autograft as a root rather than using the subcoronary technique (Gerosa 2000). The first freestanding aortic root was implanted by Ross (1972) for treatment of an adult with aortic root abscess. A flurry of new operative techniques were attempted in the 1970s, some of these utilising frames upon which the homografts were sewn. These frames (or ‘stents’) were similar to those commonly used in commercial xenograft manufacture i.e. plastic with cloth cover, flexible but relatively obstructive. Aortic homografts were sewn onto these stents and inverted for mitral valve replacement. Autologous glutaraledhyde treated, Facsia Lata and pericardium were also sewn onto stents and used for surgery for the aortic and mitral positions. The results for these frame mounted valves were disappointing (Ross, 1972) due to a variety of factors including technical defects made whilst the valves were hastily sewn onto frames in theatre. Tissue thickening and shrinkage away from the stent was also commonly observed after an intermediate post operative term (i.e. 5 to 10yrs). These stented valves were abandoned and instead flexible Dacron cylinders with autologous pericardium or Dacron patch (‘top hat’) were introduced for support of the homograft or autograft for supra annular mitral valve implantation or for mitral Ross procedures. Due to a shortage of large aortic homografts, pulmonary homografts were eventually used to reconstruct the right ventricular outflow tract (Kay, 1986) after extensive experimentation (Livi, 1987). Pulmonary homografts were later used to replace the diseased or infected aortic valves following further testing to confirm that there are minimal mechanical differences between aortic and pulmonary tissue (Vesely, 2000). Despite successful ‘early’ 3 to 4 yrs postoperative performance (Gerosa 1994), longer term follow up identified significant acute and chronic malfunction (Naegele 2000) and thereafter this was abandoned.
One of the most recent successful advances in homograft use is the re-introduction of the Ross procedure for replacement of the mitral valve which has been modified by a team in Syria led by DN Ross and now involves use of pulmonary homograft sewn into a cloth tube with autologous pericardial patch covering (top hat) technique (Kabbani 1999). The author was also involved in this new series and performed the sewing in theatre.

The most common use currently in the UK is for homografts (as patches or non valved conduits) for the Norwood procedure for alleviation of hypoplastic left heart syndrome (Alphonso, 2005) although the challenges of postoperative care remain now the most critical challenge.

Cardiovascular Homografts, Allografts and Autografts have been used relatively successfully for more than 30 years, there main draw being lack of availability and an accepted optimised method of preservation and storage to ensure maximum durability.

3.2.4 Advantages of using cardiovascular valve tissue

Homograft (or allograft) tissue has excellent hydraulic function and haemodynamics due to its central non obstructive flow even in small sizes since it is flexible and does not need an attached frame or stent unlike prosthetic and bioprosthetic alternatives. Recipients quality of life is therefore improved (Schmidtke, 2001). Thromboembolism and hemolysis rates are reduced without the need for anticoagulation. Patients, for whom anticoagulation is contraindicated such as pregnant females or children, can thus benefit from homograft surgery. Homografts are versatile and can be used to treat patients with a wide range of acquired diseases or infection and especially in complex congenital defects. Consequently, it is considered the valve of choice for right ventricular outflow tract reconstruction (Kay 1985). The failure mode of homografts is slow and the nature is by calcific degeneration. Hence there are very few sudden deaths in homograft recipients which relate to the valve. Calcification rarely affects the valve leaflets normally is restricted to the conduit wall and is slow enough to enable time to safely organise elective re-operation (McGiffin,1997). Homograft and autograft long term performance is generally more successful than the alternative mechanical or xenografts in both adults (Yacoub, 2006 and Ross, 2005) and in children (Kouchoukos, 2004 and Homann, 2000). For re-operations, homografts Homografts are remarkably resistant to infection compared to mechanical or xenograft valves, whether homograft implantation is for repair or replacement of the native or the prosthetic infected valve (Haydock, 1992).

The Ross procedure is now widely accepted as a potentially ‘permanent’ valve replacement therapy since its autotransplanted pulmonary valve tissue has the potential to remain viable and to grow in a child recipient (Elkins, 2001). This growth has been shown to be proportional to the somatic growth of the child.
(Elkins 2001) and is often considered the ideal valve replacement for paediatric patients. Long term systematic follow up of Ross procedures performed by many different surgeons operating in many surgical centres is co-ordinated in two centres. The original registry is in the US (Oury, 1998), and more recently a separate registry has been set up by H. Sievers in Germany (Hanke, 2007).

In developed countries of the world, homograft costs (‘processing reimbursement charges’) are now similar to that of alternative commercial valves. However, there is now increasing evidence (Jaggers, 1998) that since the homograft procedures can be performed safely, with short hospital stays, decreased morbidity, this results in an overall cost effective option.

In some countries where mechanical and biological valves are relatively expensive such as India, Africa, Brazil and Syria, homografts are utilised as a low cost option. These countries also have a higher incidence of mitral valve disease since diseases such as rheumatic fever have not been eradicated. Using homografts offers a means of avoiding the anticoagulation therapy which is mandatory for all patients with mechanical valves. Patients in these poorer countries cannot often afford to purchase these anticoagulation drugs nor can they afford to attend the necessary blood clinics necessary to monitor the lifelong anticoagulation therapy. Hence in India, Brazil and Syria, stent mounted homografts are still commonly used for mitral valve replacement despite the expected poor long term performance. 10 year acceptable valve performance is their aim and is most often achieved. The Syrian team have so far implanted the largest series of Ross procedures to the Mitral position using top hat autologous pulmonary valves with excellent short term results (Kabbani, 1999).

### 3.2.5 Disadvantages of Homografts

In addition to the lack of availability of human valves despite progress in tissue banking methods, there are reasons why homografts may not always be the optimal valve. Valve repair or replacement using homografts often takes at least 30 minutes longer on bypass than the stent mounted or cloth covered mechanical or biological prosthesis alternatives currently available. Thus surgeons are reluctant to use homografts when prolonged myocardial ischaemia may jeopardise the patient recovery, such as for patients with unstable angina and those with coronary artery disease and poor left ventricles.

The relative long term failure of top hat aortic homografts which were inverted and inserted for mitral valve replacement for a period commencing 1968 was due to increased early degeneration (Mankad 1986). Mitral valve replacement with mitral homografts was first reported in 1994 (Acar) and tricuspid valve replacement with mitral homografts first performed in 1993 (Pomar). Since then there have been very mixed results using
various operative techniques with limited early success (Acar, 1997, Kumar, 2000 and Doty, 2001). Mitral and tricuspid repair with homograft leaflet or Dacron patches is now preferred (Osman 2005).

One of the main disadvantages with the Ross Procedure is that the surgical technique is initially very difficult and surgeons have reported that there is an associated ‘learning curve’ with this form of transplant which was evident in the early pioneering series (Chambers, 1997 and Oury, 1999). There are others which cite mismatch of the aortic and pulmonary roots as a concern (David, 1996). Aortic valve replacement (AVR) using the Ross procedure requires longer cross clamp (41%) and longer bypass times (43%) than conventional homograft valve replacement. However, now the technical risk factors relating to the anatomy of the septal artery seem to have been identified (Geens, 1971), results of a prospective randomised trial of Ross procedure versus homograft AVR (Aklog, 2000), showed that the greater complexity of the Ross procedure did not result in significantly more bleeding, longer recovery or more complications. Post operative echocardiography confirmed excellent function in autograft valves, but early signs of degeneration in the homografts, therefore illustrating that the Ross procedure may be advantageous compared to homograft replacement, especially in children, despite the more complex technical surgery.

One of the issues with very fresh (‘viable’) allografts is that it seems likely that they may elicit donor-specific immune response (Jashari, 2004 and Bechtel, 2001) particularly in paediatric patients. This subject will be discussed in a later section.

3.2.6 Evolution of homograft disinfection methodology

The processing and preservation techniques for cardiovascular tissue have changed greatly since the first human heart valve was processed was processed and used in Guys Hospital, London in 1962.

The two main centres of homograft use in the first ten years of use of the homograft valve were operating independently. Donald Ross’s team working in London experimented with a variety of methods whilst the New Zealand team led by Brian Barratt-Boyes remained fixed upon one method of preservation. Detailed experimental studies and evidence of encouraging results reported by the Australian team led by Marc O’Brien, eventually led both the London and New Zealand teams to switch to ‘viable’ cryopreservation techniques of homograft processing.

The first cardiovascular tissue valve used was disinfected in London using a broad spectrum, relatively toxic, antibiotic cocktail (Longmore et al 1966). More harsh disinfection treatment methods using betapropolactone, gassing in ethylene oxide and gamma irradiation were all subsequently used in parallel for a period of time.

The main aim of the London team (confirmed by Donald Ross, Robert Parker) was to ensure adequate disinfection since the tissue was often procured in the mortuary environment under clean but unsterile
conditions. The New Zealand group started using homografts at the end of 1962 and they used a relatively low dose antibiotic cocktail (Barratt-Boyes 1964). Due to increasing evidence of early homograft failure with some of the more harsh alternatives, the London team compared the disinfection regimens of London valves (which yielded typically more than 80% sterility), against tissue they immersed in the New Zealand low dose antibiotic cocktail (unpublished study). Despite reports that the New Zealand disinfection rate was satisfactory, the London team did not find that this effectiveness could be reproduced, and so the London team changed back to their antibiotic disinfection regimen in 1968 (Lockey et al 1972). Magdi Yacoub also working in London started a large series of homovital homograft transplants. His homografts were exclusively procured in the operating theatre from living heart transplant donors or brain dead multi organ donors and were usually transplanted within 7 days of procurement (Yacoub, 1995). Initially Yacoub preserved this tissue in nutrient media only, but despite the procurement aseptic theatre conditions, this tissue showed signs of positive contamination and disinfection antibiotics were therefore added to the media to minimise the risk of transmission of this infection to the recipient (Lund, 1999).

3.2.7 Evolution of Preservation methodology

The first human aortic valve tissue was transplanted into the descending aorta by Murray (1956) and was a fresh antibiotic nutrient media preserved valve. The first aortic homograft valve transplanted into the aortic position for total aortic valve replacement was performed by Ross, in London (Ross, 1962) and was sterilised by ethylene oxide and then preserved by freeze drying and stored at room temperature in the homograft bank. Reconstitution in theatre involved first adding distilled water and then leaving for thirty minutes and was then followed by transfer to isotonic saline before it was ready for use (see figure 6a & b ). This method was used until 1967/68 by the London team.
Figure 6a. A Freeze dried aortic homograft valve which was processed in the London bank in a sealed glass container under vacuum. b. Freeze dried valve showing label confirming that it was procured, sterilised in ethylene oxide and then freeze dried in 1967 before being kept at room temperature in the homograft bank.

The second valve was implanted to replace the aortic valve several months later in New Zealand and it was an unpreserved 'homovital' valve retrieved from a heart transplant recipient (Barratt-Boyes, 1964). Thereafter, the New Zealand team predominantly used nutrient media which they stored at 4°C for 7 days and in which they added their low dose disinfecting antibiotics. Following the freeze drying preservation period the London team changed in 1967, to uncontrolled rapid freezing (Ross, 1968). The protocol for this freezing simulated the empirical cryopreservation method which was then being used successfully for cells and applied extensively throughout medicine and biology. The publication documenting the validation for this procedure (Al Janabi, 1974) cited references of original clinical experience (Smith, 1951, Lovelock, 1953b and Lovelock, 1959) and other more updated and relevant publications (Wolsteholme, 1970), none of which appeared to be directly related to cardiac tissue. The technique seemed not to have been optimised for cardiovascular homograft tissue, but some details are documented in the validation written by Al Janabi. The freezing procedure they designed comprised of using tissue which has been procured within 24 to 48 hours of death and after dissection, the valves were immersed in the London nutrient media with antibiotic cocktail and calf serum (Lockey, 1972). If the valves were not used, or in excess of demand, then they would be frozen within 4 weeks of death. The tissue chosen for freezing would be placed into their freezing medium which consisted of 85% antibiotic nutrient media and 15% DMSO and pH was adjusted using sodium bicarbonate to pH 8.0. The valves were then cooled slowly (-1°C per minute) using an automatic ‘Medical Scientific Company’ freezer from ambient temperature down to -70°C. They were then transferred to the vapour phase of a liquid nitrogen refrigerator and stored at -195°C, usually for up to 3 months. Their procedures for thawing and removal of the DMSO also did not seem to have been optimised for this cardiovascular tissue application.
Their protocol was to transfer the tissue directly to thaw in a water bath at 37°C and this was followed by two washes in Hanks solution. The validation for this freezing method (Al Janabi et al 1974) was based upon the London group’s previous related studies. The first study (Al Janabi, 1972) illustrated that the morphology of valves removed within 48 hours of death and sterilised with antibiotic mixture could be shown to contain living fibroblasts. The second study (Al Janabi, 1972) showed that the valve leaflet fibroblasts were capable of synthesising DNA and they later showed that these fibroblasts could also synthesise protein (Al Janabi, 1973). The rate of decrease in the number of viable cells in tissue stored in nutrient media rather than Hanks was investigated in their next publication (Al Janabi, 1973) and resulted in a change to store tissue in nutrient media which extended the ‘shelf life’ from 18 days to 18 weeks. The London team hose to assess viability of the frozen tissue by means of autoradiography using titriated thymidine (Al Janabi, 1972). They postulated that the uptake of titriated thymidine may be a direct indicator of the utilisation of thymidine (which is a precursor of DNA) by dividing cells as previously described (Taylor, 1960 and 1965). They found that there was a decrease in mitotically active fibroblasts which related to the increasing time that the tissue was in antibiotic cocktail pre freezing. They suggested that, so long as the period of pre freeze antibiotic storage did not exceed 4 weeks, then these valves (they called them ‘living valves’) could be safely frozen for up to 3 months with >50% preservation of mitotically active fibroblasts.

The London team then felt able to introduce this freezing method as a satisfactory prolonged storage option which would maintain the viability and the structural integrity for aortic valve tissue sterilised in antibiotics and then frozen with 15% DMSO. They applied this method to those valves unused in the first 4 weeks after procurement or found to be in excess within that time. They used this method of freezing until 1970/71 after implanting more than 93 of these frozen valves. A post operative patient study 11 years afterwards (Ingegneri, 1979) reported results which were very disappointing since the valve degeneration was more than predicted. It was, however, slightly better than that of the freeze dried valves, the report showed that the onset of degeneration of the frozen valves was delayed compared to that of the freeze dried valves. The only encouraging data was that the patient survival was still relatively good (77% probability of survival at 11 years).

There was, however, similar Internationally accepted evidence of poor long term durability seemingly resulting from the various combinations of sterilisation and preservation methods attempted (O’Brien, 1987, Angell, 1973). In response to this uncertain long term homograft performance, and following a variety of detailed post operative and experimental studies performed by Al Janabi and her team in London (Al Janabi, 1972, 1973, 1973 and Gibson, 1973), the London team returned to fresh viable valve homografts in 1970 and ‘conformed’ with the rest of the International homograft banks. They also returned to storage in tissue
culture medium (TC 199 or Hanks media) in order to improve preservation of tissue ‘viability’ (Al Janabi et al 1973) with their less harsh high dose antibiotic disinfecting cocktail. This move to return to antibiotic nutrient media preservation, however, allowed only very short term storage and homograft availability worsened. The London team then added bovine calf serum to the nutrient media in the early 1980’s which was meant to improve and extend the tissue preservation. However, it was soon observed (Bodnar, 1989) that valves immersed in media with this calf serum suffered degeneration (by ‘primary tissue failure’) sooner than those without. Also, they noticed clinically significant immunological affects in this serum treated tissue and so its use was withdrawn. Upon investigation using the London homograft processing records, it has been discovered that in the late 1970’s and early 1980’s, when there was a surplus in valves of a certain size, to avoid wastage, a small number of these surplus antibiotic disinfected homografts were rapidly frozen (seemingly approximately 15% of the total number of stored valves). The London team were aware that this frozen tissue was of less ‘viability’ than the antibiotic nutrient preserved tissue but believed them to still be superior to the mechanical and xenograft alternatives, and so this temporarily helped them to alleviate the shortage of homografts and were transplanted when no other homograft was available.

3.2.8 Introduction of ‘viable’ cryopreservation

In order to preserve tissue for longer periods and thereby increase the number of banked tissue available, an alternative longer term storage method was still therefore required. The ‘solution’ was then found to be that of freezing using a ‘new’ method of controlled cooling and low temperature storage cryopreservation. ‘Viable’ cryopreservation was introduced in the late 1970’s (Angell, 1976, 1987) and expanded in the 1980’s based upon the data illustrating enhanced long term performance reported independently by the Angell and O’Brien’s teams in San Deigo, California, United states and Brisbane, Australia (Angell, 1989 and O’Brien, 1987) which retrospectively compared series of antibiotic sterilised and refrigerated homografts with their later series of viable cryopreserved homografts. O’Brien’s method of cryopreservation was first used in his unit in 1975 and was designed to permit maintenance of tissue viability. It was applied to tissue that had been procured in theatre within 24 hours of death and then disinfected for 24 hours followed immediately by cryopreservation. The cryopreservation protocol consisted of slow controlled cooling with 10% dimethyl sulphoxide and storage in liquid nitrogen vapour phase at -180 to -190°C (O’Brien, 1987). O’Brien claimed that his tissue was viable after transplantation although initially, the evidence for this is ambiguous. On the basis of O’Brien’s claims and the fact that he reported good performance from this tissue (Mc Giffin 1988, O’Brien, 1991) most centres worldwide quickly switched paradigms to begin this new form of cryopreservation. This rapid acceptance of O’Brien’s claims seems to have ignored some of the concerns.
which perhaps should have been raised at the time, such as comparing two series of data from different eras
is questionably less acceptable than prospective randomised trials (Bodnar, 1979). Also, O’Brien’s claims of
viability following cryopreservation should have been defined, justified and then confirmed by different
viability assessment methodologies as recommended by leading cryobiologists at the time (Pegg, 1989 and
Bank, 1989). Homograft tissue after cryopreservation was tested later by different methodologies and found
to produce serious damage to cytosolic and mitochondrial function of both endothelial cells and fibroblasts
and caused the collagen metabolism to become degradable (Kitagawa, 2001) which was likely to lead to
valve failure. O’Brien’s claims that, after explant, the presence of cells of donor origin was established, was
based upon one case only (O’Brien, 1987) and was later to be contradicted by many other researchers
(Mitchell, 1995 & 1998, Schoen 1995 and Koolbergen, 2002). This seemingly would alleviate the shortage of
homografts by enabling a ‘bank’ of cryopreserved tissue to be built up and safely stored for at least 5 years.
O’Brien suggested that long term performance of homografts ought to be expressed in terms of viability in
future studies. Dimethyl sulphoxide gradually replaced the use of glycerol as cryoprotectant and controlled
rate freezers were predominantly utilised. This longer term storage also facilitated more detailed viral testing
and more extensive donor record examination. Since antiviral disinfectants were not included in the
disinfection regimes, this helped to minimise risk of transmission of viral disease to recipient patients.
Following examination of explanted viable cryopreserved tissue, O’Brien found a significant amount of
degeneration which was confirmed by histological examination (O’Brien, 1988) despite there having been a
standard protocol in place for 12 years. O’Brien found that the leaflet viability varied considerably (O’Brien,
1988) and that valves with proven pre implant viability were not always perfectly maintained after
cryopreservation and implantation and this had led to subsequent leaflet degeneration. O’Brien then began
to recommend that markers for degeneration should not just measure viability, but should also monitor post
operative allograft valve incompetence and histology of valve leaflet tissue. He suggested increasing pre
implant viability by lowering the antibiotic dose used for organ donor tissue, shortening the period between
death and procurement and increasing the number of organ donors, and shortening the antibiotic disinfection
period and even also cryopreserving within 12 to 18 hours of death. Many of these ideas however, were
practically difficult to implement but tissue bankers seem to have striven towards them over subsequent
years.
In hindsight, it seems that the main difference between the old London method of cryopreservation (or flash
freezing) and the latter O’Brien and Angell viable cryopreservation techniques, was that the old London
tissue was likely to be ‘non viable’ prior to implantation (due to the increased time between death to
procurement and freezing and the more toxic antibiotic disinfection regimen).
In order to enhance the quality and safety of cryopreserved tissue intended for therapeutic use, it is important to collate evidence of quality tissue banking experience in order to identify best practice and to fully understand the effects of cryopreservation on the tissue’s living, functional cells and on the extracellular matrix of the tissue.
3.3. UK CARDIOVASCULAR TISSUE BANKING REVIEW - GENERAL FEATURES

A review of the organisation, role and status, of UK tissue banks has been performed and differences are highlighted and discussed.

3.3.1 Organisation of UK Tissue banks

There are currently 6 banks in the UK and one bank in Dublin, which process and supply cardiovascular tissue for therapeutic use (see fig 2). These banks are often known as ‘heart valve banks’. The original heart valve bank was opened in the UK in the 1960’s at the Guys Hospital in London and it was from here that Mr Donald Ross FRCS obtained the human valve which he used in the first human heart valve transplant in the world. This bank was relocated first to the National Heart Hospital and then was based at The Royal Brompton Hospital in London. In the same year another bank was opened in Oxford at the John Radcliffe Hospital using a similar ethos and processing techniques. Bone banks in Sheffield and Wakefield also expanded to add a small cardiovascular tissue processing service. Several years later, other heart valve banks were opened in Harefield Hospital in Uxbridge, at Southampton General Hospital and at Birmingham Children’s Hospital. These banks were all set up in response to a local clinical demand which is why they were located and funded by the cardiovascular surgery centres within each hospital and sourced donors from their local area for use predominantly in patients from that same area. Another bank opened shortly afterwards at the Bristol Eye Hospital adjacent to the Eye bank in order to supply human cardiovascular tissue to the Bristol Royal Infirmary. Later, the National Blood Service (NBS) expanded their blood products service to begin processing musculoskeletal tissue and eventually added the processing of cardiovascular tissue to their portfolio. Likewise the Scottish National Blood Service (SNBTS) and the Irish Blood Service (IBS) began to process cardiovascular tissue.

The National Blood service initially had several centres around the country processing mostly bone tissue, but following rationalisation and the introduction of EUTCD, they subsequently closed their East Anglia, Wakefield and Wrexham banks where their cardiovascular tissue processing service was based and recently has further consolidated
their service to only one centre for all tissues in Liverpool. The banks at Harefield and Southampton have also now closed following failure to secure funding to upgrade facilities in order to comply with EUTCD. The catchment area for NBS banks is variable and often cardiovascular tissue retrieval accompanies and is dependant upon whether musculoskeletal tissue is also retrieved. The SNBTS however, retrieve and supply only within Scotland. The IBS retrieve multiorgan donors from their local area but do not retrieve any tissue from deceased donors. Northern Ireland refers donors to, and receives tissue from, the London and Oxford banks. The organisation of the tissue banks in EU and US are also variable. Some banks are based in Hospitals and others collect and supply tissue from a number of hospitals. The Rotterdam heart valve bank retrieves tissue from the Netherlands, Germany and Belgium, whilst also in Germany and Belgium; some hospitals have their own heart valve banks. It seems that surgical centres with busy paediatric cardiac surgery programs are most likely to have their own heart valve banks in order to secure tissue for their patients who require tissue transplants on an emergency basis and thus require a 24-hour heart valve bank service. The independent, hospital-based banks are self funding where as the Blood Centre banks remain subsidised by the Blood Service. In order to achieve ‘self –funding’ status, most banks will supply tissue to other surgical centres and will invoice the centre afterwards with a fee which is annually set at a level equivalent to re-imburse the processing costs. Scotland does not currently invoice any surgical centre; instead their reimbursement is funded centrally. UK legislation (HT Act 2004) strictly prohibits anyone involved with the supply of human tissue to make a profit from buying or selling tissue. One of the main benefits of the hospital based banks seems to be that they are embedded within a cardiac clinical service and can thus use the services of specialist consultants for donor selection advice and for confirmation of tissue rejection criteria and will inevitably be more clinically aware of emerging trends and changes in clinical needs of cardiac surgeons. They are therefore more competent at offering advice to those ordering valves since they are experienced in valve transplantation issues and can ask for consultant advice if it is necessary. The organisational structures also facilitate NHS
driven priorities and healthcare pressures. A typical independent hospital based cardiovascular tissue bank (bank 1) organisational chart is attached (see Appendix III).

3.3.2 The Role of UK cardiovascular tissue banks

UK cardiovascular tissue banks provide a service which includes five key roles. Some banks participate in all five functions and some have a more limited service. All functions performed by third parties on behalf of the tissue bank must be strictly controlled and responsibilities clearly indicated in Service level agreements or Third Party agreements (see Appendix IV). Some UK banks perform all key functions (see Appendix V). The five key functions are:-

a) Consent
b) Procurement
c) Processing
d) Testing
e) Distribution

a) Consent

Some banks have their own team of nurses trained to take consent for donation and they are known as ‘Tissue Co-ordinators’ (banks 3, 4, 6). These tissue co-ordinators normally interview bereaved families by telephone in order to obtain consent and this is supported by taping the conversation. Other banks (banks 1, 2, 5, 7) instead normally prefer to use the services of the regionally based National Organ Donor Transplant Co-ordinators who normally meet the bereaved families personally. In the EU there is a variety of co-ordinator systems in place; however, the most efficient (in terms of increasing donor consent rates) is that of the ‘Spanish model’. In Spain, a co-ordinator is based in each major hospital and will then be present to daily identify and follow up potential donors and interview potential donors and their families. As previously described (section 3.1.2) In order to increase UK donation rates of cardiovascular tissue, the UK banks that currently do not have their own co-ordinator service, are now contemplating adding a
co-ordinator to their staff team, however, this involves a considerable financial commitment. This is being considered in response to the fact that the scope of the taskforce does not include that of tissue donation (except corneas) as previously discussed (section 3.1.3) and that there is a shortage of human tissue in the UK. The EUTCD requires that UK tissue banks have service level agreements with all co-ordinator teams detailing the expectations and responsibilities of both parties in relation to gaining informed consent for cardiovascular tissue donation and highlighting the donor selection procedures required (as previously discussed in section 3.1.1).

b) Procurement

All UK banks currently organise the procurement of their cardiovascular tissue. If a multi organ team is present to retrieve organs, then they will retrieve on behalf of the tissue bank. If however, a post mortem examination is to take place, then some UK banks (banks 1, 3, 6) will attend and retrieve the tissue themselves, if possible at the start of the examination. The other banks (banks 2, 4, 5) will rely on the pathologist to retrieve on their behalf, but this normally is then performed without sterile kits and without aseptic technique. Often the tissue may be damaged or cut too short by the pathologist. One bank (bank 7) does not retrieve tissue from deceased donors. Cardiovascular tissue may also be retrieved when there is no post mortem, and, for this to happen, the tissue bank needs to send out their retrieval team with all necessary procurement equipment and consumables. The retrieval centre may be a Hospital operating theatre, a mortuary a tissue bank retrieval suite or in a very small amount of cases a funeral parlour or hospice. The conditions for retrieval are described and discussed in a later section.

C) Processing

All UK banks are involved in the processing of the tissue and currently all cardiovascular tissue is processed within each bank. Some musculoskeletal tissue however, is sterilised by gamma irradiation at sites outside the UK tissue banks. In some banks in the EU, cardiovascular tissue may be despatched outside the tissue bank to cryogenic storage
facilities. This seems to be due to the high purchase price and the relatively large amount of space required for storage of tissue in nitrogen refrigerators and the relatively high running costs. Commercial cryogenic storage facilities allow tissue banks to store their tissue safely and inexpensively off-site with the cost of a monthly storage charge only.

d) Testing
Most banks in the UK send out tissue or blood samples to external laboratories for testing. These laboratories routinely deal with large volumes of samples and are regularly inspected and accredited by either the Public Health Laboratory or a similar inspectorate. The labelling, packaging and transportation of these samples is strictly controlled. Tissue banks have to have service level agreements (SLAs) with every bank that is used to test their specimens. This is similar in both EU and US.

e) Distribution
All UK banks are actively involved in the distribution of the processed tissue. The Department of Health (DH) has funded an organ transplant waiting list which is regularly updated to monitor the details of patients waiting for all types of organ transplant. Unfortunately, the DH has only funded a waiting list for patients awaiting cornea transplants and has no plans to expand this list to include other tissues, despite the fact that there is a National shortage of donor tissue (particularly skin and cardiovascular). Consequently, surgeons requiring cardiovascular tissues for their patients have no choice but to directly contact the heart valve banks. The heart valve bank will then despatch tissue on a ‘first-come-first-served’ basis. If one heart valve bank is unable to supply the type or size of tissue requested, the bank staff will suggest another UK bank who may be able to help instead. This is based upon a BATB CV SIG incentive to keep a bi-weekly list of cardiovascular tissue available in the UK. This list is updated with co-operation of all UK banks and is intended to facilitate timely distribution of cardiovascular tissue. One bank (bank 1) keeps a list of requests for tissue for patients waiting for cardiovascular tissue transplants (as previously described in section 3.1.3).
In the EU there are again similar problems with supply of tissue (except in the countries where there is an ‘opt-out’ scheme). In some areas in Europe, surgeons request from their local heart valve banks and in other areas, where there is no bank, they will direct their request to a large organisation that will source the tissue for them. Bio implant Services (BIS) operate as a distributor of organs and tissues within Europe. In the US, most tissue is supplied directly from the heart valve bank.

One US bank, (‘Cryolife’ Inc.) has opened a facility in the UK to import cardiovascular tissue from their bank in the US to any surgeon in the EU. This is a new development and has not yet been utilised in the UK but BIS has been used by some UK surgeons when there has been no tissue available in the UK. However, if a UK surgeon decides to use a distribution ‘agent’, then they must arrange an SLA with the distributor and also hold a HTA license for storage of human tissue.

3.3.3 Inspection and Licensing Status of UK tissue banks
Currently all UK tissue banks have achieved HTA or IMB accreditation status and have licenses for storage of human tissue for therapeutic use. The HTA licensed banks appear on the HTA register for banks storing human tissue for therapeutic use (see Appendix II). Initially this was achieved by a ‘phase one’ inspection made by HTA following review of the self assessment made by each bank and this has been followed up in all six of the UK banks, with a ‘phase two’ on-site inspection. The Irish Medicines board perform on-site inspections only.

There is no doubt that the processing details vary between the licensed banks, although there are many similarities which reflect areas where the EUTCD and HTA Directions are detailed or where there is accepted clinically based validation. The inspections conducted by the competent authorities (HTA or IMB) have audited tissue bank organisation and practice strictly against only the requirements. The Irish Medicines Board has already inspected the cardiovascular section within the Irish Blood Centre and this was seemingly more stringent than the equivalent HTA inspections. The IMB recorded non-compliances
for lack of SLA’s with surgeons which is a new requirement introduced very recently and not yet fully enforced in UK banks. They also recorded a non compliance for the lack of continuous monitoring within the processing facility during operation and again this is a very recent standard introduced with EUTCD that UK tissue banks are unable yet to achieve. This will be a financially costly process and the physical installation of the systems required is relatively difficult since it requires wiring to be placed through the entire sealed aseptic facility. The HTA inspections however, using the same criteria, did not issue non compliances on inspection of the other UK banks. This variation in inspection was predictable since, prior to the formation of HTA; all UK banks were inspected by the IBM equivalent - The Medicines and Healthcare Regulation Authority (MHRA). Both the IBM and MHRA routinely inspect pharmacy manufacturing premises and blood transfusion laboratories within, and outside the UK according to the criteria set out in the good manufacturing guide (Rules of Pharmaceutical Manufacturing Premises). The UK banks that have so far been inspected by HTA (banks 2, 3, 4, 5) have reported that they felt that the inspectors seemed to have limited tissue banking experience and background knowledge and their inspections felt less stringent than previous MHRA inspections. This has been reflected by HTA’s recent drive to use experienced persons who currently work in UK tissue banks to act as independent assessors and accompany the HTA inspectors at each UK tissue bank inspection. However, since the assessors are actively employed in other tissue banks, they may therefore not be presumed to be innately ‘independent’. Other banks in the EU and US seem to be even more strictly controlled. This appears to be due to the fact that their countries had previous enforced even higher standards than EUTCD and in this case, these higher internal standards must be maintained. The EUTCD is meant to be a minimum standard. In the US, the Food and Drug Authority sets National guidance which is seemingly more stringent than EUTCD. In Germany, human tissue is classified as a ‘medical device’ and, as such, the required standards for processing are extremely high. The European Commission is currently aware of the difference in standards of inspection set by member state responsible authorities. It has therefore set up a project known as ‘Eustite’ to achieve
consensus for best practice in inspection, and to develop guidelines and a training program for inspectors to ensure that this will be more consistent in the future. Eustite will also be expected to harmonise adverse event and reaction reporting and management across member states.
3.4. TISSUE PROCESSING PRACTICE

A review of the procedures used for obtaining consent and for donor selection, procurement of donor heart tissue, dissection, quality assessment, disinfection, testing and test result reporting has been performed and is described and discussed.

3.4.1 Consent & Donor Selection

Currently, each UK Tissue Bank relies upon donors families volunteering their relative’s tissue after death or instead relies upon individual healthcare professionals in hospital units approaching bereaved families to initially offer the option of donation. If there is a positive response toward donation, then a team of specifically trained nurse co-ordinators will be asked to liaise with the bereaved families to complete the strictly controlled tissue donor consenting process. Only three banks (banks 3, 4, 7) have their own co-ordinators, the remaining tissue banks use the services of the National Organ Donor Transplant co-ordinators which are regionally based and managed by the United Kingdom Transplant Authority and are members of the UK Transplant Co-ordinators Association.

All tissue banks in the UK are required to have a service level agreement in place with the co-ordinator teams. Following the consent procedure, the co-ordinators check the past medical, behavioural and social history of each donor. They then establish whether the donor has signs of malignant or chronic diseases by interviewing the closest donor relatives or partner (or, using HTA terminology, ‘the highest ranking person’). They will also interview the donor’s General Practitioner, and review medical notes and interview clinicians if relevant. If there is a post mortem examination, they will also organise that the report is sent to the tissue bank. If the donor is also donating organs, then the co-ordinator will pass on the virology reports to the tissue bank. The donor will then be referred to the tissue bank. The tissue bank is then responsible for making a decision to accept or reject the donor on the basis of the completed information and then the tissue bank will organise the retrieval of the tissue and the collection of a blood sample. The Donor Selection criteria used by each bank varies, however, most banks use the National
Blood Services standard as the broad basis for their acceptance/rejection criteria before then using their own teams of medical advisors to look at unusual individual cases.

3.4.2 Procured Donor Tissue types

The procedures the tissue banks use for procurement, packaging and transportation of the donated hearts to the tissue establishment and their subsequent dissection are relatively similar. Most banks have two types of donors, Deceased donors (or ‘non heart beating donors’) and ‘Heart Beating’ donors (or multi-organ donors whose death has been certified by two sets of brain stem death tests). The former are retrieved in the mortuary during post mortem examination by pathologists, or by the tissue banks specialist retrieval team (approximately 60%). The latter are retrieved by multi-organ retrieval teams in operating theatres, when the heart has been found to be unsuitable for heart transplantation (approximately 39%). A small amount of donors are live donors who have had a heart transplant (less than 1%). There seems to have been a sharp decline by all UK tissue banks in the retrieval of hearts from this type of donor (a heart transplant recipient who donates the tissue from their explanted hearts). Heart Transplant Recipients hearts were initially thought to have been a ‘guaranteed’ source of viable cardiovascular tissue (known as ‘homovital homografts’) which would be retrieved in operating theatres and therefore controlled and with sterile equipment and trained staff, and with easily accessible donor information (Yacoub, 1995). However, since the mid 1990’s, this type of donated tissue was also found to be of greater likelihood of being unusable due to cardiomyopathy or acquired disease (tissue may be stenosed, calcified, or degenerated) which reflects the average age of the donor being above 60 years. There was also found to be increased risk of transmission of viral infection (Donoso, 2005) and this led to recommendations that myocardial tissue from these donors should be subjected to polymerase chain reaction (PCR) analysis for enteroviruses, adenoviruses, human cytomegla viruses (HCMV), paroviruses B19 (PVB19) and influenza viruses. This would be practically difficult and costly and since there are no antiviral agents used in the disinfection of tissue and, for most banks the inconvenience and risks outweigh the
benefits of using this type of tissue. For some international banks such as Brazil (da Costa, 2001) and Bangkok (Vajaradul, 2001) however, heart transplant recipient’s hearts continue to be their main source of cardiovascular tissue. Other banks, such as the one if Cracow, Poland, routinely retrieves the majority of their cardiovascular tissue from deceased donors during routine autopsies (Stolinski, 2006).

3.4.3 Time limits for Procurement

The focus of procurement techniques for all UK banks is to minimise the morphological and metabolic changes consistent with hypoxic damage during the period of time to, and during retrieval, and to preservation in nutrient media. Warm ischaemic time (WIT) is known as the time from death to body refrigeration, and for all UK banks, is currently normally 6 hours, and if this is the case, all banks currently require retrieval of the donated hearts within 48 hours of death which constitutes cold ischaemia (CIT) or time after body refrigeration until heart valve tissue is dissected and refrigerated (See Table 1). If the body is not refrigerated within 6 hours then the retrieval must be within 24 hours of death. It has been shown (Stegman via Warwick 2007) that there is no microbiological evidence to limit retrieval to 24 hours. They showed that the unavoidable clinical parameters such as pre-mortem antibiotic treatment, cause of death (cardiac/other), ventilation, length of hospitalisation, time of body at room temperature after death had no significant influence upon positive hemocultures. This has been confirmed by a UK bank (McGowan, 2007) who reviewed their contamination rates of tissue procured in UK mortuaries over a five year period. They went on to show that their rates of contamination and of ultimate tissue discard (after decontamination) reduced over the 5 year period as their methods of training, cleaning and preparation of the procurement environment and technique improved. Neither study however, looked at the effect of time to retrieval and the other clinical parameters upon tissue quality. Other evidence illustrates, however, that pre-processing ischaemic time reduction is one of the foremost determinants for recipient patient’s freedom of need for re-operation (Kiraldy, 1994, Jashari, 2004).
Table 1. Comparison of UK tissue banking processing time limits for procurement, and dissection and fluids used for transportation and rinsing of procured cardiovascular tissue. The sections highlighted show areas where there are significant differences.

<table>
<thead>
<tr>
<th>Bank</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time limit to procurement</strong></td>
<td>48 hrs</td>
<td>&lt; 72 hrs</td>
<td>&lt; 48 hrs</td>
<td>&lt; 48 hrs</td>
<td>Same as 4.</td>
<td>&lt; 48 hrs</td>
<td>Paediatric: &lt; 12 hrs Adults = multi organ donors</td>
</tr>
<tr>
<td><strong>Transport solution</strong></td>
<td>Buffered isotonic solution</td>
<td>Isotonic solution</td>
<td>N/A – transported dry, wrapped in Hydrex-soaked gauze</td>
<td>Hartmans</td>
<td>Same as 4.</td>
<td>Saline</td>
<td>N/A (Dissected at multi organ donation)</td>
</tr>
<tr>
<td><strong>Time limit to dissection</strong></td>
<td>&lt; 72 hrs</td>
<td>&lt; 72 hrs</td>
<td>24 hrs after removal</td>
<td>&lt; 72 hrs</td>
<td>Same as 4.</td>
<td>24 hrs after removal</td>
<td>Dissected in theatre at multi organ donation</td>
</tr>
<tr>
<td><strong>Rinse solution during dissection</strong></td>
<td>Buffered isotonic solution</td>
<td>Isotonic solution</td>
<td>Buffered isotonic solution</td>
<td>Hartmans</td>
<td>Same as 4.</td>
<td>Buffered isotonic solution</td>
<td>N/A</td>
</tr>
</tbody>
</table>
3.4.4 Donor Age limits

Cardiovascular tissue has been retrieved for UK banks from donors of at least 32 weeks gestation to maximum age limit of 65 years. Only two banks (banks 1, 3) retrieve tissue from full term babies of ‘normal’ birth weight and size, other banks retrieve tissue from donors of 6 months of age or more. Tissue was previously retrieved in the UK from babies of 32 weeks gestation; however the demand for this tissue promptly diminished following the Bristol Heart Surgery enquiry. The Bristol enquiry illustrated that, performing some forms of complex congenital cardiac surgery on babies, may be not in the best interest of the child or parents since the operative risk is very high and the prognosis very poor. Some high risk surgical procedures therefore were abandoned and alternative surgical or palliative treatment adopted instead. Very small baby donor valves (< 5mm internal diameter valve size) were therefore not required for transplantation. However, valves from babies of full term weight and size (>8mm internal diameter) are still required and they are currently transplanted into babies of a similar age and size (see fig 7a, b & c). There is an increasingly large demand for cardiovascular tissue from donors of ages 6 months to 15 years but there is always a National shortage of this type of tissue. The National population census statistics indicate that the ‘pool’ of potential donors of this age group is also very small; currently, in the average population of the UK, there are many more deaths of babies within the first month of life than in a child of any age. Tissue banks hence take very special care to ensure all paediatric donor tissue is procured with maximum efficiency, using specialised equipment and personnel.
Fig 7 a. Clinically transplantable baby donor aortic and pulmonary valves of 5-6mm internal diameter. Fig 7 b. external view of adult donor pulmonary valve of 25mm internal diameter. Fig 7c. Internal view of Adult aortic valve of 23mm internal diameter.

The upper age limit used by tissue banks for cardiovascular donors is variable; Most UK banks (banks 2, 3, 4, 5, 6) have an upper limit for cardiovascular tissue set at 60 years. One bank (bank1) has an upper limit for aortic valve tissue set at 60 years but has extended the limit to 65 years for pulmonary valve tissue. This difference in upper age limit between aortic and pulmonary tissue is common amongst EU and US banks (Grosse, 2008). The concessionary older donor pulmonary valve tissue is useful since it is normally less degenerated than the equivalent aortic tissue and it is mostly used for pulmonary valve reconstruction or repair surgery. The average population above 50 years of age are likely to experience a degree of increased blood pressure and their aortic roots will become larger and more friable or calcified (Barzilla, 2006), however their pulmonary valves, functioning under lower right sided pressures, will remain virtually unaffected. Pulmonary tissue used to be implanted into both left and right ventricular outflow tract since it was shown that the pulmonary valve was mechanically similar to that of the aortic (David, 1994) and early post operative outcome reports were acceptable (Gerosa, 1994). However later recipient follow up reports documented unsatisfactory degeneration of pulmonary tissue implanted into the left side (Naegele, 2000;Jashari, 2004) resulted in a cessation of use of pulmonary tissue in the left ventricular outflow tract. Pulmonary tissue is predominantly now implanted into the right side, therefore pressure induced tissue degeneration is likely to be less for pulmonary tissue following transplantation. Pulmonary tissue from older donors is thus likely to be as durable as younger aortic tissue.
3.4.5 Procurement technique & infection risk

Delayed removal of tissue from the deceased donor may result in increased tissue contamination with anaerobic and spore forming bacterial pathogens which are often more resistant to disinfection (Eastlund, 2006). It has been shown (Suh, 1999) that WIT is more important than CIT in maintaining viability of the cardiovascular tissue. It seems that most UK and EU banks aim to procure tissue within 24 hours of death but allow up to 48 hours maximum to minimise this risk (see table 1). There is no dispute either that Donor’s blood samples must be collected within 24 hours of death since relates to the limit stipulated by the commercial testing kit licenses.

It has been shown (Haponiuk, 2001) that the technique used for retrieval of cardiovascular tissue has up to 200% effect upon the amount of clinically useful tissue procured (paediatric retrieval being most effected).

One UK Group (McGowan, 2007) have observed that when only cardiovascular tissue was retrieved by their procurement teams, it was less likely to be contaminated then if it were retrieved by the same teams during multi tissue retrievals. This is most likely to have been affected by sequencing of tissue retrievals when the effect of translocation of intestinal contaminants by not procuring the cardiovascular tissue first, may be significant.

During skin procurement, the body is turned over and to procure musculoskeletal tissue, there is considerable physical movement of the donor.

Most banks retrieve whole donor hearts except one tissue bank team (bank 1) that routinely retrieves heart blocks or valves (see Fig 8.a, b, c, & d). Bank 1 believes that this enables more thorough inspection, washing and more rapid cooling before transporting back to tissue bank and also reduces WIT (unpublished data).

One bank (bank 1) argues that retrieval of tissue rather than heart at the procurement facility also allows the majority of donor valves which are deemed physically unsuitable for transplantable to be returned immediately to the donor’s body rather than disposed of as clinical waste and this maybe considered ethically more acceptable and more sensitive toward the Donor’s family.
Fig. 8. a. Procurement of Cardiovascular tissue using aseptic technique, 8 b. Rinsed donor heart packaging at retrieval centre, 8. c. heart block illustrating position of valve to be harvested, 8. d. cardiovascular tissue successfully procured from one donor (from left to right, pulmonary valve, aortic valve, aortic descending aorta conduit and carotid conduit).

3.4.6 Procurement facility & conditions

The environmental conditions for retrieval are relatively similar for all UK banks. They are performed either in a mortuary (non heart beating donors) or in an operating theatre (heart beating donors).

However, one tissue bank (bank 3) has recently opened a retrieval suite at their tissue bank. The aim was to cut down retrieval times and to perform their retrievals in a cleaner air quality environment. However, it has been shown (Davies, J., et al, 2007) that the air quality in the operating theatre and mortuary environments is acceptable by EUTCD standards since a review of particulate and microbial air quality levels is comparable to that of the pharmaceutical manufacturers guidance level ‘C/D’ air quality in mortuaries and ‘C’ in the operating theatres. The Rules and Guidance of Pharmaceutical Manufacturers 2007 (known as the ‘Orange guide’) publish the air quality guidance for UK tissue banks. A likely disadvantage with the tissue bank facility is that the donors have to be moved to the retrieval facility which can induce movement of post mortem contaminants within the donor’s body and therefore increase contamination risk to the tissue before it is retrieved as previously described. Additional disadvantage of the tissue bank retrieval suite is that there is currently substantial public concern that moving bodies long distances across the country, may be unacceptable to donor families since it could delay relative’s viewings and funerals, and this may reduce the donation rate. Legally,
there is also a concern; The Rules of Her Majesty’s Coroners currently prevent the movement of bodies of people who are to undergo a post mortem from one jurisdiction to another. There is, as yet, no plan to change the relevant sections of these Coroner’s rules.

Other UK tissue banks are not currently planning to develop their own retrieval centres. The tissue bank who has the retrieval centre (bank 3) has confirmed (Kerawala M., et al 2007) that they have found a very high bacteriology test failure rate (38%) which will be referred to in the following section. However, it has also been shown (Davies, 2007) that the type of infective agents in a 2 year review of donated cardiovascular tissue to bank 1 is quite different to that found in the environment of the retrieval centre and, in fact, most contaminants found were likely to have originated from the donor’s intestine after death.

Another UK bank (McGowan, 2007) has also found that the most pathogenic organisms they found in a five year review of mortuary procurement were of donor origin and introduced during heart retrieval rather than derived from the procurement environment. This may be explained by the fact that the epithelial mucous membrane of the intestine is very fragile and is sensitive to ischaemia and thus, after death, there may be a degeneration of the intestinal barrier facilitating escape and spread of luminal bacteria toward the tissue to be retrieved. This sequence has been termed bacterial translocation (Steffen, 1983; Berg, 1995). Retrieval of the tissue, in situ at the Donor Hospital, prior to post mortem examination, may therefore minimise this contamination spread.

EUTCD requires EC banks to register the retrieval centres and the training of retrieval personnel. This is new to the UK banks and is still not completed, however in the EU; tissue banks had previously been required to register their centres and also to use their own tissue retrieval team unless a multi-organ team is involved. However, the majority of banks in the US are not actively involved in the procurement process instead; they use the services of a procurement agency.

3.4.7 Procurement and processing equipment

There is a plethora of Department of Health CJD risk guidance issued following advice from the Spongiform Encephalopathy Advisory Committee. This includes ‘Variant
Creutzfeldt-Jacob Disease (vCJD); minimising the Risk of Transmission – Health Services Circular 1999/178, NHS Executive 13/08/1999’ (www.dh.gov.uk). The guidance warns that there are difficulties in decontamination of infected medical devices or equipment and risks of transmission if the device or equipment is re-used. Hence, the equipment and consumables used by all UK banks is sterile and predominantly for ‘single-use-only.’ The only reused items are traceable.

3.4.8 Packaging & Transport of procured tissue

All UK banks prepare the donor hearts (or heart tissue) by first rinsing at the retrieval site in sterile isotonic media and placed in sterile containers in a validated container at 2-8 °C and transported to the tissue bank facility (see table 1.). It has been shown in one study (Krs, 2006) that washing in saline will damage the endothelial coating of the valve tissue; however, all UK banks (except bank 3) continue to soak the tissue at retrieval and in transit to reduce the bio burden of pathogens. Five banks transport the retrieved heart/heart block to the tissue establishment in sterile isotonic solution. One bank (bank 7) retrieves and dissects the donor tissue in theatre by cardiac registrars (air quality is not monitored). The other bank (bank 3) has recently changed their procedure to wrapping tissue in alcohol soaked gauze. It is believed that this may help reduce the bio burden of incoming heart tissue but, however, it potentially may not keep the tissue as moist during transportation. This bank has confirmed that they have a very high bacteriology test failure rate for their tissue. Surrounding fatty adventia on the heart becomes hardened if it is not soaked during transportation and is then inevitably more difficult to dissect successfully and this may cause tissue to be damaged at dissection.

Transportation of the donor hearts to the tissue bank from the retrieval centres and the despatch of processed tissue to the surgical centres are routinely performed by specialist couriers in validated packaging with documentation and labelling in accordance with EUTCD requirements. Tissue banks use different couriers and have slightly different transportation requirements. One bank (bank 1) only uses a specialist ambulance service in order to ensure that the tissue is collected and despatched by trained emergency...
ambulance drivers in one vehicle, in one journey, and collected/delivered to the door personally. Other banks (banks 2, 4, 5) allow tissue to be left in a warehouse overnight. Couriers must be aware of the methods of safe handling of tissue stored in dry ice (@-80°C) or on dry shippers (@-<170°C). Whenever tissue is despatched abroad, a specialist air handling company must be used to facilitate Customs declaration and clearance. It seems that there has never been any donor tissue imported for processing in the UK in the last decade, and there is rarely any processed tissue imported into the UK for use, but, if it is, it must comply with the HTA’s Code of practice for Import & Export of human tissue. SLA’s between the couriers and tissue banks are also required.

3.4.9 Tissue bank facility air quality.

The tissue processing establishments have aseptic room suites with microbiological safety cabinets inside which they process the cardiovascular tissue (see Fig 9 a. & b.). These purpose-built aseptic facilities are monitored regularly and are required to achieve very high grade environmental air quality in order to comply with EUTCD requirements. For open manipulation of tissue prior to decontamination (which takes place at dissection), the acceptance criteria required by EUTCD is for cardiovascular tissue to have a grade ‘A’ air quality in the working zone (this is achieved within a class II microbiological safety cabinet) with a grade C background in the surrounding room. The air quality and room grades refer to the published definitions in the Rules and Guidance of Pharmaceutical Manufacturers 2007 (known as the ‘Orange guide’). For post decontamination processing (this occurs during cryoprotection) of tissue that cannot be terminally sterilised, the requirement is higher - grade ‘A’ air quality within a microbiological safety cabinet with a grade B background in the room. The requirement for tissue which is terminally sterilised, such as bone, is less stringent.

New EU requirements stipulate that the air quality has to be continuously monitored (only one UK bank as yet can facilitate this - Bank 6). UK legislation does not yet demand this. All UK banks strive to continually achieve these air quality conditions and have regular monitoring procedures in place.
Fig. 9. a. Technicians working in the grade B background tissue bank aseptic room, fig 9.b. Technicians processing tissue in a microbiological safety cabinet inside the grade C background aseptic room.

3.4.10 Dissection, Quality Assessment, sizing and grading.
Dissection is performed by all UK banks within 72 hours. The BATB previously had guidelines for these time limits which may be why all UK banks still work within the same maximum time limits. The dissection is performed in the tissue banks aseptic facility inside a microbiological safety cabinet. The cabinets aim to achieve at least a grade ‘c’ air environment, with all but bank 2 using cabinets and working within a positive pressure room. The cabinets act to protect the dissecting member of staff from potential infection from the tissue, although the tissue has already been deemed low risk via previous donor screening protocols. Most banks have one technician to dissect the heart and the same person will then perform the internal and external examination of the tissue or the ‘quality assessment’. One bank (bank 1) always has two members of staff independently assessing quality, the first at dissection and the second assessment being immediately prior to addition of cryoprotectant. The main reasons for rejection of tissue following dissection were found to be on the basis of unacceptable morphology, cuts which have been inadvertently made at retrieval post mortem or dissection, and contamination (see fig 10 a & b.). This is the same in other EU and US banks (Jashari R et al 2004).
Fig 10 a. Internal inspection of aortic valve showing cut in aorta following procurement error which would be noted on valve information record. 10.b. Internal inspection of aortic valve showing acquired aortic valve disease and calcium seed which would be reason to reject valve for clinical use.

One UK bank (bank 1) and two European banks use an explicit tissue quality grading scheme which is non-subjective. All other banks use various protocols and consumables with wide ranging disparity in results. One bank (bank 1) routinely has technician re-assessment every three months. As a result of this review, already, the BATB cardiovascular SIG, has held two technician workshops with all UK banks to harmonise their sizing and grading procedures. The results were presented at BATB/EATB/AATB ASM in Nov 2008.

### 3.4.11 Tissue Disinfection

Cardiovascular tissue is currently disinfected in all UK, EU and US banks with a comprehensive antibiotic/antimycotic mixture in a nutrient medium in order to aim to minimise the contamination of the tissue which may have been due to donor ante mortem or post mortem contamination or due to the tissue banks retrieval, transport and processing procedures. The composition of the antibiotic disinfection cocktail varies however (see table 2.). Disinfection however, can never be claimed to successfully sterilise or decontaminate the tissue, despite the fact that high concentrations are used (doses are well above the levels used clinically by microbiologists). No anti-viral agents are used in the tissue disinfection process which is why tissue viral infection transmission from explanted hearts remains a high risk (Donoso, 2005).
Other, more efficient methods of sterilisation such as irradiation and ethylene oxide gassing has been used over the years but was later proven to be damaging to the integrity and function of the tissue (as previously described in section 3.2.6). Tissue transplantation is now routinely successful and yet there still remains a risk of causing transmission of infection in the recipient. The risk of viral infection is the most concerning (Donoso, 2005). However, very few reports yet have specifically concluded that, although there are a growing number of case reports of transmission of infection via other tissue transplants (Eastlund, 2006); infection originating from human donor cardiovascular tissue is extremely rare. It has been confirmed (Tyras, 1978; Rumisek, 1985) that several patients developed Mycobacterium chelonae endocarditis following implantation of porcine xenograft valves contaminated during production at the same manufacturing facility. One case of a patient developing Candida albicans endocarditis and fungemia was reported to the Center for Disease Control in USA (Kuehnert, 1998) and this was transmitted from the donor despite the tissue being culture negative after routine disinfection. Several cases of military tuberculosis have been tenuously linked to the donor tissue transplanted (Rumisek, 1985; Anyanwu, 1976). The donors of these suspect tissues would now be excluded from donation due to their age or past medical history. Additionally antibiotics which are effective against tuberculosis are included in all UK banks cocktails thus reducing greatly the current risk of transmission of tuberculosis by cardiovascular tissue transplantation (Yankah, 1988). A survey was recently undertaken of 32,289 donors tested for TB, with participants from all UK banks, and some Europe and US banks (Warwick, 2008 in print). Although no results were found to be TB positive, a small number of non-tuberculosis mycobacterium (24) were detected and some of them were identified to be in clusters. Further investigation of the clusters proved that these results (all on tissue from one bank) were not associated with infected donors but instead related to a contaminated water bath used in the thawing process.
<table>
<thead>
<tr>
<th>Bank</th>
<th>Disinfection cocktail used</th>
<th>Incubation Temperature</th>
<th>Time of Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ticarcillin, Polymixin B Sulphate, Cefuroxime, Amikacin, Vancomycin, Nystatin, made up in Hapes buffered Medium 199</td>
<td>25°C</td>
<td>18 hours</td>
</tr>
<tr>
<td>2</td>
<td>Cefuroxime, Gentamycin, Ciprofloxacin, Colistin, Vancomycin, Amphotericin made up in Medium 199</td>
<td>Room Temperature</td>
<td>24 hours</td>
</tr>
<tr>
<td>3</td>
<td>Imipenem, Gentamycin, Polymixin B Sulphate, Vancomycin, Nystatin made up in Hapes buffered Medium 199 (Commercially manufactured media)</td>
<td>4°C</td>
<td>24 hours</td>
</tr>
<tr>
<td>4</td>
<td>Imipenem, Gentamycin, Polymixin B Sulphate, Vancomycin, Nystatin made up in Hapes buffered Medium 199 (Commercially manufactured media)</td>
<td>22°C</td>
<td>21-23 hours</td>
</tr>
<tr>
<td>5</td>
<td>Imipenem, Gentamycin, Polymixin B Sulphate, Vancomycin, Nystatin made up in Hapes buffered Medium 199 (Commercially manufactured media)</td>
<td>22°C</td>
<td>21-23 hours</td>
</tr>
<tr>
<td>6</td>
<td>Imipenem, Gentamycin, Polymixin B sulphate, Vancomycin, Nystatin made up in Hapes buffered Medium 199 (Commercially manufactured media)</td>
<td>37°C</td>
<td>24 hours</td>
</tr>
<tr>
<td>7</td>
<td>Metranidazole, Gentamycin and Fluocoxacillen made up in normal saline</td>
<td>4°C</td>
<td>Minimum 12 hours</td>
</tr>
</tbody>
</table>

Table 2. Ingredients of the Antibiotic & antymycotic disinfection Cocktail used for disinfection of cardiovascular tissue with the relevant incubation (or ‘exposure’) time and temperature conditions used by each bank to optimise disinfection. The sections highlighted show areas where there are significant differences.
The survey concluded that, the donor selection protocols in place to exclude donors at risk of TB were effective when combined with routine culturing for mycobacterium and recommended that, clusters of positive non tuberculosis mycobacterium results should be investigated to determine possible sources.

All UK banks (except banks 2 & 7) use nystatin as their antifungal agent (see table 2.) since it has been shown (Aguirreguiacoa, 1989) to be more effective and less toxic than the alternative amphotericin B. The other banks vary with one bank (bank 2) continuing to use Amphotericin B and the other (bank 7) not using any antifungal agent in their disinfection media. The reports of potential transmission mycobacterium transmission, encouraged some banks (banks 1,3,6) to have a list of pathogens for which their presence at pre-disinfection would constitute an automatic tissue rejection and it also encouraged banks to re-examine the effectiveness of their anti fungal disinfection regime. Some non-UK banks still do not use any antifungal treatment but most instead rely upon a very strict rejection criteria for incoming tissue (in Belgium and Australia).

There is also evidence that the strength of antibiotic mixtures currently used in UK banks is in such a high concentration that it may be considered toxic and may render the tissue less viable after disinfection. One Belgian tissue bank (Jashari, 2007) has recently modified their antibiotic cocktail in order to reduce its concentration and toxicity. They achieved this by removing Cefoxitin after proving that there was no significant difference in the level of decontamination between the two cocktails. Some non-UK banks such as the Czech and Slovak transplant centre (Spatenka, 1997), Madras Medical Mission in Chennai, India (Verghese, 2004) continue to use amphotericin B as their antifungal agent despite its cytotoxicity. It was found (Aguirreguiacoa, 1989) that nystatin had little detrimental effect on the viability of cardiovascular tissue fibroblasts but treatment with amphotericin B in contrast, was rapidly cytotoxic even at 4°C and 12% less effective as an antifungal. Disinfection with a cocktail incorporating amphotericin B is likely to result in little residual fibroblast viability and, as previously mentioned, also in decreased fungal growth decontamination.
One commercially available antibiotic disinfection cocktail is used by four banks (banks 3, 4, 5 and 6). The other banks use their own selected cocktail and mix this immediately prior to its use. The dissected tissue is immersed in the antibiotic cocktails and incubated allowing for an ‘exposure’ time which ideally optimises disinfection without harm to the tissue. The incubation temperature and time varies between banks, ranging from time of 12 – 24 hours at temperatures set at between 4 – 37˚C (see table 2.). All UK cardiovascular tissue banks have previously participated in a UK survey conducted by the National Institute of Standards and Control (NIBSC) (data presented but not yet published). The commercially marketed media performed poorly compared to media used by the other UK banks. It is still however, used by the four banks. Only one bank (bank 1) has independently validated their antibiotic decontamination media and procedure (Validation protocol no.HVB/VP/8.1) and results were by the author at BATB/EATB/AATB meeting Nov 2008.

3.4.12 Microbiology in-process testing of tissue segments
All banks test tissue wall segments following different stages of the tissue processing. (See table 3.). One is taken prior to disinfection; a second is taken following decontamination and another following cryoprotection. Only one bank sends samples for testing after cryopreservation (bank 1). The timing of obtaining these samples is similar in all UK tissue banks, but not identical. Some banks (banks 1, 6) test a sample of filtered transport fluid, to observe incoming pathogens. One bank (bank 3) swab the outer surface of the incoming heart instead, however, swabbing allograft surfaces has been shown to have a much lower detection rate than that of other methods observed (Vehmeyer, 2001). Only one bank (bank 1) routinely sends ‘spiked’ samples (standards of known organisms) through testing procedure to ensure procedure remains effective (validation protocol HVB/VP/8.1). No banks ‘rinse off’ the disinfection media from their tissue samples prior to testing. However, one bank (bank 1) has shown (validation protocol HVB/VP/8.1) that one ‘rinse’ with one aliquot of fluid is not enough to rinse off the disinfection media. They showed that, even 3 rinses with up to 500ml are insufficient.
### Microbiology testing in UK CV banks

<table>
<thead>
<tr>
<th>Bank</th>
<th>Do you do a Transport Fluid qualitative test?</th>
<th>Do you do a Transport Fluid quantitative test?</th>
<th>Do you do a pre disinfection test on tissue segment?</th>
<th>Do you do a post disinfection test on tissue segment?</th>
<th>Do you do a post Cryoprotect ion test on unfrozen tissue sample?</th>
<th>Do you do a post Cryoprotect ion test on tissue sample after freezing?</th>
<th>Do you routinely keep an archive sample of tissue?</th>
<th>Do you do a routine histology test on tissue segment?</th>
<th>Do you do any other tests with tissue?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>yes</td>
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<td>yes</td>
<td>no</td>
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<td>Yes</td>
<td>Yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

Table 3. Comparison of the various stages that UK banks collect their microbiology & histopathology specimens. The sections highlighted show areas where there are significant differences.
They then ensure that all decontamination fluid is removed from tissue samples before they are tested by neutralisation of the antibiotics in the decontamination fluid (using aerobic and anaerobic ‘Bactec’ bottles). Further work is currently being performed by bank 1 to quantify how much rinsing would be sufficient using HPLC studies (Validation protocol HVB/VP/14.1). Final sterility testing has been shown to be unreliable (Eastlund 2006; Ireland, 2005), especially if antibiotics have not been effectively removed from the tissue samples prior to testing since residuals can interfere with the detection of some virulent microbes, especially spore forming organisms (where only the vegetative forms may be eliminated by antibiotics and not the spores). Also the report of Candida transmission (Kuehnert, 1998) suggested that there was evidence of emerging resistance after disinfection but, the results may also have suggested that the post disinfection testing technique had not been sufficiently validated.

Three banks (banks 1, 3, 6) attempt routine quantitative testing (see table 3.). One bank (bank3) performs this by swabbing the outer and inner surface of the heart and uses these results to assess bio burden on the tissue (Kerwala, 2007) but this has limited value as a direct quantitative correlation (Vehmeyer, 2001). The other two banks (bank 1, 6) that attempt quantitative testing use pre disinfection heart transport fluid (post filtration). Additionally, during antibiotic media and testing regime validation studies and also during routine test practice, one bank (bank 1) challenges their media and testing regime with a known number of colonies (simulating a ‘large’ and small ‘bio burden’) of their commonly found contaminants. They use organisms chosen as a result of a review of a two year review of contaminants. The AATB standards stipulate that the effectiveness of any disinfection process is affected by the amount and the type of micro organisms present on the incoming tissue (the bio burden). One study (Forsell, 2000) compares data regarding potential sources of increased bio burden from seven US banks. Unfortunately, although they identified the major risk factors, they were unable to use statistical methods to compare data because each tissue bank’s procedures and techniques were responsible for most of the variability. They did however; suggest appropriate methods for each bank to monitor their own data.
The techniques for testing regime within UK banks vary considerably, most banks use a combination of enrichment broths and incubation onto blood agar plates for aerobic and anaerobic incubation and additionally they use saboraud plates for fungal culture. The incubation temperatures and times vary (5 – 14 days and up to 9 weeks for mycobacterium incubation). All organisms isolated are then identified to species level in order to exclude specific pathogenic organisms. The predisinfection testing, whether it is just on tissue or on transport fluid, is subjected to the same regime and is used to exclude virulent pathogens at the earliest stage of processing.

A recent study has been completed (Gupta, 2008) in order to introduce a new form of identification of tissue fungal contamination. The new method investigated was a quantitative polymerase chain reaction assay (qPCR) and results showed that the method was successfully validated against the existing routine protocol. Samples spiked with fewer than 10 colony forming units (CFUs) of Aspergillus and Candida species were able to be detected in a much reduced time period. This method is likely to be implemented in tissue banks in the near future, provided its availability and costs are acceptable.

All UK banks frequently recover pathogenic organisms from cardiovascular tissue samples, which may or may not be successfully decontaminated with antibiotics. This may be due to the in-effectiveness of the decontamination medium or to the bio burden of the tissue. However, if all tests are analysed together (the final product and intermediate processing testing, the bio burden monitoring and the predisinfection organism identification), then this then help to ensure a safe and reliable product.

3.4.13 Donor Serology testing

Blood samples are retrieved by all banks within 24 hours of death of the donor since, as previously mentioned, the licensing of blood test kits stipulate this. All UK tissue banks aim to achieve this prompt sample collection and, if they cannot for some reason, they will use the results of another bank or they will reject the donor tissue rather than use results from blood collected after the 24 hour period. This may be particularly challenging
if it must precede procurement and may have to be collected by a member of staff looking after the donor in the mortuary or even after brain death but prior to multiorgan procurement by a member of the intensive care team. All blood samples are tested by UK banks for the mandatory markers detailed in the UK guidance of the Microbiological Committee for the Safety of Biological Tissue for Organ or Tissue transplantation (MSBTO) since this is actually more stringent than the minimum EUTCD requirements (Human T lymphocyte Virus testing is not required by EUTCD but it is by MSBTO). Other EU and US countries have more stringent guidance for blood testing than EUTCD and the banks in those countries maintain testing using the more strict country specific requirements. Additional tests are performed as required, such as malaria testing for those donors who have travelled within a malaria risk area in the 12 months preceding death or have ever lived there. All tissue banks have the responsibility to pass information to the donor’s family about confirmed positive blood test results if relevant.

During the consenting procedure, consent has to be given for blood testing and collection of medical records of the donor before the referral is accepted and donor families of other persons giving consent are warned that they may be informed of positive results if relevant.

Blood testing is required of the mothers’ blood of baby donors of ages less than 18 months or for baby donors that are older but breast fed within 12 months of donation. This maternal sample has to taken within 7 days of the donation and repeated at 180 days afterwards in order to eliminate the risk of false negative results from babies whose infectious disease markers may not yet have been developed. The two banks that retrieve tissue from baby donors of less than 6 months of age (banks 1,3), aim to collect blood both from the baby and their mother but, they have described cases where no blood could be retrieved from the baby, or that it was haemolysed prior which precluded testing. In these cases, the banks medical and microbiological advisors will individually assess the risk of using tissue from the baby donor and, since there is a shortage of

3.4.14 Paediatric Donor serology testing

Blood testing is required of the mothers’ blood of baby donors of ages less than 18 months or for baby donors that are older but breast fed within 12 months of donation. This maternal sample has to taken within 7 days of the donation and repeated at 180 days afterwards in order to eliminate the risk of false negative results from babies whose infectious disease markers may not yet have been developed. The two banks that retrieve tissue from baby donors of less than 6 months of age (banks 1,3), aim to collect blood both from the baby and their mother but, they have described cases where no blood could be retrieved from the baby, or that it was haemolysed prior which precluded testing. In these cases, the banks medical and microbiological advisors will individually assess the risk of using tissue from the baby donor and, since there is a shortage of
these small donor valves, they may write a concession for emergency use only. The implanting surgeon has to be made aware of the details of the concession before choosing to transplant such tissue. Many EU banks also collect blood cultures for microbiology testing but this is not performed in UK tissue banks. This seems to be since UK tissue banks advisors consider such results as unrepresentative of the donated tissue infectivity, believing that virtually all deceased persons would expect to show signs of infection in blood cultures after death.

3.4.15 Additional serology testing

Donors who used non-injected illicit drugs may be at higher risk of viral infectivity such as Human Immunodeficiency Virus Type 1 (HIV-1), Hepatitis B Virus (HBV), Hepatitis C virus (HCV), Human T lymphocyte Virus Types I & II (HTLV I & II) and syphilis. This adds to the risk of infection transmission through transplantation of donor tissues since the incidence of positive infectious disease markers is not always sufficient (Gocke et al 2005). As a result many UK banks use Nucleic acid testing (NAT) to reduce the risk of transmitting infectious disease in addition to the mandatory testing. Disadvantages with NAT testing appear to be that only a few testing centres are able to offer this service and this is at an additional cost and also that there is an issue with the quality of post mortem samples affecting the test. Haemolysis of deceased donor samples, despite being taken within 24 hours of death, may have the potential to produce invalid NAT results due to inhibition of amplification reactions. There has not yet been an analysis of the extent of inhibited results observed in the UK or EU tissue banks, but one EU bank (bank 8) has reported (so far unpublished) in excess of 20% of their NAT results are ‘inhibited’. One US report (Strong et al 2005) estimated numbers of inhibited results and described techniques that could minimise inhibition to 1% of tests.

3.4.16 Other supplementary tests

Despite the donor selection procedures in place in tissue banks, given the number of significant viral Creutzfeld Jacoub Disease (vCJD) cases in the UK, and the fact that the
agents inducing the disease (abnormal, prion proteins) are particularly resistant to physical and chemical inactivation, there is sufficient concern to introduce testing of deceased donors for vCJD disease or latency (Pauli, 2005; Warwick, 2005). However, there is still no available licensed assay for blood or donor tissue. A pilot project has been started in the UK in April 2006 which uses reticulo-endothelial system (eg tonsil) samples from deceased donors, collected at tissue retrieval, Biopsies from these tonsil samples are being used to test for vCJD and this data is currently used in epidemiological studies of incidence of vCJD in the UK (Purkis, 2007). This is not yet available for all donors and necessitates additional consenting of donor families and training of tissue bank retrieval staff, but it seems to be a step closer to developing a routine vCJD assay.

3.4.17 Testing of thawed tissue samples

Only one bank (bank 7) encourages a routine test of remnants cut during transplantation. Other banks report that there is in the region of 9% failure rate of these theatre remnant tests and that the interpretation and significance of these results is questionable. Failures may reflect upon the theatre air quality or quality of aseptic technique used by operating team rather than true contamination of the tissue. The condition of UK operating theatres is not ideal, due to the design, age, lack of maintenance and lack of funding to repair or replace existing theatres. This is generally known and it has now been shown, that theatre air quality has compared poorly to retrieval centres and tissue bank suites (Davies, 2007). The aseptic technique used by theatre staff is less stringently monitored than in tissue banks, and theatre staff aseptic training is often a ‘once-only’ event.

3.4.18 Interpretation of tissue sample results & rejection criteria

After review of all the processing test results, a decision will be made by the tissue banks medical advisory team whether to accept or fail the test status of the tissue. One of the banks (bank 3) which uses the commercial decontamination cocktail, presented at the BATB Annual Scientific Meeting 2007 (Kerwala, 2007) reporting a 41%
bacteriology failure rate for their tissue. This is much higher than the typically reported 1-15% by other UK and International banks. The UK rates have only been recently reported to HTA, and a published report is imminent. International cardiovascular banks tissue rejection rates are compared amongst other data, at the World Congress (last one held in Brazil 2005, next one in Kuala Lumpur in August 2008) but these are not formally published.

The fact that some decontamination cocktails (such as the commercially produced ‘Sigma’ cocktail) are not as effective as the other disinfection media used in the UK, has been reported by NIBSC but reasons why have not yet been established. One bank (bank 1) reported that they trialled and later rejected a similar mixture because they found it to be damaging to the tissue (this was thought to be potentially due to chemical effects such as synergic reaction going on between the highly concentrated components of the media). Another issue is that the cocktail is incubated at 4°C which is not an ideal working temperature for most antibiotics. The commercial cocktail may also be less effective due to the fact that it is only distributed as a frozen product and stored at -20°C and this may have some detrimental effect and reduces the effectiveness of each component as decontaminants. One EU bank however, reports an even higher post-disinfection infection rate (Spatenka, 1997) of donor tissue harvested at post mortem NHBD (43%). This may be related to the fact that they continue to use the less effective amphotericin B in their antibiotic cocktail and incubate at 4°C. A Belgian bank (Tabaku, 2004) has reported similar high pre-disinfection rates of infection, their MOD rate was 36%, their NHBD rate was 78.1% and they had also tissue from heart transplant recipients RHT with pre disinfection rate of 21.6%. However, following processing they achieved a sterility rate of 94% and did not observe any difference in decontamination rate when exposure to antibiotic cocktail time was varied, but they did find that a considerable proportion (40.4%) of NHBD tissue was contaminated with more than one germ compared to 83.3% and 88.5% of MOD and RHT. The most predominant germ they found was Coagulase Negative Staphylococcus. In contrast, a bank based in Senegal (Ba, 2001) reported a contamination rate of 25% essentially due to Pseudomonas species.
Each UK bank has had to file a productivity report to the HTA in 2008 as indicated in section 3.1 & fig 1. The data collected cannot yet be satisfactorily used to compare banks since the rejection criteria employed by each bank varies considerably. Most banks will normally accept one positive out of a series of three results (depending upon the type of the contaminating organism). However, other banks may reject the tissue from which only one failed result has been reported. Another bank may additionally reject all other cardiovascular tissue from the same donor. It was found difficult in this study to get detailed information about this aspect of each banks procedures, since often this is looked at on ‘an-individual-case-basis’ but, it is obvious that each bank has very different acceptance/rejection criteria. Once this HTA reporting system is refined, it may be possible to compare statistical data, such as sterility rates, from individual UK banks.

It has been stressed (Ireland, 2005) and now widely practised in the UK that a specialist microbiologist must be involved in the interpretation of the significance of the culture results. All results for one donor must be collated; the identity of the organisms in the tissues must be established, whether it is a spore former and whether there was growth before and after disinfection. The microbiologist must also be cognizant of the processing methods used, the nature of the disinfection media and be aware of the stage each sample was removed for testing. They must also take into account the potential for effective disinfection of fungi and spore formers and the potential for false negative results due to residual antibiotics or cryopreservation media. All but the latter seem to be part of routine practice in the UK.
3.4.19 SUMMARY OF SIMILARITIES & DIFFERENCES IN UK BANKS

SIMILARITIES

• Most banks have a purpose built or re-designed aseptic facility for tissue processing.
• They all use similar donor selection criteria and consent procedures.
• All UK banks retrieve blood samples within 24 hours of death of the donors
• All banks procure the CV tissue within a maximum of 48 hours, transport it to their tissue bank on ice and then dissect within 72 hours
• All banks disinfect with an antimicrobial cocktail

DIFFERENCES

• Many variations in practice were found.
  - The organisation to which the bank belongs varies from NHS cardiac surgery units to blood services or an eye bank & the Role varied with only 3 banks performing all stages of ‘processing’ i.e. Consent, Procurement, Processing, Storage, Testing, Allocation & Despatch to Implanting Centre.
  - The quality assessment & sizing protocols varied and are reported differently, from schematic computer print out to individual hand drawn representation.
  - Different antimicrobial disinfectant cocktails, exposure times & temperatures are used (6-24 hrs @ 4-25°C)
  - Microbiological sampling methodology, rejection criteria and the ultimate rates of rejected contaminated tissue are considerably different.

In summary, the processing procedures in many banks have many similarities but there were also some areas where there were found to be considerable variation, and this may indicate potential differences in resulting tissue quality. However, the general overview of the cardiovascular tissue processing stages used by UK banks was, for the first time, identified (see Figure 11).
Fig. 11. Overview of current UK Cardiovascular Tissue Processing Practice

- Donor Referral
- Transplant
- Serial dilution
- Rapid thawing
- Aseptic procurement <48hrs
- Heart transported in isotonic fluid on ice
- Dissection <72hrs
- Aortic & pulmonary valves QA & Sizing
- Disinfection in antibiotics
- Cryoprotection in 10% DMSO
- Packaging in 2 sterile pouches
- Storage in electric freezers or vapour phase liquid N₂
- Slow controlled freezing @ -1°C/min
- Despatch to theatre in dry ice or dry shipper
- Rapid thawing
- Serial dilution
3.5. REVIEW OF UK CARDIOVASCULAR TISSUE BANKING - CRYOPRESERVATION

ISSUES

There were found to be many variations in the cryoprotection, cryopreservation, storage, re-warming and dilution protocols of the UK cardiovascular tissue banks and also at least one significant difference between UK practice and that of other EU and US banks. The general overview of UK cryopreservation methods currently in use is summarised in figure 11. However all UK banks and the great majority of banks in Europe and US use cryopreservation for long term storage of their cardiovascular tissue. Very few International banks choose to preserve their tissue in refrigerators at 4°C now since there is increasing evidence to illustrate that there is significant degeneration of the quality of tissue after several weeks’ storage at this temperature (see section 3.2.6). However, low temperature preservation of cardiovascular tissue does require purchase of the costly liquid nitrogen refrigerators or ultra low temperature electric freezers. The International Atomic Energy Authority (IAEA) has an on-going project to provide funding for multi tissue banks in developing countries which use radiation sterilisation methodology in their tissue processing (mostly musculo-skeletal tissue). This has helped multi tissue banks to add cardiovascular processing to their regimen, which is why there is less difficulty in these countries in setting up the expensive cryopreservation facilities. However, there are still some banks in poorer countries such as India, which continue to use refrigerated tissue stored for a short time. The evidence for use of refrigerated tissue stored for up to three weeks at 4°C remains a satisfactory solution (Lang, 1994) but inevitably results in a significant amount of discard of unused tissue. Figure 11. Summarises the overview of current UK tissue bank that was identified during this study. The variations in current practice which were related to cryopreservation issues are discussed in the following sections.
3.5.1 Cryoprotection time limits

The maximum time limit to cryopreservation varies between different banks (see table 4). Since the banks cannot control the death to dissection times, except by setting a maximum time limit, some banks have opted for standardisation of the rest of the processing. Banks 3 and 6 aim to begin cryoprotection 24 hours after the tissue disinfection stage of processing and this will inevitably be within 96 hours due to their disinfection time limit of 72 hours. Other banks (banks 1, 4, 5) instead aim to cryoprotect the tissue within 96 hours of death no matter when the disinfection stage started. One bank (bank 7) aims to begin cryoprotection within 72 hours of death. However, the final bank (bank 2), will cryoprotect their tissue any time up to 2 weeks following death. This extended time limit may be linked to this bank being one of several UK banks (also bank 1) who have always been committed to the ‘National-Heart-Hospital-belief’ that ‘non-viable’ tissue is more appropriate for transplantation - this will be mentioned later in the Discussion section.

3.5.2 Rinsing tissue prior to Cryoprotection

Some banks ‘rinse off’ the disinfection media from their tissue prior to cryoprotection and this is performed with one ‘rinse’ in either buffered isotonic media, saline or tissue culture media. However, one bank (bank 1) has shown (validation protocol HVB/VP/14.1) that a ‘rinse’ with one aliquot of fluid is not enough to rinse off the disinfection media. They showed that, even 3 rinses with up to 500ml are insufficient. Further work is currently being performed by bank 1 to quantify how much rinsing is sufficient (HPLC studies described in following Discussion section). Obviously, if disinfection media is still present in the tissue it may lead interfere with test results and potentially lead to false negative reporting as previously mentioned. It has been reported (Gall et al 1995) that there remain residual antibiotics in postoperative tissue, which may be beneficial in assisting the reduction of, and resistance to infection in the immediate post operative period.
<table>
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<th>Time limit for cryopreservation</th>
<th>Bank 1</th>
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<th>Bank 3</th>
<th>Bank 4 &amp; 5</th>
<th>Bank 6</th>
<th>Bank 7</th>
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</thead>
<tbody>
<tr>
<td>&lt; 96 hrs after death</td>
<td>-2 weeks</td>
<td>24 hrs after disinfection</td>
<td>&lt; 96 hrs</td>
<td>24 hrs after disinfection</td>
<td>&lt;72 hrs</td>
<td></td>
</tr>
</tbody>
</table>

| Solution for media Rinse | n/a | n/a | Buffered isotonic solution | Sigma rinse solution | Saline | RPMI |

| Cryo media | Hanks and DMSO (10% w/w) | DMSO (10% w/w) (or glycerol when DMSO not available) | 10% DMSO in Hanks | DMSO & Hanks (Sigma) | 10% DMSO in RPMI 16/40 | 10% DMSO in RPMI |

| Method of adding cryoprotectant | Drop by drop addition (in 4 mins) to 16% and then 2nd stage one step dilution to 10% DMSO. | One stage | One stage | Two stages. 50ml total volume, pour out solution | One stage | Open inner bag, drop straight into 100mls |

| Total amount of cryo media with valve | Total 100ml (Valve and cryomedia) | No cryomedia added | 100ml | 50 ml cryomedia | Depends on size of valve | Approx 100mls |

| Type of packaging | Two Teflon pouch | Gauze tray, nylon tube, tri aluminate sachet | Fresenius Hemofreeze bags (Kapton/Teflon) | Nylon pouch, foil pouch | Nylon inner – trilaminate outer & eva bag | 2x teflon bags |

| Controlled rate freezer program | One program * | One program ** | One program | One program for 1 valve, one program for 2 valves | -1°C @ 100 mins. One program | One program. One Program |

| Dummy sachet used? | During each run | Yes, every 6/12 run | During each run | No | No |

| End temp of program | - 150°C | -65°C | -150°C | -110°C | -150°C | -180°C |

| Rate of cooling | Approx. -1°C per minute | Approx. -1°C per minute | Approx. -1°C per minute | Approx. -1°C per minute | Approx. -1°C per minute | 4°C for 2 hrs 20mins |

Table 4. Comparison of the Cryoprotection, Packaging & Cooling procedures used in each of the UK cardiovascular tissue banks. The sections highlighted show areas where there are significant differences.
3.5.3 Cryoprotection media

The cryo media used by each bank is similar. Most banks use 10% DMSO in Hanks nutrient media or other nutrient media. Most banks appear to pre-cool the DMSO in order to reduce its toxicity since it is known that this increases with temperature. Although it was used in UK banks in the past, only one UK bank now uses 15% glycerol (bank 2) and they only do so when DMSO is unavailable. It was difficult to find other EU and US banks using any different cryoprotectant but, in other parts of the world such as South Africa (Lindeque AM et al 2005), and India and Syria, glycerol is still routinely used as a cryoprotectant for cardiovascular tissue banking. Its main disadvantage with respect to cardiovascular tissue cryoprotection is that it seems to require 24 hours to permeate the tissue; however, it is cheaper and easier to source and handle.

3.5.4 Cryoprotectant addition

The protocol for the addition of cryoprotectant varies between UK banks. One bank (bank 1) aims to keep tissue and media at 2-8°C during the cryoprotection process. Most banks (banks 2, 3, 4, 5, 6, 7) now immerse their tissue directly into the 10% cooled cryoprotectant (see fig 12). However most of these banks (banks 3,4, 5) now use a commercially prepared 10% DMSO media (banks 3,4, 5, 6) and this coincided with their change from a previous two step addition method to the new one step addition (they do not prepare any DMSO solutions themselves any more). Bank 1 however, employs a gradual drop wise addition of DMSO to 16% followed by a second stage to achieve a final 10% DMSO mixture. The same method is used in some EU banks. The only UK bank which appears to have maximum time limits for cryoprotection to commencement of cryopreservation is bank 1 and it aims to ensure that the cryoprotected tissue is cryopreserved within 45 minutes of cryoprotection. This limit is again similar to several EU banks.
One article (Birtsas et al, 2005) suggested that there was no significant difference (at least in the parameters that they monitored); between the one step or two step protocols provided the antibiotic/antimycotic rinse in Hank’s solution stage was introduced. However, a more extensive study (Hu et al 2002) describes the pattern and rate of penetration of DMSO into porcine cardiovascular tissue valves. This shows that the penetration is non-linear and time dependent and may take up to 120 minutes to achieve a tissue concentration of 0.7mol/litre tissue water volume. This paper suggests that the cryoprotection techniques used in most banks may not have been fully optimised. UK banks seem to have little understanding of chilling injury but they are aware that cryo-medium needs to be pre-cooled since the toxicity of DMSO increases with temperature.

An analysis of the viability of porcine valvular tissue (Vasquez et al, 2008), suggested that examination of the factors affecting apoptosis are necessary for optimisation of cardiovascular processing. This may be particularly relevant to improvement and standardisation of cryoprotection techniques.

3.5.5 Cryoprotectant total volume

The final volume of cryoprotectant media with the tissue varies between UK banks from 0 – 100mls. This may be of significance when comparing re-warming and dilution procedures and validation protocols. Only two UK banks (banks 1, 3) retrieve cardiovascular tissue from donors <6 months of age. The total volume for most banks is the same (total for banks 3, 6, 7 is 100mls and total for banks 4, 5 is currently 50ml) regardless of donor age or type of tissue cryopreserved. Baby valves range from 5-10mm
internal diameter and 20mm length and adults normally are between 22 – 25mm internal diameter and 50mm length. Type, and therefore also quantity of tissue, can range from aortic valve with aorta root attached, to single 20mm pulmonary artery wall patch. Only one bank (bank1) chooses to vary the total volume depending upon the size of the tissue to be cryopreserved. They achieve this by measuring volume of media with tissue and making it up to a standard total 100ml. One bank (bank 2) cryopreserves their tissue without cryoprotectant media – they let it ‘drip-dry’ before wrapping it in a gauze lined foil tray.

3.5.6 Tissue packaging prior to cryopreservation

All UK banks employ at least a double packaging system to provide greater security against leaks, having an outer package which is intended to resist mechanical damage and an inner pack that can be placed directly into the sterile field in the operating theatre (see fig 13). Most UK banks (banks 2, 3, 4, 6) place their tissue and cryoprotectant into an inner sterile nylon tubing bag, taking care to remove air bubbles. Then the nylon bag is carefully closed with a disinfected Hulme Martin electrical heated wire Heat sealer which is in situated in the aseptic room adjacent to the microbiological safety cabinet. They then add an outer sterile plastic coated foil sachet which is also sealed with a modified (thicker wired) Hulme Martin heat sealer at a higher temperature. A labelled cardboard box is then used as a protective outer layer for long term storage. In some cases (bank 3, 6), an additional outer waterproof bag is added for transportation to surgical centre. Only two UK banks (banks 1, 7) use purpose designed hemofreeze cryopreservation bags made from Teflon. Many EU banks and US banks have been found to also use hemofreeze bags. During validation of the long term storage capabilities of the packaging, one bank, (bank 1) have shown that on a small number of inner nylon bags which have been stored for >5 years @ <-130°C which have been inspected have been found to leak at the seals (Validation HVB/VP/16.1).
Theoretically this should not be a danger if this was found in a tissue despatched for transplantation since package insert instructions sent with each UK banks despatched tissue, does instruct implanting surgeons to check the integrity of the packaging (i.e. look for holes and leaking seals) before use. However, surgeons only normally have one piece of tissue and open its packaging in theatre during the transplant (since UK banks warn that tissue must be used within 1 hour of thawing and transplants normally last up to 4 hours). It therefore may not always be possible for surgeons to then stop the surgery mid way through the transplant because this could be a significant risk to the patient. The likelihood of finding damaged inner packaging may be small but if it occurs, the consequences may be very serious. Furthermore, one bank (bank 1) found neither the manufacturers of the nylon or the aluminium foil packaging would confirm that their packaging had been validated (or would be appropriate) for long term storage @$<-130^\circ$C. The advantages of the foil sachets is that they have improved heat transfer across the metal layer and that it is tough enough to resist mechanical damage. Adverse incident reports have illustrated that mistakes by technicians packaging the tissue have occurred which have potentially caused risk to patients. On more than two occasions, from different UK banks, it has been shown that a valve has been mixed up with another from the same donor and labelled incorrectly (i.e. an aortic valve has been labelled as a pulmonary valve) and this has not been discovered until the sachets were opened in theatre. On two occasions the wrong valve then had to be used (since it was too late to despatch a second valve) which caused increased risk to the transplant recipient.
(pulmonary valves are not ideally suited for implantation into the higher pressure left side of the heart and hence tend to fail more quickly). One bank (bank 1) has introduced a safety checking procedure so that the hemofreeze bags contents and labelling can be confirmed by a second technician after packaging and before cryopreservation to reduce this risk of mixing valves. Obviously this would not be possible in banks which use foil sachets. The disadvantages with using hemofreeze bags are that they are relatively costly (at least £30 per bag) and that they have to be sealed at a temperature of approximately 300°C which is a challenge for commercially produced heat sealers. The only available heat sealer which can reach such temperatures is one produced by the hemofreeze bag manufacturers (Hemofreeze NPBI Heat Sealer) which is much more costly than regular heat sealers (approximately £3000 versus £300). Considering all above issues however, it may seem more appropriate to use purpose-designed Teflon bags for packaging of cardiovascular tissue for cryopreservation.

3.5.7 Cryopreservation equipment & dummy sachets
All UK banks aim to achieve an overall slow rate of cooling (approximately -1°C per minute), however, it is not obvious that this is what is actually happening in practice. Most UK banks use the Planer Biomed Kryo 10/16 series 3 programmable freezers, whilst one bank (bank 1) and other EU and US banks use a variety of other freezers – bank 1 uses the Sylab, Ice Cube programmable freezer. Most UK banks use one program for all cryoprotected tissue regardless of size and amount of each piece of tissue, and also regardless of how many pieces of tissue are frozen at one time. One bank (bank 3) has a different program for freezing two pieces of tissue which is slightly different to their program for freezing one piece of tissue. UK banks freeze between 1 and 10 pieces of tissue at one time. The maximum amount has been determined by each bank and, in some banks, seems to reflect how many can physically fit in the freezing chamber at one time, rather than on any experimental evidence based work which identifies how the nitrogen flow in the chamber is affected by the amount of tissue sachets in the chamber during each freezing run.
Some banks (banks 1, 3, 4, 5,) have porcine or non clinical human valve prepared in cryoprotectant media (a ‘dummy sachet’) which has a platinum resistance thermometer inside its lumen in the banks standard volume of cryoprotectant which extends into a socket inside the freezer chamber. Although it cannot be sealed using a heat sealer, the dummy sachet is closed carefully with tape to avoid leakage. This dummy sachet is used in the chamber for each freezing run in some banks (banks 1, 3, 6) in order to measure, display and record the conditions that each piece of tissue experiences during freezing (see fig 3.1.14). Other tissue banks only use a dummy sachet for validation review every 6 months or annually. This may not be significant, but having a dummy sachet during each run, does enhance safety since any deviation from expected program outcome can be identified immediately, monitored and acted upon and the tissue affected may easily be traced. A graph is printed out by all banks who routinely have dummy sachets set up for each freezing run, these print outs are checked against acceptance criteria and then filed in donor tissue processing records. Typically, the acceptance criteria for bank 1 is that the profile shall show an overall freezing rate of between -1 to -2°C per minute, has reached an end temperature within an expected time period and that the shape of the freezing profile conforms to a recognised and ‘standard’ profile (see fig 14).
Figure 14. Freezing profile computer record of a dummy sachet which identifies the cooling rate achieved in a cooling program. Handwritten notes on the profile are comments made by the tissue banker confirmed that they have checked the profile and it is meets the acceptance criteria set out in their cryopreservation protocol.
3.5.8 Cryopreservation programs

Each bank uses a program to achieve slow cooling which is slightly different (apart from banks 4 & 5 which use identical programs). The programs used over the last decade had initially been based upon the Planer application note (no. ANB-04.1 – April 1991) written by Dr John Kearney. Since then, two UK banks (banks 1, 3) have modified this program to achieve a more uniform cooling rate. The cooling programs have been combined onto one graph in this study to illustrate the differences between all of the UK cooling programs (see figure 15.a.). A correction has then been made to remove initial hold stages to align each program to allow a more detailed comparison of the cooling rates implemented by each bank (see figure 15.b.).

The length of the programs varies from 75 minutes to 100 minutes and contains from 4 to 12 freezing, warming and hold settings. Some banks have their freezing chambers pre-equilibrated at the start temperature of 4°C (banks 1.6). The dummy sachet and the sachets with clinically suitable tissue are placed vertically in the chamber with their thinnest sectional area (their long side) facing the incoming flow of nitrogen vapour. Bank 2 has up to 3 sachets on each of 3 shelves placed horizontally with thinnest sectional area (their width) facing the nitrogen flow. The dummy sachet is normally a replica of the packaged, cryoprotected tissue (but not necessarily the same size and type of tissue). However, it will cool at a different rate to the chamber and typically lags about 10-15 minutes behind. Use of dummy sachets therefore closely mimics the tissue cooling profile in terms of both time and temperature.

All banks except two (banks 4, 5) begin their programs at 4°C and have a holding period of between 4 to 15 minutes. The DMSO therefore has had the time following cryoprotection plus the extent of holding time at the start of the freezing program to permeate the tissue, inevitably a minimum of 30 minutes. Banks 4 & 5 uses exactly the same program which starts at 0°C and has no initial holding time. The aim of each bank is to cryopreserve the tissue at a uniform rate of -1°C per minute (+/- 1°C). The programs are set up to initially rapidly cool the chamber in order to remove of the latent heat of crystallisation which is produced as the tissue begins to freeze.
Figure 15.a. Comparison of the cooling programs of all UK cardiovascular tissue banks. Note: bank 3 has two programs, having a modified program (3b) for cooling two valves at the same time. Data was plotted after viewing each individual banks freezing profile.

Figure 15.b. Comparison of the cooling programs of all UK cardiovascular tissue banks following alignment of the start of the cooling program.
This is achieved using the still commonly used 1991 Planer instructions, by following an 11 step program. First, the chamber is cooled at a rate of -10°C per minute to a temperature of -15°C and this is followed by a hold period of 2 minutes, followed by reduced cooling rate of -3°C per minute to -34°C and then, an increased rate of -30°C per minute during which time the chamber is rapidly cooled to -100°C. The chamber is then allowed to re-warm to the region of -35°C which is achieved by warming at 15°C per minute and then holding for 8 minutes, by which time the latent heat of crystallisation of the tissue should have been safely removed. The time needed for this to happen is in the region of 25 minutes after the 4°C hold phase at the start of the program. This relatively complicated programming prevents the tissue remaining at freezing point until all of its latent heat has been removed which may take some considerable time. If the chamber was allowed to cool at -1°C per minute, the sample would stay at its freezing point until all latent heat would be removed and, by that time, the temperature of the chamber would have reached a very low temperature. The sample would then plunge from its freezing point down to the chamber temperature and this would be likely to be very damaging to the tissue.

The critical range of temperature within which there is the most risk of cryogenic injury to the tissue is between -10 to -30°C. As long as the tissue is below this critical temperature (i.e. when chamber is less than -60°C and thus tissue is at -40 to -50°C) it is safe to increase the cooling rate. The next phase of the program is to slowly reduce the chamber temperature from -35°C to -60°C using a cooling rate setting of -0.8°C per minute which is achieved in a further 30 minutes. The cooling rate is increased to -3°C per minute to cool the chamber to -90°C (taking 10 minutes) and finally it is quickly cooled at -10°C per minute to an end temperature of -150°C. One bank has introduced minor variations to this old program (bank 1) which enables the sample freezing profile to consistently be more linear. One bank does not set up a re-warming stage but does still add a rapidly cool step to speed up the removal of latent heat of crystallisation (bank 2). Others do not appear to have a rapid cooling phase to speed up the latent heat removal nor do they have a re-warming stage (banks 4, 5, 7). Coincidently, these banks are the ones previously
identified that do not routinely have dummy sachets inside the chamber for each run. It is therefore unclear as to what their tissue freezing profile is routinely like and thus whether damage is occurring to the tissue and if this varies depending on type and quantity of tissue frozen in each run. One bank has a varied program for cryopreservation of two valves (bank 3) which starts at the same temperature and ends with the same programmed steps. The initial cooling rate however is lower (-6 and then -8°C per minute to -30°C). The chamber is then rapidly cooled to -70°C at a rate of -30°C per minute. The chamber temperature is then held for 7 minutes and re-warmed to -50°C, cooled to -55°C, and again re-warmed to -40°C. The time taken to perform this middle phase of the program is the same as the original planer program (i.e. 25 minutes). The validation for this change has not been published but it would seem to suggest that the total amount of tissue in the chamber has an influence on the program requirements for latent heat removal. If this is so, then this could be increasingly worse if there were more than two valves frozen – as previously explained, one banks freeze up to 10 valves at one time (bank 2). There ought to also be a difference in whether baby or adult valves are frozen. One bank (bank 1) has reviewed the over 5 years of freezing profiles and tried to identify any observable trends between number of pieces of tissue frozen at one time and also in whether there is a difference between freezing adult and baby valves (see validation HVB/VP/12.1). They have shown that there is no significant difference in either grouping.

The end temperature of the freezing programs varies between -65°C (bank 2) and -180°C (bank 7). Some Banks (banks 4, 5) set their end temperature to be at -110°C and the remaining banks (banks 1, 3, 6) set their programs to end at -150°C. There may be a risk of harm to tissue when it is only frozen to end temperature of -65°C or even possibly to -110°C, in that physical transfer to the storage refrigerator after freezing takes several minutes since it requires sachets to be carefully removed from the programmable freezer, placed into labelled cardboard boxes and then positioned in predefined rack positions. Whilst this happens there is inevitably some re-warming of the sachet taking place which may adversely affect the tissue (see Fig. 16 a & b & c). The bank which freezes to -180°C,
may not actually be achieving their desired end temperature, since platinum resistance thermometers used to measure this ultra low temperature, are extremely difficult to calibrate for use at such temperatures. It seems that the tissue should not be damaged if it is frozen safely below the ice crystallisation temperature of – 130 °C. The banks which use a program which ends at -150°C will ensure that their tissue has at least cooled to -130°C (bank 3) (see fig.15. a & b). Two banks have a hold period after end temperature to ensure the tissue eventually reaches -150°C (bank 6 holds for 30 minutes at -150°C and bank 1 holds for 15 minutes). The programmed end temperature of -150°C may ideally provide technicians with enough time to safely transfer tissue from programmable freezer to labelled cardboard boxes and predetermined rack positions in ultra low temperature storage at < -130°C and therefore minimise the potentially damaging temperature gradient.

Fig. 16.a. ‘Sylab’ Controlled rate freezer, Figure 16.b. Sylab freezer screen during freezing showing the cooling profile of the run, 16.c. valves being transferred from controlled rate freezer into ‘in Quarantine’ storage into the vapour phase of a liquid nitrogen refrigerator.

3.5.9 Preferred type of long term Storage

Most banks (banks 1, 3, 4, 5, 6, 7) use nitrogen refrigerators as their preferred long term storage option (see table 5). They store the tissue in the vapour phase of nitrogen in the temperature region of < -160 °C. One bank (bank 2) uses electric ultra low temperature freezers for storage of their tissue at approximately -140 °C. The banks which don’t use electric freezers cited cost, reliability, lack of necessary space and the fact that a nitrogen back up was additionally required as their main reasons. Although it has been reported (Rendal et al, 2004) that there was a greater viability among valves stored in (or ‘under’) liquid nitrogen versus within the vapour phase, there remains a strong consensus in the UK to avoid storage of cardiovascular tissue in liquid nitrogen. This seems to be

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due to the belief that submersion of the tissue sachet in liquid nitrogen may induce hairline cracks on the valve surface (Adam et al., 1990). Also, should there be any leak in the packaging; submersion in liquid would facilitate potential cross contamination between tissues and also pooling of contaminants in the base of the nitrogen refrigerator.

A rare case of Hepatitis B contamination has been reported (Hawkins et al 1996) which seemingly had been acquired during liquid nitrogen storage. To investigate the likelihood of cross contamination between cardiovascular tissues stored in nitrogen refrigerators, one bank (bank 1) has validated the cleaning of their nitrogen refrigerators (see validation HVB/VP/18.1). Their two refrigerators were decommissioned (valves safely transferred to different refrigerators) and allowed to re-warm. The remaining fluid and swabs of the top, bottom and sides of the refrigerators, storage racks and spine chillers were tested using bacteriological and viral testing procedures. There were, as expected, a wide variety of contaminants found in the test samples. This bank uses sterile purpose designed Teflon hemofreeze double bag packaging for their tissue and they also routinely have the packaging seals checked by a second technician. They therefore could only speculate that the contamination may have potentially originated from the cardboard boxes in which the double packed sachets were placed, or from the nitrogen refrigerator or the nitrogen vapour, from the protective gloves or finally from the air when the refrigerator was opened.

Although the bank subsequently disinfected thoroughly the base, top and sides of the refrigerators, they reported that it was very difficult to disinfect the inside of the racking system. This highlights the need for extreme care to ensure that all of the tissue sachet seals are intact and that the sachet surfaces must be disinfected prior to the start of re-warming in theatre and also that the storage sachet itself must be specifically validated for long term storage in nitrogen vapour. Some EU banks (Barcelona and Czech) and many US banks (Cryolife Inc.) continue store their tissue in liquid nitrogen however, they use the purpose designed sachets and most also have strict packaging checks in place. The disadvantage of the potential cross contamination risk is therefore reduced.

However, they have to program their end temperature to be as close to -196°C as possible and transfer the tissue rapidly to the storage refrigerator and when putting in or
removing tissue sachets they have to take care not to cause splashing of the liquid nitrogen.

The advantage of storage of tissue at or very close to liquid nitrogen temperatures versus storage in the nitrogen vapour phase does not seem to have been proven, but it would seem likely that the main advantage would potentially be that the lower the storage temperature (and therefore the closer -196°C), the least possible damage may occur during long term storage. Since it was first postulated in 1990 (Adam et al., 1990), no further reports have been published suggesting that storage by submersion in nitrogen may increasing risk of micro cracking. It is now more likely that it is too rapid warming from liquid nitrogen that is most likely to induce micro-cracking (Norfolk et al 1991; Wassenaar et al 1995).

Since there is therefore a significant difference in protocol between the UK and many EU and US banks, it would be interesting to determine if there is any significant and quantifiable difference in the quality of this tissue.

3.5.10 Storage Maximum time limit

All banks except one (bank 3) store their tissue at <-135 °C for a maximum time period of 5 years. Bank 3 stores their tissue in nitrogen vapour for up to 10 years. It does seem however, that there is no scientific evidence to support either 5 or 10 years storage. Historically, there seems to have been no major incidents directly related to long term storage but, in patient post operative follow up studies, there are no reports that the time of storage prior to despatch has ever been studied as an independent risk factor. Very few banks have tissue that needs to be stored for longer than 5 years since the demand for cardiovascular tissue is generally greater than the supply. Despite the increasing demand, there remain some types of cardiovascular tissue for which there is a limited surgical need. One bank (bank 1) reports that they have some very small valves from baby donors of 32 weeks gestation which have exceeded the 5 years storage period. This is due to the sudden cessation of transplantation upon a particular group of babies with congenital heart valve defects of less than full term weight and size following the
Bristol Hospital Heart surgery enquiry. Bank 1 reported that, following the enquiry, there were no subsequent requests for donor valves of less than 7mm internal diameter (previously 1-2 per month). In order to determine if this was a short term response to the media coverage, they performed a UK and then an EU survey of paediatric cardiothoracic surgeons and found that the demand for such tiny valves was likely to be non existent in the long term. As a result, Bank 1 do not now retrieve tissue from donors less than full term birth weight and size from which the valves will be more than 7mm internal diameter. The only other tissue found to be stored for periods approaching 5 years are reported to be tissue which is slightly damaged during retrieval or dissection or those with some form of abnormality which may restrict their use to only a limited type of transplant.
<table>
<thead>
<tr>
<th>Preservation &amp; Despatch</th>
<th>Bank 1</th>
<th>Bank 2</th>
<th>Bank 3</th>
<th>Bank 4 &amp; 5</th>
<th>Bank 6</th>
<th>Bank 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stored in</td>
<td>Liquid nitrogen vapour phase @ approx – 170°C Or Electric freezer @ -80°C</td>
<td>Electric freezer @ -130 +/- 10°C</td>
<td>Vapour phase nitrogen</td>
<td>Liquid nitrogen vapour phase @ approx -170°C</td>
<td>Liquid nitrogen vapour phase @ approx -170°C</td>
<td>Liquid nitrogen vapour phase @ approx -170°C</td>
</tr>
<tr>
<td>Maximum storage time</td>
<td>5 yrs @ approx -170°C Or 3 months @ -80°C</td>
<td>5 yrs</td>
<td>10 years in nitrogen storage</td>
<td>5 yrs</td>
<td>5 yrs</td>
<td>5 yrs</td>
</tr>
<tr>
<td>Packaging for transport</td>
<td>Dry ice Or Thawed and stored @ 4°C (for own theatre) or Dry Shipper (if return likely)</td>
<td>Dry ice</td>
<td>Dry ice</td>
<td>Dry Ice</td>
<td>Dry shipper within local area Outside area in dry ice</td>
<td>Dry shipper</td>
</tr>
</tbody>
</table>

Table 5. Comparison of procedures used by UK tissue banks for type and extent of low temperature storage and type of packaging used for despatch of Cardiovascular Tissue. The sections highlighted show areas where there are significant differences.
3.5.11 Packaging for distribution

Each UK bank is required to package and label their tissue appropriately to ensure quality, safety and traceability whilst retaining the anonymity of the donor. The packaging documentation must include details of the processing techniques used to prepare the tissue as well as instructions for surgeons for the handling the tissue upon arrival at the surgical centre. The handling instructions include a warning for the surgeon to check the integrity of the packaging and instructions for re-warming and dilution. Each bank validates their arrangements for packaging and has service level agreements with the despatch couriers and with the surgeons (or their NHS Trusts) to ensure effective communication of responsibilities.

Most banks despatch their cryopreserved tissue at approximately -80°C in dry ice (solid carbon dioxide pellets) (banks 1, 2, 3, 4, 5, and 7) (see table 5). One bank routinely issues the tissue in a dry shipper in nitrogen vapour (bank 6) and one bank (bank 1) offers this option when a surgeon is in doubt that the tissue may be actually used to prevent wastage of thawed tissue.

The despatch of human cardiothoracic tissue has obvious health and safety implications for handling by couriers (and airlines) during despatch and for surgical theatre staff and so appropriate safety warnings, handling instructions and declarations for customs officials accompany each piece of tissue in transit.

Tissue is despatched for transplantation into a single patient by a registered medical practitioner, the cardiothoracic surgeon. The service level agreements prepared by the tissue banks in order to comply with HTA and EUTCD requirements, point out the responsibilities of both parties. The responsibility of the surgeon begins following receipt of the tissue at the surgical centre. The surgeon is reminded that they must assess the risks of transmission of disease from donor to recipient and explain the significance to the recipient or their family prior to consent. The surgeon is given information about the antibiotic disinfection of the tissue, the cryoprotection treatment, details about the donor and microbiological test screening of the donor tissue samples. They are also specifically warned about the lack of testing available for variant Creutzfeldt Jacoub disease and
warned about the potential, but low, transmission risk. Details of testing of the particular donor tissue supplied are listed in the documentation that all banks insert with the tissue. Forms are also supplied with each piece of tissue to collect initial post-operative event details. One bank (bank 1) also adds a surgeon satisfaction questionnaire with each piece of tissue supplied. Surgeons are reminded to add clearly record the use of human donor tissue in the patient’s medical records and reminded of their responsibility to inform the tissue banks promptly should they observe any significant adverse incident potentially attributable to the implantation of the donor tissue.

3.5.12 Re-warming & Removal of Cryoprotectant

It is the intention of all UK banks to thaw their cardiovascular tissue rapidly and to remove the cryoprotectant slowly by dilution, however the procedures performed in each bank vary somewhat and may appear to be relatively uncontrolled (see table 6). There has been a significant change in the last few years in the re-warming instructions issued by each bank. All UK banks now stipulate that there is a potential risk of surface micro cracking of the tissue if it is placed in a 37 °C water bath directly following storage at <-135 °C. This is in response to a small series of research articles (Wassenar et al, 1995) which described the nature of micro cracking and traced origin of this damage to the re-warming protocols from tissue despatched in dry shippers (at <-135 °C ) which were initially 'too rapid'. This evidence based reasoning then led to the rejection of the previously held hypothesis (Adam et al 1990) that micro cracking was due to nitrogen submersion.

Although many UK banks never actually witnessed this happening to their despatched tissue, This was understood as being due to the fact that only one UK bank routinely used dry shippers. All other UK banks routinely used dry ice despatch at -80 °C and seemed to have had no reported incidence of tissue cracking. This only led to acceptance of the ‘new’ hypothesis that the micro cracking damage was induced by the rapid
### Thawing procedure

#### Bank 1
1. Place unopened in Water bath @ 37°C for 15 mins
2. Open in 100ml sterile isotonic solution for 5 mins
3. Add further 200mls for 5 mins
4. Add further 400ml for 5 minutes
5. Transfer to new container

#### Bank 2
1. Place unopened in water bath @37°C for 10 mins
2. Swab and open
3. Add sterile isotonic fluid @37°C for 15 mins

#### Bank 3
1. Place unopened in waterbath @ 37°C until pack is soft
2. Wipe pack with alcohol and air dry on sterile field
3. Cut open outer pack and remove inner pack
4. Empty contents into sterile bowl and add 100ml sterile saline and leave for 5 mins
5. Add a further 200ml sterile saline and leave for 5 mins
6. Add a further 400ml sterile saline and leave for 5 mins
7. Transfer to a fresh bowl and rinse in further saline

#### Bank 4
1. Place unopened in Water bath @ 35 - 40°C until soft
2. Swab and open
3. Place in sterile bowl and allow ice to melt
4. Add 500ml Hartmans for 3 mins in new bowl
5. Same as 4.

#### Bank 5
1. Place unopened in Water bath @ 40°C until valve preservation solution inside bag had begun to melt
2. Swab outside and open
3. Place valve in saline at 40°C until all ice has melted
4. Transfer valve to fresh saline @40°C for 3 mins
5. Transfer and store valve in antibiotics until required

#### Bank 6
1. Place unopened in Water bath @ 37°C for 15 mins
2. Swab and open outer Eva bag
3. Open trialuminate bag and place contents in 100ml saline for 3 mins
4. Add further 200ml and leave for 3 mins
5. Add further 800ml and leave for 3 mins
6. Remove valve after further 3 mins
7. Transfer valve to 1 litre saline for 3 mins

#### Bank 7
1. Place unopened in Water bath @ 40°C until valve preservation solution inside bag had begun to melt
2. Swab outside and open
3. Place valve in saline at 40°C until all ice has melted
4. Transfer valve to fresh saline @40°C for 3 mins
5. Transfer and store valve in antibiotics until required

Table 6. Comparison of Procedures recommended on instruction notes sent with each valve issued by UK banks for the Re-warming and removal of Cryoprotectant from Cardiovascular tissue to be performed in the operating theatre
increase in temperature gradient inadvertently achieved when tissue was transferred from temperatures of approximately <-135 °C to those of the water bath at +37 °C.

Some banks warn in their thawing/diluting instructions to surgical staff, that the cryopreserved tissue is vulnerable to damage if handling inappropriately during the re-warming phase.

All banks recommend that their tissue is placed in theatre unopened in a water bath for 10-15 minutes, or in one banks instructions (bank 3), ‘until melting begins’, or in another banks words (bank 4) ‘until soft’. These instructions by bank 3 and 4 have been reported by surgical staff as being considered too ambiguous a phrase considering both of these banks supply their tissue with an outer packaging consisting of an opaque foil sachet. Surgical staff thus feel encouraged to prod and handle the tissue during this initial water bath warming phase to determine when the tissue is ‘melting’ or ‘soft’.

All UK banks then recommend that their tissue outer packaging be swabbed with disinfectant, opened and transferred to a sterile field into sterile saline or isotonic fluid. The amount of fluid and number of dilutions which is then recommended by each bank varies. Four banks recommend one dilution only (banks 2, 4, 5, 7) and three banks (banks 1, 3, 6) recommend a three step dilution protocol ending with final transfer to a separate bowl of fresh diluent. Only two banks (banks 1, 7) finally recommend transfer of their tissue to antibiotic media, but this is then on a local hospital basis only. However, the presence of residual antibiotics after disinfection before cryopreservation, or following soaking post dilution, has been shown (Gall et al 1995) to assist in the reduction of, and resistance to, infection in the immediate operative and postoperative period. This is supported by the low incidence of endocarditis in the first postoperative 3 month period.

The tissue’s antibiotic permeability has also been cited (Yankah et al 2005) as the major reason why it’s use for left ventricular outflow tract replacement with aortic root abscess results in low recurrent infection rates and low overall morbidity and mortality.

In response to surgeon’s requests to reduce cryopreserved tissue preparation time, one bank (bank 4) performed a study to determine if there were any significant differences
between porcine cardiovascular valve tissue prepared by shortened one step protocol (intended to be completed in 3 minutes) or a three step dilution protocol (typically performed in 15 minutes). The results of their study (Armitage et al 2005) seemed to show that neither the rate of warming, not the rate of dilution of DMSO had any influence upon the viability of the leaflet fibroblasts. They went on to suggest that it was therefore acceptable to reduce the thawing and dilution time from >20 minutes to <10 minutes and thus perform a one step dilution. However, this study has not since encouraged other UK, EU and US banks to change to recommending a one step dilution protocol. In fact, surgeons performing tissue transplants have at least one hour whilst the patients are being given anaesthetic and have their chest opened, before the open heart stage of the surgery begins which necessitates the use of the tissue. Therefore they should have ample time to re-warm the tissue and perform three step dilution of the cryoprotectant. Reducing the number of steps and time of dilution phase therefore seems to the other banks to be an unnecessary and potentially less safe incentive. One bank (bank 1) has performed basic validation of the removal of cryoprotectant by dilution (see validation HVB/VP/14.1) and found it to be more difficult to remove cryoprotectant than initially thought. Using high performance liquid chromatography methodology they were able to show that a significant amount of DMSO still remains in the tissue after a 3 step dilution protocol. An extensive study monitoring the removal of DMSO from thawed porcine valve tissue (Hu et al 2002), illustrates that it may take up to 50 minutes at 4°C for tissue concentrations of DMSO to be reduced to 0.24mol/litre tissue water volume, which is far in excess of an expected equilibrium suspending solution. The authors suggest that DMSO removal protocols in current use may not provide sufficient time to permit the cryoprotectant to diffuse from the tissue prior to clinical transplantation. Since DMSO is known to be a sensitising substance, it is likely that the dilution protocols in use may need to be extended rather than reduced.
3.5.13 Tissue Storage at the Surgical centre

The tissue which is despatched for transplantation is normally used immediately after re-warming and dilution. However, should the despatched tissue be thawed but then unused, all banks stipulate that it must be discarded. The range of time limit to discard varies between banks 1 – 24 hours and this does not seem to be based on sound scientific evidence (see table 7). One bank however, has made an exception for special provision in their own surgical unit (bank 1). This bank thaws a very small number of pieces of tissue annually, immerses it in antibiotic nutrient media and stores it in their cardiothoracic theatre refrigerator at 2-8°C to provide a 24 hour emergency stock which is intended for unscheduled paediatric transplants. This emergency stock is monitored very closely by the bank and is discarded within 4 weeks of removal from nitrogen vapour storage. Several banks in the EU operate a similar local service and one bank (bank 8) routinely despatches containers of antibiotic nutrient media with every piece of tissue they issue. It is intended that a dry shipper capable of storing the tissue at <-135°C for more than 1 week will be used by bank 1 in the near future instead. The health and safety implications and necessary training of theatre staff filling and using this dry shipper storage container has been the main hurdle in commencing this alternative emergency stock arrangement.

Most banks insist that following transport at -80 °C, the tissue may be stored in the short term at -80 °C but that it should not be returned to storage below -80°C. The time limits UK banks stipulate for maximum storage at -80 °C varies from 3 months (banks 1, 3, 4, 5) to 6 months (bank 2, 6). One bank also suggests that tissue can be stored in surgical units in a ‘standard’ (circa at -20 °C) freezer for up to 1 week.

One study (Brockbank et al 1992) recommended that tissue be stored below -130°C to prevent gradual tissue deterioration which occurs at warmer temperatures due to chemical and physical processes which are not totally inhibited until storage is below -130°C. The study showed that there was loss of viability in tissues stored in mechanical freezer at -80°C and hypothesised that this was due to recrystallisation. They believed that, during cryopreservation, very tiny ice crystals were formed which then fused or grew...
### Storage & Return of Unused Tissue

<table>
<thead>
<tr>
<th>Bank</th>
<th>Max storage after thawing</th>
<th>Max storage if tissue is still frozen</th>
<th>Are returns accepted</th>
<th>Is tissue ever put back into storage @ &lt;= 130°C after transport in dry ice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bank 1</td>
<td>Use within 4 hrs (if @ 4°C)</td>
<td>In -80°C freezer, use within 3 months</td>
<td>Only if prearranged &amp; security tagged. Data loggers or dry shipper also used.</td>
<td>No. Put into -80°C Freezer and used within 3 months by same centre</td>
</tr>
<tr>
<td>Bank 2</td>
<td>Use within 24 hrs (@ 4°C)</td>
<td>In Standard freezer, use within 1 week</td>
<td>No returns accepted except in local area in dry ice</td>
<td>Yes</td>
</tr>
<tr>
<td>Bank 3</td>
<td>Use within 1 hr @ 4°C</td>
<td>In -80°C until date of expiry on label</td>
<td>No. Unused tissue is always discarded.</td>
<td>N/A</td>
</tr>
<tr>
<td>Bank 4 &amp; 5</td>
<td>Use within 1 hr @ 4°C</td>
<td>In -80°C freezer, use within 6 months</td>
<td>No returns except local hospital in dry ice</td>
<td>Yes if &lt; 6hrs in Dry ice</td>
</tr>
<tr>
<td>Bank 6</td>
<td>Within 2 hrs @ 4°C</td>
<td>-80°C until date of expiry on label</td>
<td>Yes if data logger and dry shipper used</td>
<td>Yes as dry shipper used</td>
</tr>
<tr>
<td>Bank 7</td>
<td>Use within 4-6 hrs @4°C</td>
<td>-80°C until date of expiry on label</td>
<td>Yes in dry shipper (local area only)</td>
<td>Yes as dry shipper used</td>
</tr>
</tbody>
</table>

Table 7. Comparison of Storage and Return protocols recommended by and used in UK banks regarding Cardiovascular Tissue which has been delivered and subsequently unused. The sections highlighted show areas where there are significant differences.
into larger crystals when warmed or held at -80°C. They suggested that temperatures <-135°C were superior to -80°C and therefore that long term storage in liquid nitrogen vapour was acceptable but storage at -80°C was only acceptable for one week. The same author (Brockbank et al 2007) now has compared traditional frozen valves stored at -130°C to vitrified tissue stored at the same temperature and then compared these to vitrified tissue stored at -80°C. Following 6 months implantation into sheep, their results demonstrate that all frozen valves showed signs of structural deterioration but the vitrified valves were free from ice-induced matrix deterioration. None of this vitrified tissue has yet been implanted into humans but future human application is likely. Potential future long term storage of vitrified tissues at -80°C would enable transportation in dry ice and would be advantageous logistically and financially.

3.5.14 Return of tissue to the tissue bank
Most banks do not accept returns of tissue after it has been despatched (see table 7). This seems to be due to the worry that, refreezing thawed tissue or cycling of tissue from storage at <-135 ℃ to transport at -80 ℃ and then return to storage at <-135 ℃ is obviously not ideal and not encouraged by any bank. Since there is a national shortage of human tissue even for adult donor tissue for aortic valve and root transplants (see fig 17. a. & b) this seems to be wasting tissue which could (by using dry shippers or data loggers and short term -80°C storage) be deemed unnecessary. There are certainly an increasing number of emergency paediatric patients for whom a transplant is now potentially the only acceptable form of treatment. Recently, for example, there has been a considerable increase in the demand in the UK for cardiovascular tissue for transplantation into neonates born with hypo plastic heart defects (see fig 17. c & d.). The post operative results of this type of transplantation is reportedly improving greatly which is very encouraging since, only 10 years ago, these patients would have been classified as ‘inoperable’. However, due to the extreme nature of their congenital deformity, it remains difficult to schedule their surgery electively. Many UK paediatric centres do not
have their own tissue bank and therefore do not have a rapid tissue order and delivery service. They may therefore be forced to postpone surgery and thus potentially increasing their patient’s risk of mortality, or they may have to use a commercially manufactured alternative prosthetic graft which may often be an even greater risk to the patient. In addition, the HTA have recently specified that, surgical centres storing human tissue for more than 48 hours, will have to apply for a HTA and comply with the requirements of the EUTCD (this requires a great deal of staffing effort and funding of at least a £7500 per annum fee to HTA).

Figure 17.a. Human Cardiovascular Valve transplantation, b. Aortic valve and root transplant for endocarditis infection, c. adult donor aortic valve being prepared for transplant, d. aortic patch being implanted into a child recipient.

In the current donor situation where there is undoubtedly a National Shortage of cardiovascular tissue, it may be considered unethical to discard thawed tissue when there is little sound evidence to quantify the additional damage this may induce upon the tissue integrity, especially when the intended use is for short term, first stage paediatric surgery and it is known that the tissue will be removed after only several months or years at the second stage surgery. It may therefore be timely to determine the adverse effects of storage after despatch at -80°C.

Consequently, four UK banks will accept the return of tissue on a limited basis (see table 7). Some base their acceptance criteria upon the amount of time it has been at a temperature of -80°C (banks 4, 5). These banks will allow returns within a <6 hour deadline and then place the returned tissue back in the nitrogen vapour phase storage. Two banks will allow returns within 24 hours (bank 1, 2). Bank 2 will return tissue within 24 hours of despatch to a -140°C electric freezer. Bank 1 will only accept returns in strictly
controlled circumstances. They insist upon having a return declaration form completed and signed by the ordering surgeon and will insist that the return transport is by their own contracted courier. Upon receipt of the returned tissue, they will check their own transport container security seals are intact (indicating the container bank 2 will return them to a -140°C electric freezer bank 2 will return them to a -140°C electric freezer has been unopened) and they will then normally return the tissue to -80°C electric freezer. If they are pre-warned about a possible return at time of ordering, they will add data loggers to the container or use a dry shipper instead. One bank (bank 6) sends out all tissue in a dry shipper with data loggers and so has no problem with returns even to vapour phase nitrogen storage. However, the potential risk of damage in transit or the ‘non-return’ of the expensive dry shipper remains a considerable worry.

3.5.15 Recall of tissue
A form is included within the packaging of each distributed cardiovascular tissue for the implanting surgeon to document the operative outcome and early performance of the tissue (within 30 days) and to ensure traceability of tissue from donor to recipient. The surgeon is also advised to report any adverse events or reactions to the tissue bank immediately. A copy of the tissue information sheet is placed in the recipient patient medical records. HTA have recently introduced new requirements for tissue banks to report any adverse reaction or events. HTA are required by EUTCD to report the National data annually to the EC. HTA alerts will be distributed to UK Tissue banks following any significant adverse events or incidents and by circulation of the anonymous details they will aim to minimise recurrence. Other EU and International countries have similar adverse reaction and incident reporting systems in place (Khoie et al 2008). The EU funded EUSTITE project is also developing a EUTCD detailed protocol for monitoring adverse event and reaction reporting systems. Traceability systems in place in tissue banks enable the tissue bank to alert the surgeon and recipients about late high risk information about the donor or about subsequent unacceptable performance of other tissue from the same donor. US and UK media
recently has uncovered and publicised details of a procurement agency retrieving tissue in the US without consent (tissue from donor Alistair Cook). The recipients of the tissue of all tissue retrieved without consent was subsequently traced by the tissue bank which processed the tissue – a great deal of the tissue was implanted in countries outside the US, including the UK. One bank (bank 1) has reported that, a referring donor transplant co-ordinator highlighted that a kidney recipient developed kidney cancer some considerable time after transplantation. The detailed case information however, was communicated to the bank 1 medical advisory team and found to be not connected to the donor. A risk assessment was made by bank 1 and the cardiovascular tissue was accepted for clinical use. The tissue adverse reaction reporting systems enable the quality and extent of adverse reaction reporting so that tissue banks may promptly take appropriate actions to improve safety and quality. The traceability and communication systems in place in tissue banks seem to facilitate co-ordinated investigation between regulatory authorities, tissue banks, transplant co-ordinators, transplant surgeons and recipients.
3.5.16 Summary of similarities and differences in Cryopreservation in UK Banks

SIMILARITIES

- All UK banks cryopreserved using slow cooling with dimethyl sulphoxide cryoprotectant.
- All UK banks store at <-140 °C in vapour phase nitrogen to minimise cross contamination between valves during storage.

DIFFERENCES

- Time to Cryoprotection varied (72hrs to 14 days) & protocols varied (1 step immersion or slow drop-wise addition of cryoprotectant).
- Aluminium sachets or Teflon pouches are used for packaging valves.
- End temperature & time taken to cool varied (-65 to -185°C & 75 to 100 minutes).
- Freezing chambers are pre-equilibrated @ 4°C & programmed to minimise risk of ‘super-cooling’ in some banks.
- Tissue is transported by most banks in solid carbon dioxide ‘dry ice’ whilst others use a dry shipper.
- Re-warming and cryoprotectant removal instructions indicate fast warming followed by either 1 or 3 step dilution.
- All banks warn not to re-freeze tissue but maximum preservation time for thawed tissue varies (1 to 24 hours).
- Some banks permit controlled return of unused tissue for short term storage @ -79°C to alleviate National shortage of tissue.
3.6. REVIEW OF UK CARDIOVASCULAR TISSUE BANKING
- QUALITY ASSESSMENT PROTOCOLS

The EUTCD Directives and HTA Directions do not focus their detail upon the clinical aspects of tissue processing; instead they focus on quality management and general safety guidance. Quality assessment and control procedures are expected to have been performed but there is, as yet, no guidance to describe what is required. Consequently, the quality assessment and control protocols currently in place in UK tissue banks have been found to seemingly be inconsistent and limited.

3.6.1 Types and 'values' of published quality assessment methods

As previously explained, cardiovascular tissue is clinically useful only if, following cryopreservation and re-warming, it has maintained its ability to provide mechanical support. This may need also to include the presence of living, functional cells retained within its intact extra cellular structure. Although it is not agreed that viable cells are required in the cardiovascular tissue to ensure good performance (see section 3.2.10) their presence may still be considered an appropriate indicator of the effectiveness of tissue matrix preservation. Often Internationally used quality assessment assays typically monitor the 'viability' of cells, with some types of assay seemingly more appropriate than others for use with cardiovascular tissue. A combination of assays facilitates a more effective evaluation of viability. 'Viability' in this context ought to be used to describe cells/tissue which are able to show signs of life which are specifically relevant to a required function of cardiovascular tissue. Traditionally there have been a variety of assays documented in the literature which appear to assess 'viability' which have been used in validation studies. A suitable assay for routine use with cardiovascular tissues should be non destructive and practically simple and rapid, but many methods typically involve expensive equipment and considerable time and effort. The 'value' of some of the published 'viability' assays used to attempt to monitor quality of cardiovascular tissue may not actually be truly representative of the likelihood of tissue durability post transplantation, since the function measured may not actually be required in the tissue for effective performance. These assays can be classified into groups with the most sophisticated (and most sensitive) assays being those that measure more than one single characteristic quality
parameter – physical integrity assays therefore least and in vivo function most representative of the tissue quality.

- Physical Integrity
  e.g. by looking at gross appearance and physical properties and by microscopic examination using light or electron microscopes

- Metabolic activity
  e.g. by assessing uptake of metabolites, production of catabolites, labile metabolites, enzymatic reactions (intracellular or membrane transport)

- Mechanical activity
  e.g. by monitoring motility, phagocytosis, contraction, attachment or aggregation

- Mitotic activity
  e.g. by monitoring mitotic index, DNA synthesis, 'plating tests', growth and development (in tissue culture or embryonic)

- Complete in vitro function
  e.g. by monitoring fertilisation and development or by monitoring recipients following transplantation (of cells, tissues or organs)

A wide range of assays were found in the literature to have been relatively recently used for cardiovascular or related tissues. These included those which involve the counting of cells after staining with a vital dye (Nozynski, 2001, Wusterman, 1999), MTT staining (Zeltinger, 2001), Resazurin assay Perrot, 2003), Trypan exclusion assay (Cimini, 2002), Neutral red uptake (Modha, 1993), organ culture and staining (Allison, 2004) and outgrowth of valve leaflet fibroblasts (Armitage, 2005). Measurement of DNA synthesis using radioisotope incorporation has also been widely used such as Tritiated thymidine incorporation assays (De Fries, 1995). Good combinations of viability assay methodology were found (Adulius, 2002) but this involved considerable lengthy and costly experimental study. No one method was shown to be enough on their own to be an effective measure of tissue quality.
3.6.2 Quality Assessment principles currently imposed in UK banks

It was no surprise to find that, the results of the review of UK cardiovascular tissue banks showed that there was only minimal routine quality assessment processes currently employed (see lack of methods listed in Appendix V). It was found that the processing techniques seemed to have been initially validated, or were historically developed, to imitate the regimen used by others.

The tissue banks were found to currently depend upon these ‘empirically designed’ protocols and they have since remained vigilant to the standard operating procedures and hence, they ensure that tissue is processed in a standardised, consistent manner. It is believed that, by conforming to donor selection procedures, physical examination procedures at procurement and performing an examination of the tissue following dissection should be sufficient, provided that the rest of the processing and cryopreservation procedures remain standard. They do not therefore perform any ‘end stage’ quality assessment assay or procedure to ensure tissue quality. Very few appear to systematically measure the performance of the tissue post transplantation.

3.6.3 Quality Assessment methods used routinely in Cardiovascular tissue banks

Donors with known unacceptable abnormalities will be excluded during the donor selection procedure. The quality assessment procedures performed routinely are predominantly restricted to subjective checks of gross appearance of the donor’s body at procurement which is part of the physical examination procedure used by most banks (banks 1, 3, 6). This is then followed, in the tissue bank, by a detailed external and internal examination by naked eye performed after dissection. This is mandatory in all banks except bank 3, who does not perform an internal inspection for fear of damaging endothelial cells (however this risk has not been validated).

Gross appearance checks are undertaken in the form of one check in most banks whilst others have two checks performed by two different operators (bank 1). However all UK banks were involved in a recent cv tissue sizing and grading workshop at the BATB CV SIG meeting in Liverpool in January 2008 and this illustrated the risk of missing internal
abnormalities if no internal inspection is completed (results were presented at BATB ASM Nov 2008) (see Fig 18 a & b & c).

This is then followed by a simple physical integrity check (competency testing) which is carried out by filling the valve with fluid and checking for leaks which may relate to holes cut at procurement or dissection or to unacceptable regurgitant valves (see Fig 19 a & b ).

One bank in Belgium has a more sophisticated method using larger, measurable pressure (method unpublished).

The only processing ‘end stage’ routine checks made in the tissue bank post cryopreservation are to inspect the labelling and seals of the sachets before placing tissue into storage and also to check the packaging of authorised valve tissue before despatch to the surgical centre. The tissue banks include in their instructions to surgeons (which accompany every despatched tissue) warn the surgeon’ team to inspect the outer and inner sachets carefully before and after thawing, and the tissue banks recommends
that the tissue should not be used if any damage is found in case its sterility is compromised. No quality assurance checks on the tissue are performed in any UK tissue bank currently. However, one bank (bank 1) is currently investigating the use of routine metabolic assay using resazurin dye (early experimental data has been presented at the BATB ASM 2008 and Society for Low Temperature Biology meeting, 2008).

3.6.4 Histopathological examination of tissue samples

Some banks perform a routine histopathology investigation of valvular wall tissue specimens (bank 9) and one bank has a routine check of all heart remnants after valves have been removed, all others banks only send histopathology samples for testing if there is an underlying concern (previous history, unusual dissection observation). The significance of routine histology checks is not fully understood and agrees. Without an indication of pathological disease, the intention of such investigation is to ensure that the tissue is ‘normal’. Histopathologists will often not agree to advocate light or electron microscopy for such an ‘open-ended’ investigation (for example in bank 1).

3.6.5 Mechanical activity testing

Mechanical integrity testing methods involving comparison of elastic properties, stress/strain, stress/relaxation and breaking stress have been used by several banks in during the design of processing methods or to validate changes in existing procedures. These methods are not used routinely however in any UK bank due to the cost and lack of availability of the equipment required. Uniaxial testing procedures have been used with UK banks cardiovascular tissue and reported in student thesis (banks 2, 3,). Banks 1,8,9 have published articles about such mechanical testing (Langerak et al 1997) and one bank (bank 1) has further investigated the differences between aortic and pulmonary cv tissue using mechanical testing methodology (Vesley et al 2000). One European bank has also uniquely developed a quantitative method of investigation of the contractile properties of porcine cardiovascular tissue leaflets in order to verify the consequences of alternative methods of tissue preservation (Wassenaar, 1997). The study involved the
use of a number of endogenous vasoactive compounds such as potassium, 5-hydroxytryptamine, nor-adrenaline, endothelin-1 and prostroglandin F2 and monitored their contractile effects upon segments of tissue wall treated by different preservation methods.

3.6.6 Tissue culture methods of quality assessment

One bank (bank 4) has recently used methods of tissue culture to measure outgrowth of viable cells after changing methods of cryoprotectant addition and removal (Birtsas et al 2005; Armitage et al 2005). However, this was used only to validate changes in thawing procedure and was used without any other confirmatory assessment methods. Conclusions eluded to ‘no change’ in fibroblast outgrowth in tissue treated by a new faster one step dilution protocol compared to a slower multi step dilution protocol. Since maintenance of fibroblasts is not a required function for long term durability, this may not have been an ideal method used in isolation. The benefits of slow and staged dilution of cryoprotectant have been described (section 1) and this study may require repeating before any other UK or International banks will use the one step method advocated by this study.

3.6.7 Complete in vitro function studies

*In Vivo* function of the tissue has been monitored, at least historically by most UK banks. As previously described, early post implantation data is forwarded by the implanting surgeon to the UK tissue bank which supplied the tissue using the packaging recipient outcome forms sent with each despatched valve. All UK banks supply such a form, but not all chase up missing forms from surgeons and analyse the data. Although, early adverse reactions and events should therefore be reported and recorded by UK banks, it is unlikely that late recipient (and tissue) progress is monitored unless there is a significant untoward event. This is since many patients do not return to their surgical centre for follow up clinics after their transplants, they have this further treatment locally with their nearest cardiologist. It is therefore difficult for independent banks which are not
associated within a surgical centre to obtain good late cardiovascular tissue performance data (Jashari 2004). Since the first cardiovascular valves were transplanted, implanting surgeons have followed up their recipient patient outcomes usually for a particular indication or surgical procedure. Some banks have instead attempted to follow up their whole series of despatched tissue but there are huge risk factors in the analysis of such wide groups such as accuracy of documented tissue and recipient patient data, varying surgeon technique and experience, variety of surgical procedures, recipient age and concomitant surgery. Wide ranging data produced can therefore be misleading (Askovich 2007). Therefore cardiovascular tissue banks monitoring of in vivo function has had to be cautiously performed. One UK bank (bank 1) reported 0% early post operative transmission of infection from more than 1400 issued cardiovascular tissues (presentation at NIBSC 2004, proceedings were unpublished, article attached in appendix). One bank has successfully compared tissue performance that had been preserved by different methods (Takkenberg 2002) and then, in another study, compared an aortic valve replacement cohort of patients who had human cardiovascular tissue to patients receiving other biological valve alternatives (Takkenberg 2007).

Echocardiography of recipient patients has also been performed by some banks (bank 1 & European & New Zealand banks) to evaluate performance of the tissue post operatively (Hoekken, 1997; Palka, 2002; David 1994).

3.6.8 Study of Explanted cardiovascular tissue
Analyses of explanted valves have been performed by several banks in order to assess durability of the cardiovascular tissue and to develop the understanding of the factors that lead to its eventual degeneration or early inflammatory response. One European group (Koolbergen 2002) in The Netherlands studied 40 cardiovascular tissue explants and used techniques such as macroscopy, light microscopy and immunohistochemistry and found tissue cellularity and normal tissue architecture was rapidly reduced within the first year of implantation but only a low-grade inflammatory response was observed. The bank in New Zealand which made every effort to ‘ensure’ leaflet fibroblast viability, also studied
explants (O'Brien 1988) but found a variety of degrees of cellularity, even in leaflets explanted after only 10 months. However, this group observed viable cells in some explanted leaflets and they were able to show that they could culture fibroblasts from these explanted leaflets and, in one of them, demonstrate that there were persistent donor cells. The only UK bank to examine explants (bank 1) has so far not written and published a report since they do not have enough explants gathered (<10 samples collected so far).
3.7. REVIEW OF UK CARDIOVASCULAR TISSUE BANKING

- VALIDATION PROTOCOLS

3.7.1 Overview of UK Cardiovascular tissue banks’ Validation Protocols

It was found more difficult to collate validation information for the UK banks. There may be several reasons for this. The main reason was that the EUTCD requirements have not yet issued guidance or stipulated extent of detail needed in validation reports for processing methodology used in UK tissue banks, nor to do the EUTCD insist that these validations are published. Many of the validation reports written by UK tissue banks are only circulated within their own bank. Also, it appeared that some banks were unsatisfied with the amount of detail and the unscientific content of some of their validation reports, some banks had validation reports which were quite old and had not been reviewed and some banks didn’t seem to have written up all of their validation reports and were thus reluctant to share them with the author. Others had been presented at BATB annual scientific meetings and abstracts published in the society’s Cell and Tissue Banking Journal (Springer).

The UK tissue banking review confirmed that, following the set up of processing standard operating procedures which seemed to have been an empirical approach, simple validation studies seem to have been written and used in most tissue banks for the introduction of changes in the procurement, processing and despatch processes (banks 1,3,4,6,7). They had been performed using simple calibrated equipment such as data loggers.

3.7.2 Validation reports recently compiled in UK tissue banks

Validation report examples include those relevant to packaging and transport boxes used for transport of the heart to the tissue banks (for maintenance of the heart at 2-8°C) and also the packaging and boxes used to despatch tissue to surgical implantation centres for maintenance at <-79°C in solid carbon dioxide or at <-130°C in liquid nitrogen vapour.
phase have been validated. All tissue banks’ aseptic facilities were calibrated, monitored and validated, and this was often performed by externally contracted specialists.

New and critical equipment (equipment, materials or consumables which are in contact with the human tissue) had been validated. Storage freezers and tissue containers were validated to assess their capabilities for safely housing the tissue at temperatures of \(<-80^\circ C\) or \(<-130^\circ C\) and for a maximum storage term but however, the tissue itself was not validated to ensure it was still of similar quality after maximum storage time. New and significant procedures had been validated to ensure standardisation and consistency. For example, most UK banks print out the graph showing the controlled rate freezer performance after each freezing run and these are checked to see if they are consistent with the desired program variables (freezing rate and endpoint and shape of curve). The tissue itself however, was not validated after freezing or after thawing in theatre.

Only one bank (bank 1) has performed disinfection cocktail validation studies (Validation protocol HVB/VP 8.1) and has validated the neutralisation of the antibiotics prior to bacteriology testing. This has been presented by this author at the BATB ASM 2008. This bank is also the only one to routinely submit known positive spiked samples through the testing system to ensure calibration is sufficient. None of the banks have validated removal of the cryoprotectant following thawing and dilution of the tissue even though this may affect subsequent testing although this is due to be reported by bank 1. It has therefore been included in the future work section.

Validation of the toxicity of the disinfection media used in each bank and the affect this has on the viability of the tissue with a lower antibiotic dose has been recently reported by one of the European banks (Tabaku, 2004 and Jashari, 2007). None of the UK banks has yet validated the toxicity of their disinfection cocktails.

Validation of tissue containers has been performed by two of the UK banks (banks 3 and 6) such as plastic screw cap containers (Eagle, 2008) and nylon pouches (in house validation by bank 6). Other banks prefer to purchase and use specifically designed (and often more expensive) products for tissue storage such as Teflon cryogenic pouches (bank 1 buys these from Fresnius). One bank has introduced the concept of transporting
donors to a specialised procurement facility and has simulated the temperature of the body during transport using a doll dressed in a diving wet suit (bank 3). Although this sounds as if it is a crude simulation, it apparently has been used in the validation of the transport and several donor bodies have been already transported and tissue procured on this basis. Validation of the procurement centre air quality has been reported by two banks (banks 1 and 6) as previously described.

3.7.3 Validation of long term clinical performance of the tissue

Validation of cardiovascular tissue performance has not recently been systematically completed by any of the UK banks. Early post operative tissue performance, as previously described, is only monitored in six out of seven UK banks by way of an information questionnaire sent out as a package insert on despatch of the tissue. This is often poorly completed or not returned at all and only monitors implant and immediate post operative history. One bank (bank 1) routinely monitors (each month) this data and the surgeon satisfaction and recipient patient outcome data after the early post operative (30 day) period. This complete in vivo function has been presented at BATB ASM (Davies, J., et al, 2003). Several published articles written by surgeons include data supplied by cardiovascular tissue banks but their focus is on recipient surgical technique and outcome and processing variables cannot be distinguished. Surgical technique is a major element of risk in many tissue transplantation procedures and this is makes it difficult to extract valuable tissue performance data from these published articles. Alternatively, articles published with the detailed collaboration of tissue banks often unfortunately collate too many risk factors for truly accurate monitoring of post operative performance. For example, human cardiovascular tissue is often used for babies and children where it is intended that the tissue remains in situ for several months or years until a further staged operation is performed. The data from these patients is often grouped together with that of other paediatric patients who have a tissue transplanted which will be intended to last for the patient’s normal lifetime, and may also be put in the same group as adults with congenital aetiology and adults with acquired disease or infection. This
obviously makes it difficult to assess tissue in vivo performance accurately. It is difficult and costly (especially in terms of ‘manpower hours’) to collect the long term recipient data since many patients are not based in the hospital where their operation was performed (they instead return after surgery to the care of their local hospital cardiologist). Also, the statistical and database software packages designed for mechanical valve follow up studies are not immediately applicable and thus have to be altered to input human cardiovascular tissue data. Consequently, tissue banks report that they only now collect implantation and immediate postoperative results (Jashari et al 2004). One UK tissue bank is currently collecting data for a larger study of post operative performance of is cardiovascular tissue and the data collection has been designed to parallel that of stentless xenograft trials performed in the banks hospital (bank 1). Small differences in processing variables are to be monitored independently such as warm ischaemic time differences and cold ischaemic time. However, it would be interesting to compare different types of tissue to add to this study, if there are sufficient numbers.

A presentation of the preliminary findings of the review of the methods used by UK Cardiovascular Tissue Bankers was completed for the 2008 Society of Low Temperature Biology (SLTB) Annual Scientific meeting and British Association for Tissue Banking /European Association of Tissue Banking (BATB/EATB) joint congress 2008. The abstracts will be published in the Cryoletters journal (SLTB) and in the Journal of Cell and Tissue Banking (BATB/EATB),
3.8. UK REVIEW OF CARDIOVASCULAR TISSUE BANKS - IDENTIFICATION OF ISSUES OF CONCERN

3.8.1 Overview of issues of concern amongst UK tissue bankers

Each UK cardiovascular tissue bank was asked to complete a table to list their concerns regarding cardiovascular tissue procurement, testing, processing or despatch. Some banks were willing to participate anonymously, but others felt that they would prefer not to confirm this in writing yet so this table represents a summary of verbal shared concerns only.

Many UK tissue bankers are relatively unconcerned about their tissue banking methods used since, the tissue that they have issued seems to have functioned acceptably. However, as previously mentioned, the evidence for long term function or durability of the tissue which is the best form of quality assessment may be unreliable.

Unsurprisingly, the main issues which do concern tissue bankers regarding processing are related to stages where there has been found to be considerable variation in procedure or little evidence based validation.

3.8.2 Examples of Issues which currently concern UK Tissue bankers

Fewer banks seem to be concerned about maximum storage limit since there appears to be more evidence to accept 5 years or longer as the limit. However, all banks seem to be concerned about the maximum storage time @-80°C since there does not seem to have been any published data to describe maximum limit. Some of the banks (the ones who currently accept returns of unused tissue back to their banks) are concerned about the potential damage of ‘cycling’ of storage temperature of returned tissue. The banks who feel that this is not acceptable, are the banks which do not accept returns and do not use dry shippers or data loggers. Most banks are concerned about the maximum storage time
after thawing. This would seem to reflect the fact that a range of different maximum times are stated in each bank’s thawing instructions.

Only a few UK and EU banks are concerned about returning tissue to nutrient media for short term storage if despatched and unused in theatre. But one UK bank and a few EU banks currently do so in limited circumstances to avoid wastage of unused tissue. There is some published evidence suggesting that this is acceptable and even beneficial (as previously discussed) but this has not yet thoroughly been investigated (no maximum limit). With more evidence, this may be an acceptable solution for cases where transplantation is temporarily postponed and this would help to alleviate the National shortage of tissue.

Not many banks have a definite maximum time from death to cryopreservation and surprisingly few are concerned about this. Some are happy to cryopreserved as quickly as possible whilst others would like to determine the relevance of a maximum time using further experimental study. Few UK cardiovascular tissue bankers are worried about controlling the conditions of cryoprotection. Even less understand the concept of chilling injury.

Only a small amount of tissue bankers are concerned about their thawing procedure. Only one uses the one step dilution protocol, the others preferring to dilute the cryoprotectant more slowly. None of the UK banks have successfully measured their rates of warming and are relatively unconcerned despite the reports of cracking caused by too rapid warming.

More of the banks are concerned about the consistency of quality assessment determination by different individuals in their banks. The recent BATB CV SIG sizing and grading workshops have helped to highlight this issue. However, only one bank yet has more that one member of staff checking this assessment.

Several banks are concerned about the variations in details on valve information forms which are given to surgeons to describe the donor valve. This seems to relate to some of them receiving negative feedback from surgeons.
Only a few banks worry about the lack of recipient information details returned by the transplanting surgeon to the supplying tissue bank. Even less, worry about the lack of further late follow up information released by surgeons necessary for long term valve performance monitoring.

Few banks are concerned about validating their antibiotic disinfection cocktail and challenging their testing methodology with known positive spiked samples to ensure testing methods are reliable.

3.8.3 Summary of Issues that currently concern UK Cardiovascular tissue bankers

- There are concerns where there is variation in procedure or little evidence based validation (e.g. methods of quality assessment, disinfection and testing protocols).
- Only a few banks are concerned about the cryoprotection, re-warming and dilution procedures.
- there is concern about maximum storage time at -80°C since there is a lack of relevant published experimental study.
- most banks are worried but unaware of the degree of damage incurred by ‘cycling’ storage temperature of returned tissue
- most banks are unconcerned about the maximum storage time after thawing & haven’t validated this.
- Only one UK bank has performed retrospective post operative patient studies, however, this was problematic due to lack of guidelines & numbers within matched patient cohorts.
4.0 DISCUSSION

Although human cardiovascular tissue seems to be successful, there is increasing evidence that early acute failures in paediatric patients are observed following use of viable tissue. There is a lack of systematic experimental research into significant unresolved issues such as, whether viability or live cells are required for effective long term performance of tissue, and what is the etiology of early degeneration or failure of viable valves transplanted into paediatric patients. Discussion of these issues needs to involve an understanding of the perceived successes and failures of past methods of processing, the need to maintain viability following preservation, an examination of the mechanisms of early failures and an understanding of what constitutes acceptable 'viability' assessment.

4.1. Comparison of early processing techniques

The London homograft team led by Mr Donald Ross, tried all variety of disinfection and preservation combinations between 1960 to the mid 1970’s and so were able to retrospectively compare the performance of the various groups of treated tissue through patient performance studies (Bodnar, 1990). Very little significant difference was found, however, and prospective, randomised trials were never attempted. The experimental data was available but could not be extrapolated directly to the clinical use of this tissue. Consequently, a variety of hypotheses for improving tissue performance were postulated throughout this period and directly applied to the clinical treatment of the tissue. Overlapping of these experimental methods prevented the direct comparison of each Individual technique and thus techniques which resulted in favourable outcome were not able to be highlighted such as irradiation sterilisation without ethylene oxide gassing or rapid freezing (Bodnar 1990). This would not be possible today as more detailed experimental evidence would first have to be gathered prior to clinical application. It may have seemed that the London team were rather recklessly trying untested methodology of preservation/sterilisation in this late 1960’s to 1970’s period. However, their recollection is such that all methods were first experimentally validated, predominantly by the research team (which included Ross, Al Janabi, Wain, Bodnar and Parker). In hindsight, one of the man drawbacks
seems to have been that animal studies did not extrapolate as expected into use in the human clinical field (Bodnar, 1990). The other drawback was that they did not, at the time report each different group of treated tissue separately, but instead combined all groups which masked good performance of at least some of their novel ideas. Their insistence on the hypothesis that viability was required only as a marker for extra cellular matrix preservation, seemed, at the time, to be in contradiction to the New Zealand team and many other cardiovascular surgeons worldwide. With the growing amount of reported anxiety about homograft durability, less homografts were implanted from the early 1970’s for a period of time, apart from in the London and New Zealand centres where homografts continued to be used.

Nevertheless, the London tissue, no matter what the disinfection and preservation treatment, was not significantly harmful but results were not particularly different to xenografts or mechanical valves which was therefore disappointing. The return to antibiotic disinfection and preservation at 4°C in the early 1970’s was consistent internationally. However, a more detailed examination of the published and unpublished data regarding the details of the methods used has highlighted unpublished differences such as in the composition and levels or quantities of antibiotics used in the various disinfection regime and the temperature chosen for incubation during this sterilisation. The London team used large amounts of antibiotics and incubated them at approximately 25°C which ultimately was a combination which was relatively cytotoxic to the tissue, where as the New Zealand team and Yacoub (with his homovitals), were using a low dose and storing at 4°C which was much less of a cytotoxic antibiotic cocktail, but resulted in a reduced disinfection effectiveness. Many teams used antibiotics which were would now be identified as synergically reactive to each other (such as gentimicin and imipenem). In the late 1990’s, the Oxford and Brompton bank both independently found disastrous tissue ‘leathering’ (similar to glutaraldehyde fixation effects) in tissue which had a cocktail with synergic components and had to discard all of this tissue as it soon showed durability problems post transplant (source: unpublished studies). Inclusion of the antimycotic agent amphotericin B into the disinfection cocktail is also something which has been shown to have significant harmful effect on the fibroblast viability (Aguirregoicoa, 1989) but its use in the disinfection cocktail is not highlighted in the majority of published articles and this may have significant if ‘viability’ is the
main quality parameter under consideration. Other differences may be observed upon detailed examination of the published and unpublished data is the record of time between death and procurement (often termed ‘warm ischaemic time’) in the Yacoub homovital series was less than 1 hour, for the New Zealand team homografts this was within 24 hours and with the London team it could be from 12 up to 96 hours. Hence the presence of viable fibroblasts in some of the London valves may be unlikely. The amount of ‘trauma’ following ‘handling’ of the tissue procured by the London team was considerably more since many of the donors appeared to have originated from deceased donors following post mortem examination by pathologists. Donors from the other two groups however, were predominantly minimally handled. This again would suggest that maintenance of endothelial cell layer in London tissue is unlikely. These differences should have been highlighted and their significance may have altered the conclusions of some of these processing variable comparisons. If, for example, the London group had extracted from their large series, the small number or valves procured within 24 hours and then compared these results to those of the New Zealand, Australian and Yacoub, group, this may have had a significant effect.

Also, differences in radiation dose imposed on the tissue which was sterilised by gamma irradiated was different as well as the temperature that the tissue was stored at during this irradiation. This may account for the differences in reported long term performance of published irradiated homografts. The US Team in Alabama used a 25 mega rad dose whilst their tissue was kept at room temperature where as the London team used the conventional 2.5 mega rad dose with tissue cooled in dry ice. The London team found no significant difference in their irradiated tissue when compared to other forms of sterilisation (Bodnar 1980, and Campalani 1989) and carried on this practice until 1981, but the Alabama team reported disastrous valve failures and discontinued this practice much earlier (Beach 1972). Following the US reported failures; the sterilisation by irradiation practice was discontinued. More careful application of irradiation has not been discounted by the London team, but must first be based upon a more detailed validation study, for example, an accurate dose measurement should be essential since, apparently it is now known that a intended target dose of 2.5 meg rad can sometimes vary from between 15 and 40 mega rads and these extremes may be potentially harmful rather
than beneficial to the tissue. The temperature that tissue needs to be maintained at during irradiation must also be optimised. Also, upon examination of the homograft preservation details, or lack of them, in some published reports of long term tissue performance, ‘frozen’ valves are grouped together whether they were the product of rapid uncontrolled cooling or the quite different tissue slow cooled tissue cooled in controlled rate freezers. Actuarial methods of statistical data analysis were not international used until the late 1970’s (Bodnar, 1979) and hence comparison of results used in prior publications was not standardised and may be potentially misleading.

Looking back at published data and having spoken to tissue bank staff in New Zealand, it appears that the New Zealand team actually used a small amount of freeze dried valves as well fresh antibiotic nutrient preserved valves. This was reported as early as 1964, and data of the two groups was ‘pooled’ in his results section. It is uncertain whether these valves were removed from the follow up studies in later reports, which would seem the best option. It seems that the New Zealand team may also have tried out more methodologies even though they have been remembered historically for their focus upon only two preservation methods – their antibiotic and their viable cryopreserved series.

Finally, differences in the types of ‘frozen’ valves were sometimes considerable but they were not clarified and were grouped together with dissimilar ‘types’ of frozen valves and hence this may have adversely affected the results reported. The different ‘types’ of frozen valves included the London freeze dried homografts, the rapid frozen valves which were plunged directly into low temperature solid carbon dioxide storage, the later London group of frozen valves which were directly plunged into liquid nitrogen vapour, and the more recently (post 1990’s) frozen valves which were slowly cooled with nitrogen vapour. These different types of valves were often grouped together despite being different and having had different affects on the tissue and potentially may have led to durability differences post transplantation. These frozen valves should have been independently monitored but, after reviewing old processing London valve data, it is easy to see why this has not been the case. These records show that there is no documented ‘start or end date’ when each method was introduced or discontinued, instead it seems, that two methods overlapped on occasions, such as in the late 1980’s, some valves
were slowly cooled after cryoprotectant addition and others plunged directly into nitrogen vapour. It would now seem obvious that the rapidly frozen valves in the earlier ‘flash freezing period’ (1962 – 1972) were damaging to the tissue, but so, were the valves plunged directly into the nitrogen vapour. It would be very interesting to retrospectively study these different groups of frozen valves, and as part of this study this has been attempted, but the lack of detailed record, render it to be extremely difficult unless any other form of records can be found to establish the exact type of preservation method is used. The search for this evidence will be discussed in the ‘future work’ chapter.

4.2. The perceived need to maintain ‘Viability’

Viability may be defined as the ability of a system to continue its physiological function and it may thus be reasonable to assume that a living valve post transplantation should maintain its structure and consequently continue to function for a relatively long time. Early homograft degeneration by calcification was disappointing and this was attributed to the damage incurred by the harsh sterilising chemical treatment (with aldehydes and β-propiolactone), irradiation, ethylene oxide or the crude freezing processes (Bodnar 1989 and Daly 1991). It was then generally believed that these results provided evidence that maintenance of viable cusp tissue components ought to result in optimal valve durability post transplantation (O’Brien 1991 and Kirklin 1993). Tissue processed by less harsh methods did actually exhibit enhanced performance which seemed to support this assumption, however the reasons for this improved long term tissue performance was not actually proven.

The predominant aim behind the development of the harsh methods in the London bank was to ensure effective tissue disinfection and hence minimise the risk to recipients whilst enabling the tissue to be at least as durable, if not more durable, than the alternative mechanical or xenograft valves. The London team first believed that their non viable tissue would act as an ideal frame or scaffold and that it would be repopulated by recipient host cells which would then be capable of maintaining a normal fibre structure. However, when the first explants were examined and found to be degenerated and acelluar or virtually acellular (Barratt-Boyes, 1964), they realised their hypothesis of cellular repopulation was not correct. It was then assumed that the
maintenance of pre-implant cellular viability by specifically designed cryopreservation techniques ought to enable these valves to have living fibroblasts post implant. The assumption was that viable fibroblasts within the stroma of the implanted valve might provide means of self repair, and would consequently be able to maintain a normal fibre structure and minimise homograft tissue degeneration allowing the valve to become a permanent replacement (Al Janabi, 1973). Then shortly afterwards, the London team, upon examination of their evidence showing that tissue may be viable and capable of synthesising DNA (Al Janabi, 1972) and of protein (Al Janabi, 1973), and from observing acellular explants, they decided that they would consider the presence of viable cells in the pre implant valve cusps as only an indicator or index of the structural integrity of the collagen and the elastic matrix.

As previously explained, studies comparing the wide variety of preservation methods used in London (Bodnar 1989, 1991) did not find any significant differences in the long term performance of the tissue. These studies were repeated to include comparison of methods of disinfection affecting viability in order to attempt to establish whether or not cellular viability of the implanted tissue is maintained permanently and is an important factor affecting long term tissue performance (Bodnar et al 1990). The study showed that the long term performance of cardiovascular tissue valves was not affected by whether the tissue was viable or not.

The New Zealand team stuck to their firm opinion that pre implant viability will enhance allograft durability post transplantation until 1991 when they reported that most of their valves were non viable at implant and they stated that good clinical performance of homograft tissue relates more to the maintenance of the acellular ground substance of the leaflets than to the persistent cell population (Armiger , 1997). The Australian team and many others have however, remained of the belief that enhanced viability pre implantation will ultimately enable most durable tissue performance post operatively. These groups are focussed upon improving procedures to preserve viability by shortening times between death and procurement and freezing etc. (as previously described) which some have considered aiming to achieve a ‘super viable’ valve. Despite this, currently the preferred disinfection and preservation methods aim to maintain tissue viability and remain that of antibiotic disinfection followed by cryopreservation (Aduilis et al 2002) using the cryoprotectant DMSO. but, although cardiovascular tissue has been
successfully transplanted for many years (Hickey et al 2007; Oosterhaf et al, 2006; Stolinski et al 2006; Takkenberg et al), cryopreservation processes have not been optimised and exact reasons why tissue performs well post operatively have not been evaluated. This may be because results are seemingly satisfactory and hence they are not in need of significant modification and consequently, time and funding for experimental studies to make improvements are therefore not a priority. In summary therefore, even though the clinical results of viable valves cryopreserved by current methods are generally very good, (O’Brien 1995, Goffin 2000), some of the older, non viable treated tissue were similarly effective (Bodnar 1990). Studies of tissue removed at re-operation have been examined and most seem to confirm that this tissue is grossly acellular after one year (Goffin, 1997, Mitchell, 1995, Koolenberg, 2002) and yet they have performed effectively without living donor cells. It thus seems that viable cells are not required in order to ensure effective durability of transplanted tissue. The presence of viable cells may be however used as a marker for monitoring effective preservation of the extracellular matrix which is essential for long term tissue effectiveness.

4.3. Early Acute homograft ‘failures’
There was however an increasing number of reports of tissue acute failures (Wassenaar 1997) related to tissue processing and other reports of viable tissue which may have actually induce early valve failures due to fibro calcification and valvular insufficiency in some paediatric recipients, particularly infants (Clarke 1993) which is likely to have an immunologic etiology. More recent surgical reports of early failures in paediatric patients may be related to the use of ‘viable’ tissue valves which are likely to be due to immunological effects (Dignan, 2000), however, the details of the processing methods used are often not reported. It is not clear whether the same effect is seen following transplantation with valves which may be less viable (e.g. if they are treated with higher dose disinfection regimes or if the warm ischaemic times are longer). It appears that expression of HLA class I and II antigens in the donor tissue are capable of triggering the host to cellular and humoral response (Shaddy, 2002 and Welters, 2002). Blood group incompatibility between donor tissue and recipients has thus been studied to identify the mechanisms involved with some researchers concluding it does play an important
role (Christenson, 2004) and other groups finding that it has no significant influence (Askari, 2004). Modification of tissue to reduce immunogenicity is now a rapidly developing field of study (Hogan, 2003). It is likely that antigenicity of the tissue can be reduced by decellularisation of the donor tissue (Hawkins, 2003 and Elkins, 2001). Clinical application in the form of decellularised valves such as valves treated with a patented Synergraft method (Bechtel, 2008) show signs of satisfactory performance up to 5 years post operatively but further follow up of this tissue is required before assessments of durability may be justified.

4.4. Viability Assessment assays

As previously discussed, the ‘value’ of a viability assay must be considered as the key parameter for appropriate assessment of whether cells are alive or dead within a tissue and whether the function required post transplantation is actually related to the presence of living cells (Schmehl 1989 and Pegg, 1989). After consideration of the review of post operative results following treatment with tissue processed by a variety of methods, and the seemingly unconvincing arguments for the need for preservation of living cells, the most important structure that is required for long term function of the tissue post operatively is the extra cellular tissue matrix. A suitable assay must therefore monitor this by a combination of assays to be a satisfactory marker for tissue ‘viability’. Some groups have claimed that their tissue is viable (O’Brien, 1995, Wassenaar, 1997, Fischlein 1995) but upon detailed examination of their methodology it seems that they have predominantly used single methods or looked only for the presence of viable cells. Other groups have found that, post routine procurement and cryopreservation protocols, viable cells may not be preserved (Lupinetti, 1993, Motomura, 1995). The most suitable viability assay method combination which has been found in this study for human cardiovascular tissue has been described by Aidulis (2002). However, changes would have to be made to ensure that it is optimised for application to reflect routinely processed human cardiac tissue as highlighted by the UK survey in this study. The warm ischaemic times used were very unlikely to be achieved in clinical practice (5-30 minutes post death) and this would have to be extended and recorded, also the cold ischaemia would have to be
increased from that reported (100 – 180 minutes). Thawing techniques used seemed acceptable but the thawing fluid must be changed to the more commonly used isotonic fluid. The tissue in the Aidulis study was warmed by removing from nitrogen vapour storage and placing directly into a water bath. Since this is now known to be a cause of cracking damage, a mid stage hold at dry ice temperatures would have to be now included. The medium chosen to perform the phenotype identification and neutral red uptake assays would have to be changed to the currently used tissue culture Medium 199 and foetal calf serum used by Aidulis would have to be excluded since it can cause toxic effects and thus affect results. The use of samples of aortic, pulmonary, tricuspid and mitral valve leaflet and wall tissue would be required; as they are relevant for potential routine assay (mitral and tricuspid tissue would have to be used if implemented into routine practice to avoid wastage of aortic and pulmonary tissue). Aortic and pulmonary tissue would be carefully compared since they are different in composition (Gerosa, 1990). Also, baby and adult donor tissue valves would also be compared if this study were to be completed in future.
5.0. CONCLUSIONS

Despite the difficulties encountered, this study seems to have achieved most of the predetermined objectives. Three sections were completed as planned.

5.1. OVERVIEW

The introduction describes the basic principles of cryobiology which includes a description of the concepts of direct and in-direct chilling injury, extracellular and intracellular freezing injury and thawing injury. It mentions the different types of cryoprotectants, and draws attention to the affects of Cryoprotectant toxicity and the need to consider the cell membrane permeability to water and Cryoprotectant when optimising Cryoprotectant protocols. The approaches currently used for cryopreservation are discussed such as controlled slow cooling and vitrification. The difficulties of the cryopreservation of tissues is then discussed followed by recommendations for variables which ought to be taken into account in order to optimise tissue cryopreservation such as cooling and warming rate, thawing and removal of Cryoprotectant and viability of the tissue. A number of successfully cryopreserved tissues are mentioned followed by a description of the general methodology commonly used for tissue cryopreservation. Finally, future tissue cryopreservation developments are briefly discussed such as ultra rapid cooling/ rapid warming techniques of human oocyte material and vitrification of cartilage using the liquidus tracking method.

The study then explored the personal history and experience of the author taking in the experiences gained whilst working in London for heart transplant pioneers Donald Ross and Magdi Yacoub through to the clinical and research experiences developed in Oxford.

The study has then established the current National status of cardiovascular tissue banking in the UK and highlighted the issues which currently affect UK tissue banking. It gives an overview of the recently introduced UK and European regulatory framework and existing guidance for tissue banking in the UK. It reviews the National shortage of organ
donors and then, for the first time, this study establishes data which confirms that there is a National shortage of cardiovascular tissue. It also suggests why this may be the case and describes some of the methods developed by UK banks to ease the situation and the limitations. The UK professional association for tissue banking staff (‘The British Association for Tissue Banking’) and lists other associations for tissue bankers Internationally. It briefly describes the efforts made by European heart valve bankers to harmonise processing methodology and suggests reasons for the subsequent failure. The study then highlights the EU requirement for systematic validation studies and suggests that this may need to become a priority for many UK banks which do not appear to have updated and appropriately detailed validation reports. This section then concludes with a synopsis of the recent progress with respect to the professional development and career framework of staff working in UK tissue banks.

The developments in cardiovascular tissue banking are then examined beginning with a review of the origins and major advances in the use of cardiovascular valves leading to a discussion of the advantages and disadvantages of using cardiovascular valve tissue (homografts). The evolution of the disinfection and preservation methods used for homografts is clarified and then, reasons for the introduction and the concept of viable cryopreservation is then explained. Comparisons of early processing methodology or techniques were then outlined by review of literature but this was found to be imprecise and this needed to be clarified by interview of surgeons and tissue bankers with relevant experience and old processing records. Published studies which grouped together results of tissue processed by different methodologies (due to previous lack of recorded processing detail) were therefore able to be evaluated and were found to warrant significant concern in acceptance of their reported conclusions. There was found not to be enough detail in many publications to identify this, but with the added detail gained from interview and processing record examinations performed in this study, this was able to be clarified. Furthermore, this study confirmed that long term performance of tissue may only be substantiated if it is accompanied by more detailed account of the tissue
processing treatment methodologies which enables true comparison of tissue recipient patient cohorts. This was verified by the evidence found, that effective preservation methods designed to maintain the tissue viability (if actually proven), did not result in enhanced tissue durability as expected and, in some cases, may have been responsible for early valve failure in paediatric patients. It was therefore confirmed that other factors, such as warm ischaemic time, toxicity of disinfection cocktail or Cryopreservation protocols, may contribute and even be more significant in improving tissue durability.

The study then focussed upon the UK tissue banks currently processing human cardiovascular tissue for therapeutic use. All 6 UK banks and one Irish bank, collaborated with this study. The organisational structure of these banks was first considered along with the variation in the roles that these banks perform i.e. whether they are involved in all or only several of the stages of consent, procurement, processing, testing and distribution of the tissue. The status of the banks with regard to licensing by the Human Tissue Authority (HTA) was established. The inspection process was compared to that of previous UK inspectorates (Medicines and Healthcare Regulatory Agency) and of the Irish equivalent (The Irish Medicines Board) and the new initiative to monitor consistency of inspectorate authorities was discussed (‘Eustite’). A detailed review of the protocols used by each UK bank for each stage of cardiovascular tissue processing was then completed. Consent and donor selection procedures were described including the type of staff who perform these procedures and the training and guidelines they use. The differences in the types of donor heart tissue were then highlighted and reasons for the decline of the referral of living heart donors was explained. The time limits for procurement were discussed with comparison to relevant published studies concerning the effects of warm and cold ischaemic time. The donor upper and lower age limits used were confirmed and compared to reported studies written by non UK banks. The risks affecting procurement such as heart retrieval technique and infection risk during retrieval were discussed along with details of reported and unpublished data illustrating attempts made to minimise risks involved. The published and unpublished studies regarding risk of
infection due to the procurement centre environment were evaluated and the use of specialised procurement centres was debated. The importance of using sterile equipment has been taken into consideration by all UK banks and thus they predominantly use sterile, single use equipment where practicable.

All UK banks were found to have validated their transport containers and most protocols instructed use of sterile isotonic fluid in the heart packaging containers. The cardiovascular tissue bank facilities in the UK were all found to be purpose designed or adapted for use following the introduction of National and European regulations. Since cardiovascular tissue cannot be terminally sterilised, the open manipulation of the tissue following disinfection is achieved by processing inside laminar flow microbiological safety cabinets inside aseptic clean rooms, thereby achieving a Grade ‘A’ equivalent background air quality (with reference to Pharmaceutical manufacturing premises guidelines). The extent of monitoring of this air quality varies slightly between banks. The next stage of processing was found to be managed quite differently by UK banks. The protocols used for dissection followed by quality assessment and sizing and grading of the tissue, were found to be relatively vague in some UK banks. The risk the tissue being incorrectly classed as ‘acceptable for transplant’ is considerable as confirmed by surgeon satisfaction reports collected by one UK bank. This stems from the lack of subjective grading criteria and performance review of the staff. This has been highlighted by the author and a preliminary presentation of the evidence of errors in judgement between members of staff (for example up to 7mm difference in internal diameter was recorded by technicians from the same bank) and this has been analysed and briefly presented (at BATB ASM 2008). A considerable variation in tissue disinfection cocktails, exposure times and temperatures were found to be in use in UK banks, with most using the antifungal agent, Nystatin. The strength of dose and subsequent toxicity of these disinfection cocktails did not seem to have been evaluated but appeared to be considerably different. Some banks aimed to use low dose cocktails exposed at 4°C to maximise viability whilst others preferred to maximise the effectiveness of the disinfection cocktail by increasing the number of ingredients, the dose and/or the exposure time and
temperature. The rates of rejection following microbiology testing varied considerably ranging from <5% in bank 1 to another bank (bank 3) reporting a 38% rejection rate and believing this to be not a failure of disinfection but presented this as a very cautious safety measure (exposure was at 25°C and 4°C respectively).

The times and types of samples taken during in process testing varied. Two banks do not have a pre disinfection sample tested whilst others have tissue segments removed at dissection and tested prior to disinfection to identify incoming tissue contaminants. Two banks (bank 1 and 6) also test the transport fluid in which the heart is packed in order to attempt to quantify the number of incoming contaminants. The methods of the in process testing varied with most banks using enrichment broth and incubation onto blood agar plates methodology but methods were found not to have been validated to ensure that the disinfection media was removed or neutralised before testing for contaminants.

None of the antimicrobial disinfection cocktails can guarantee to decontaminate the valves since they will not be effective against all bacteria, they may be ineffective against fungi and considerably more ineffective against viruses. The more gentle cocktails (such as bank 7) or the cocktails exposed at lower temperatures (bank 3 and 7) are less likely to be effective, but are likely to be less toxic to the live cells in the tissue.

Serology testing by UK banks is similar with some banks performing more than the required mandatory testing. Banks 1 & 3 perform the additional Nucleic acid testing or polymerase chain reaction testing for HCV, HBV, HIV on all or most donors (banks 1 & 3 respectively) including repeat testing of the mother of a baby donor (bank 1). Other tests performed by some of the UK banks are vCJD testing of tonsils. Only one bank (bank 7) recommends testing of thawed tissue, the others do not because they feel that the result may be misleading.

The results of the microbiology testing are interpreted by different banks in a variety of ways. Some banks will reject all tissue from one donor if there is only one in process test result that shows positive contamination (bank 3) whilst others will accept both valves from one donor even if one of three results for both valves has positive results (bank 1 & 2).
All UK banks now use low temperature storage as their method of long term tissue preservation in order to retrieve tissue when it is referred and preserve (or ‘bank’) it until it is needed for transplantation. Although some banks believe that Cryopreservation is used to preserve living ‘viable’ cells in their tissue (banks 3, 4, 5 & 7), other banks use it primarily to ensure effective preservation of the extracellular matrix. There are many differences however, in the actual protocols used for the cryopreservation practice which is currently performed in UK tissue banks. The time limits imposed by banks between death and cryoprotection vary from 72 hours to 2 weeks (bank 4 and 2 respectively). Only one bank rinses off the disinfection media prior to cryoprotection (bank 1). All banks now use the cryoprotectant DMSO (in a nutrient media) but it is added in a variety of methods, some using a one step protocol and others using two steps (banks 2,3,6,7 and banks 1, 4 & 5 respectively). Only one bank controls the timing and incubation of the DMSO (bank 1). Most banks make up a total fixed volume of 100ml (regardless of the amount or size of the tissue) but two banks (banks 4 & 5) use total volume of 50ml to reduce the thawing time. One bank has a completely different protocol in that they cryopreserve dry in a gauze lined tray (bank 2). The tissue is packaged in different containers, some use aluminium foil pouches (banks 2, 3, 4, 5 & 7) and others use purpose designed Teflon pouches (banks 1 & 6) which are transparent which can be quality checked after packing by a second technician. All banks use a second pouch of usually the same material and an outer cardboard box for physical protection during storage. All banks except one use a Planar Biomed Kryo 10/16 programmable freezer, the other bank uses a Sylab Ice cube freezer. The cooling programs used by each bank are different but they have been found to have all originated from an application note for a controlled rate freezer written by the cryopreservation equipment manufacturer ‘Planar’. The length of the program varies from 75 minutes to 100 minutes (bank 2 and bank 7 respectively). Some banks place a dummy sachet (or pouch) in the freezer chamber during each cooling run to simulate the cooling profile that the tissue experiences and they will add this record to the tissue processing records, where as other banks will only use a dummy sachet for validation purposes (bank 2, 6 & 7). This record will then be checked each time by some banks to ensure that
the tissue has been cooled as intended (bank 1). Some programs have an equilibration stage before the start of the cooling run whilst others start cooling immediately after the tissue is loaded (banks 4, 5 & 7).

All banks aim to achieve a slow cooling rate of approximately -1 °C per minute. Only three banks (banks 1, 3 & 6) add a more rapid cooling and warming stage when the tissue is cooling between 4 to -30°C with the intention to safely remove the latent heat of crystallisation. Some banks set an end temperature at temperatures higher than -130°C (banks 2, 4 & 5 at end temperatures of -65, -110 & -110°C respectively). Other banks feel that this is too high an end temperature and tissue quality may be damaged when the tissue is subsequently transferred into liquid nitrogen vapour phase. They thus set end temperatures below the glass transition temperature at -150 °C (banks 1, 3 & 6) or at an end temperature of -180°C (bank 7). Some banks freeze up to 4 valves at one time using the same program (banks 1,2), where as other will only freeze one valve at a time and use a different program if two valves are frozen at the same time (bank 3). There seemed to be a concern amongst a few tissue bankers that cooling profiles of freezing more than one valve at a time should be reviewed to see if cooling profile varied due to the chamber being more full and thus hampering the nitrogen vapour flow. There were also concerns about the program for baby valves and whether it should be the same as for adult valves. All but one bank (bank 2) use liquid nitrogen refrigerators for long term storage of the tissue in the vapour phase. Bank 2 uses electrical -140°C refrigerators instead. In Europe and US most banks seem to store tissue submerged in the liquid nitrogen. The UK tissue bankers believe that submerging tissue in liquid nitrogen may cause more risk of cross contamination should the packaging of contaminated tissue be leaking in any way.

Most UK banks store tissue for up to 5 years despite the common belief that 10 year storage at <-140 is acceptable and the reasons they do not do this are related to the quality of the donor selection procedures of tissue following storage for >5 years (for example the donor screening and testing methods may have varied significantly). There were found to be two methods by which UK banks package their tissue for despatch to the transplant centre. Most banks package their valves in dry ice (solid
carbon dioxide pellets) at approximately -79°C but two banks routinely use a dry shipper in which there is nitrogen vapour and a temperature of -170°C for at least 5 days (bank 6 & 7). These two units only transport within a small geographical area to their networked hospitals. One bank (bank 1) uses a dry shipper for despatch over further distances if there is a possibility that the tissue may not be used (such as in the case of a patient with infective endocarditis who may be critically ill and may not survive until the operation time or may be able to have a repair operation rather than a root replacement operation).

All UK tissue banks issue an instruction leaflet for thawing their tissue which is inserted in the package with every issued valve. Their instructions are all based upon a protocol of rapid warming. The banks which supply tissue packaged in dry ice (banks 1, 2, 3, 4, & 5) will instruct transplant theatre staff to check the valve pouch for damage, then wipe or spray with alcohol and place in a water bath for 10 – 15 minutes or until ‘soft’. The banks who supply tissue in a dry shipper will add a first stage warming on dry ice (or at approximately -79°C) to minimise risks of fractures due to excessive warming rates. Most banks will then instruct that serial dilution of the cryoprotectant is then performed using two or more steps. Two banks will however use only one step fast dilution in order to speed up the thawing time for the surgeon (banks 4 & 5). Only one bank has monitored the amount of DMSO remaining after thawing (bank 1) and this suggests that more than 30 minutes may be needed to ensure satisfactory dilution of DMSO. No UK bank seems to have systematically optimised their re-warming and serial dilution protocol.

Most tissue is used within one hour of thawing; however, when operations are postponed or the patient does not survive to surgery, the tissue may have to be kept temporarily until re-operation is scheduled or until a different transplant recipient is selected. In the UK, if human tissue is stored in a surgical centre for > 48hrs, then the centre where the tissue is actually stored must be licensed by the Human Tissue Authority.

The study looked at the advice UK banks give to surgical centres for storage after re-warming and Cryoprotectant dilution which was quite different (this ranged from keeping the tissue cool for 1-24 hours) and there seemed to be little evidence base for this. Tissue which had not been thawed was recommended by all UK banks to be kept for
no more than 3 months at -80°C which reflected old BATB standards which were again not evidence based. Return policies of UK tissue banks were found to be very different ranging from those not wanting to accept returned valves at all to those who will accept them all provided a dry shipper is used. There were found to be systems for recall of tissue in operation in all UK banks.

The study then progressed to looking at the quality assessment protocols in place in the within the processing regime of cardiovascular tissue in UK banks. Following a review of quality assessment assay methods used generally, the current procedures used in the UK were rated in terms of assessment ‘value’. Assessments were found to be limited to gross appearance checks and grading after dissection which appeared to be inconsistent in most banks and physical integrity measured by relatively competence fill testing. Only one bank performs routine histology testing. No end stage quality assessment methods are currently in place. Mechanical testing and tissue culture methods have been used but only for validation of changes to protocols.

Postoperative data has recently been studied by one UK bank but not yet published. Older studies, co-ordinated by surgeons rather than tissue bankers, appeared to have unknowingly grouped together tissue treated with different types of processing methodology and may therefore have been misleading. Explanted tissue studies have been performed in EU banks but only one bank was collecting data to produce a report at the time of this study.

It was more difficult to establish the current status of validation protocols written by UK banks despite the EUTCD requirements. Many validation reports appeared to be absent or of limited detail and this may be why some banks did not seem to want to disclose them. Existing reports seemed to be of simple procedures and most were unpublished documents. Durability (or long term clinical performance) of the UK cardiovascular tissue had not been validated or even systematically monitored.

Interviews with UK tissue bankers led to the identification of areas of processing practice where there is some concern and this is typically related to the areas where there are differences in UK practice. In these areas, UK tissue banks are keen to work towards
harmonisation. However, this study also highlighted a perceived lack of understanding of some of the concepts of cryopreservation and also a reluctance to change processes whilst the long term follow up studies (although possibly misleading) seem to suggest that the tissue durability is acceptable. This seems to be disputed however, by surgeons operating on paediatric patients with viable tissue valves, who report that there are an increasing number of early valve failures or degenerated valves (Clarke, 1993 and Dignan, 2000).

5.2. SUMMARY

This study has involved research into areas of cardiovascular tissue banking that have previously not been clarified, explored in detail or reported.

The study has identified the pressures which have been the driving forces for changes in tissue banking in recent years (such as implementation of a new regulatory framework) and has, for the first time, quantified the National shortage of cardiovascular tissue donors in relation to the increasing surgical demand.

The study has described the evolution of cardiovascular tissue banking and identified that there are inconsistencies reported regarding which tissue treatment seems to be most effective. A main source of confusion has been found to be that researchers in their reported studies, have not separated into different groups, their tissue which has been treated by different processing methods for post operative follow up reporting. This minimises the accuracy of the results and led to misleading conclusions (e.g. for irradiated and freeze dried homografts). The study identified, from direct review of old processing records, that there had been incomplete recording of the actual processing methods used (e.g. it could not always be established which of the London valves were flash frozen because it was not routinely documented in the donor records).

The study confirmed that there is no one method by which the quality of cardiovascular tissue could be measured. This obviously will hamper future optimisation studies. It was confirmed by review of literature, processing documentation and interview with tissue bankers and surgeons, that many different methods had been used in the past claiming
that the presence of living cells (or ‘viability’) should be the best quality marker for cardiovascular tissue. However, this study has shown that it is now evident that, since living cells are not present in functionally satisfactory tissue (from explant studies), the presence of living viable cells are not a pre requisite for tissue durability. Methods were therefore recommended in this study to assess quality by means of markers which monitor the effectiveness of the preservation of the extra cellular tissue matrix. It was however, confirmed that the best method of studying the effective performance of similarly processed tissue is to monitor the durability of each group in the recipients post transplantation.

Thanks to the remarkable co-operation of the UK cardiovascular tissue bankers, a detailed overview of current methodology was able to be collated which, for the first time, has established the similarities and differences in current processing practice in the UK. The study has subsequently facilitated exploration of tissue bankers concerns and lack of awareness of cryopreservation principles.

The study confirmed that there are significant and subtle differences in the processing techniques and that the processes used were indeed generally empirically based. However, as a result of the UK tissue banks vigilance in standardisation of protocols and ensuring these protocols are used at all times, tissue seems to be currently processed in a consistent manner which appears to produce tissue with satisfactory durability. There seems to be sufficient evidence, however, for a need to control, standardise and improve some of the techniques used, and this maybe achieved by in-house validation or group experimental study.
Figure 20.a. Cooling rate profile of cardiovascular tissue processed in one UK bank which is deemed to be acceptable – blue line represents the cooling of the dummy sachet and is a smooth curve as expected.
Figure 20.b. Unusual cooling rate profile of cardiovascular tissue processed in one UK bank – the blue line represents the cooling profile of the dummy sachet which is not a smooth curve as expected.
5.3. FUTURE WORK

Initial feedback has been given to the BATB CV SIG members who participated in this study and to the surgeons and tissue bankers interviewed. Pilot results have been presented at the BATB/EATB.AATB scientific meeting in 2008 and, as a result, a large number of tissue bankers from other non-UK banks have agreed to participate in the study and are keen to complete questionnaires, send protocols and participate in interviews as necessary. Cardiovascular tissue bankers in Europe, US, Australia, South Africa and Brazil will soon be joining the study.

This study may then be extended to highlight where there are differences in processing practice internationally, which should enable recording of the small differences in processing. This may enhance the accuracy of reported post operative studies which could then avoid confusion in the analysis of processing methodology which has been found to have detrimentally influenced changes in practice in the past (Bodnar, 1990)

Since this study has analysed the arguments for and against maintaining tissue ‘viability’ or the presence of living cells and concluded that living cells may not be required or beneficial for long term tissue durability, the future studies aim to monitor the preservation of the extra cellular tissue matrix. The study has however shown that the perceived viability of each banks valves is often misinterpreted (e.g. some banks believe they have viable valves even if a high dose disinfection medium is used at room temperatures or above). Furthermore, viability may vary within the tissue in each bank (e.g. with tissue from donors with long or short warm ischaemic times) and this is not currently recognised. The study has therefore made recommendations for methods which may be best used for viability assessment validation assays and schemes for cardiovascular tissue. All participants (current and future) have agreed to work together to finalise the design of this recommended series of quality assessment assays which is hoped will be used to identify
more relevant functional characteristics required in cardiac tissue for long term performance, without the confusion of mixing up the different types of processed tissue. These assays will then be used in experimental studies to optimise processing techniques and to improve the understanding of areas of processing where there has been found to be currently no systematic evidence base validation. These experiments may ultimately be grouped together to optimise the stages of cardiovascular tissue processing where there have found to be areas of concern. Also, it has been agreed that, future research and developmental projects may be jointly commissioned and funded to alleviate cost and time constraints. It has also been agreed that research tissue from each bank will be pooled for use in future projects. The lack of availability of human tissue donated in the UK for research means that pooling tissues from all participating banks which have been treated with similar processing techniques will help to build up numbers of critically needed experimental tissue.

Since the demand for tissue outweighs the availability of tissues internationally, tissue is currently imported and exported as required. In order to minimise the risk of cross-border transmission of infection, harmonisation of the methodology used will be beneficial to clarify the processing treatment that has been used, assess the ‘viability’ (e.g. has it been treated with high or low dose disinfection media) of the tissue and ultimately improve the quality and safety and long term performance of clinical heart valve tissue used in transplantation. Therefore the next stage would be to encourage tissue banks to ‘harmonise’ (or standardise) processing, at first within the UK. Discussions at CV SIG meetings and BATB/EATB/AATB meetings have already confirmed that the issues that still concern UK cardiovascular banks are similarly held in non-UK banks. The study will also enable more accurate long term recipient patient follow up by clarifying the differences in tissue processing treatment and ensuring that the tissue is grouped accordingly and using tissue from each bank participating in the harmonisation phase to increase numbers of patients in each cohort.
Completion of the study of the ‘flash frozen’ cohort of tissue processed in London which appeared to be much better when briefly analysed in this study than that which had been published (due to grouping with other processed tissue) will be performed by further detailed inspection of old processing records. This data will then be compared to existing published frozen tissue data.

A more detailed study of the current demand of frozen tissue will be analysed, as it appears that some paediatric surgeons are switching to use of porcine or bovine temporary alternative conduits (Hickey, 2008) or decellularised valves (Betchel, 2004) instead of human tissue due to the reported early acute failures observed by a minority of surgeon operating with viable tissue in paediatric patients. If it is the case that the demand is decreasing for this type of recipient, then detailed investigation of the ‘true’ viability of the tissue is to be assessed. This tissue can then be compared to other non viable tissue and if results are as expected, recommendations can then be made for tissue bankers to allocate only non viable valves to such recipients.

Finally, work is underway currently with a team currently working with decellularised human tissue (‘the Vesalio project’) to compare viable, non viable and decellularised tissue experimentally, and post human implantation. This may help to clarify unresolved issues such as whether completely non viable human tissue can be expected to perform satisfactorily after implantation, and, if so, for how long. If completely non viable tissue is found to be durable, then processing techniques may ultimately be simplified such as by changing from cryopreservation (which requires expensive equipment and nitrogen) to a more convenient immersion in 85% glycerol and stored at room temperature (Aidulis, 2002). Confirmation that non viable tissue is durable (if this can be confirmed) would enable a change from gentle microbial disinfection to more rigorous chemical treatment such as immersion in peracetic acid (Farrington, 2002) which would help to minimise risk of transmission of contamination from donor tissue to recipients and help to maximise patient safety.
5.4. REFLECTIVE NOTES

On reflection, one of the main changes that may have improved this study would have been to alter the questionnaires that were sent out to each participant. They may have been more informative in the details in each question, for example, it may have been more appropriate to use questions such as ‘explain how and when you take samples for in process testing?’ rather than ask ‘do you do a transport fluid qualitative test?’ in order to ensure that the participants fully understood the question. This also applies to the questionnaires relating to quality assessment and viability assays, where it later transpired that several of those surveyed were unsure of the types of assays which were measured in terms of ‘metabolic activity’ or ‘mitotic activity’.

Some of the questionnaires may have been improved by asking for more detail regarding some of the processing areas examined. The questionnaire collating the details of the disinfection cocktail used ideally, in hindsight, should have asked for doses of each ingredient, so that the ‘viability’ of the cocktail could be determined. Some of the questions asked for time limits (to retrieval, to dissection for cryopreservation), and it may have been prudent to explain what exactly was required such as time from stopping of the heart to time placing it in cooled transport fluid or time to start of the cooling program. Terms such as ‘warm ischaemia’ and ‘cold ischaemia’ to describe these time limits were not used initially because they have been misinterpreted in the past. The difficulties in gaining the data about validation methodology and issues of concern may have been eased by means of interviews on site with the banks in question in order to encourage them to be more forthcoming about their lack of protocols and/or understanding of principles of cryopreservation.
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PRESENTATIONS AND PUBLICATIONS

1. PRESENTATIONS


1. Davies, J. UK Cardiovascular tissue processing – an overview (presented at the BATB Annual Scientific meeting 2007)
2. Davies, J., Zhang, T., Fuller, B., and Pillai, R. To perform a pilot study to develop a quality assessment protocol for optimisation studies and for routine post cryopreservation use in cardiovascular tissue banking (presented at the SLTB annual meeting 2008)
5. Davies, J., Zhang, T., Fuller, B., and Pillai, R. To identify, Validate and apply a quality assessment protocol for routine use in Cardiovascular Tissue Banking (presented at the BATB/EATB 2008)

2. PUBLICATIONS (& IN PRESS ABSTRACTS)

ABSTRACTS ACCEPTED BY THE CELL AND TISSUE BANKING JOURNAL FOLLOWING PRESENTATION AT THE BATB/EATB CONGRESS 2008

1. Davies, J., Survey of UK Cardiovascular Tissue Banking (in press)
3. Davies, J., To identify, Validate and apply a quality assessment protocol for routine use in Cardiovascular Tissue Banking (in press)

ABSTRACT ACCEPTED BY THE CRYOLETTERS JOURNAL FOLLOWING PRESENTATION AT THE SLTB CONGRESS 2008

1. Davies, J., Zhang, T., Fuller, B., and Pillai, R. To perform a pilot study to develop a quality assessment protocol for optimisation studies and for routine post cryopreservation use in cardiovascular tissue banking

ABSTRACTS PUBLISHED IN THE CELL AND TISSUE BANKING JOURNAL FOLLOWING PRESENTATION AT THE BATB/EATB CONGRESS 2007

To perform a pilot study to develop a quality assessment protocol for optimisation studies and for routine post preservation use in Cardiovascular Tissue Banking.

Presented at SLTB 2008

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Background

It was confirmed that current cryopreservation methodology is variable, quality assessment methods are limited and showed that there is a need for standardisation and further experimental study. An assessment procedure was chosen and adapted for optimisation studies and for routine use post cryopreservation. It was then validated with porcine cardiovascular tissue and applied to assess difference in relative quality of a selection of human tissue specimens. Analysis of data was made using statistical student t tests.

Results

Assay selection criteria in this study were based upon parameters such as ease, speed, cost, sensitivity and whether it was non-destructive. On this basis, Resazurin metabolic assay was chosen as an indicator for tissue integrity. Assay validation studies produced acceptable results when analysed both spectrophotometrically and fluorometrically. Sample contamination and difficulty producing positive and negative controls were the main obstacles. Tissue sample results seemed to be more sensitive but different to porcine results. Assays were performed with a range of specimens such as baby and adult donors, valves which had been treated with/without antibiotics, valves frozen once and stored at different times and temperatures.

Conclusions

It seemed encouraging since they reflected expected viability trends. However, since there is a shortage of available donor tissue for research, not enough grouped samples were used and so further work must be completed to verify the suitability for use in the intended applications.
Abstract

Aim
To collate, compare and contrast, cardiovascular tissue banking methodology currently used within the UK.

Method
BATB cardiovascular special Interest group members agreed to participate in the review of UK tissue processing practice. A series of questionnaires followed up by written and verbal communication enabled details of all stages of processing to be collated, analysed and tabulated or graphically represented. Standard operating procedures, product information forms, instructions for implanting surgeons, freezing program lists and graphs were also collected. Data from banks outside UK were also forwarded for inclusion in the survey.

Results
There were many similarities in current practice – all UK banks retrieve tissue within a maximum of 48 hours and dissect within 72 hours, disinfect with an antimicrobial cocktail, cryopreserve using dimethyl sulphoxide and store in vapour phase nitrogen at <-140°C. Also, most banks use similar donor selection criteria. However many variations in practice were found, some of which may be significant. There are different antimicrobial disinfectant media in use. Microbiological sampling methodology varies between banks in terms of timing and type of sample used and also in protocol of testing regime. The rejection criteria and the ultimate rates of rejected contaminated tissue varied considerably. Cryoprotection and controlled freezing protocols also varied markedly. There also seemed to be at least two significant variations in re-warming and cryoprotectant dilution protocols.

Conclusions
Thanks to the remarkable co-operation of the UK cardiovascular tissue bankers, a detailed overview of current methodology was able to be collated: The survey has subsequently facilitated exploration of tissue bankers concerns and identified areas where there is thought to be a need for standardisation of methodology, more detailed/updated experimental research and even areas where staff performance assessment may be indicated. It is hoped that it will help to encourage standardisation of cardiovascular banking within the UK and thus enhance safety of implantable tissue. It has been agreed that the survey scope should now be widened to include details from European, American and other tissue banks.
Title  Oxford Heart Valve Bank Disinfection Media Validation Study

Abstract Text

• Aim
To validate the efficacy of the disinfection solution prepared by Oxford Heart Valve Bank (OHVB).

Method
A review of two years results of predisinfection tissue samples was performed to establish the genus of organisms most typically recovered. The optimal incubation conditions were identified for each constituent ingredient of the disinfection media.

The efficacy of disinfection media was assessed according to methods described in the British Pharmacopoeia 2008 (BP 2008) and European Pharmacopoeia 6th edition 2007 (Ph EUR).

In the validation control simulation (method E), 1ml of disinfection media was injected into blood culture bottles followed by an inoculation of 10-100 cfu/ml. The control simulation (method F) using Tryptone Soya broth involved membrane filtration of 180ml of disinfection media followed by inoculation of 10-100 cfu/ml of the same genus onto the filter, which was then subsequently placed onto Tryptone Soya broth.

The antibiotic challenge simulation (methods G & H) was then performed by inoculating 7 individual aliquots of 180ml disinfection media with 10⁶ cfu/ml. These aliquots were incubated under the optimised conditions (i.e. 23 ± 2°C for 18 ± 2 hours) and then tested as previously described.

• Results
All organisms inoculated in method E were successfully enumerated and identified but none were recovered following method F. Further study found that this was most likely due to the residual antibiotics masking the recovery of the low level bioburden of CFU’s. This simulation method was repeated with the inclusion of a rinse of the membrane filter after the disinfection media filtration and prior to inoculation, to wash away residual antibiotics. Results showed that 3 x 100ml rinses of buffered peptone water enabled only 51 – 54% recovery of organisms and 5 x 100mls rinses were required to facilitate >91% recovery of all organisms (>70% recovery is sufficient to confirm validity1-3).

The results of the antibiotic challenge methods G & H both showed that all 7 aliquots had negative growth which indicated a >10⁶ cfu/ml reduction in bioburden.

• Conclusions
The OHVB disinfection media is effective to reduce bioburden levels of ≤10⁶ cfu/ml.

References
Title  
To identify, validate and apply a quality assessment protocol for routine use in Cardiovascular Tissue Banking

Abstract Text

• Aim
To choose an appropriate quality assessment protocol and then validate and apply it to routine cardiovascular tissue banking practice.

Method
Data was collated to establish which quality assessment methods are currently in routine use in UK cardiovascular tissue banks. A review of published articles identified other methods used including those used in combination with other protocols.
The method chosen and equipment required was then modified for use. It was then validated with porcine cardiovascular tissue and applied to assess human non-clinical tissue specimens. Analysis of data was made using statistical student t tests.

• Results
Quality assessment methods in routine use typically consists of physical inspection methods, competency and some histology testing. Combinations of extensive quality methods used in the past for validation of processes included viability testing, physical stress/strain measurement and post-operative tissue performance studies.
The selection criteria for the assay to be used in this study was based upon parameters such as ease, speed, cost, sensitivity and whether it is non-destructive. On this basis, Resazurin metabolic assay was ultimately chosen as an indicator for tissue integrity. Although this viability assay had not been used for cardiovascular tissue, it had been successfully applied to corneas.
Porcine tissue validation studies produced acceptable results when analysed both spectrophotometrically and fluorometrically. However there were issues with contaminated samples and positive and negative controls.
Human tissue sample results seemed to be more sensitive but different to porcine results. Assays were performed with groups of specimens such as baby and adult donors, valves which had been treated with/without antibiotics, valves frozen more than once and stored at different times and temperatures.

• Conclusions
It was found possible to apply the resazurin assay for routine use and it appeared to be sensitive since, when used for similar human tissue specimens, it produced expected viability trends. However, since there is a shortage of available donor tissue for research, not enough human samples were used and so further work must be completed to verify the assays suitability for use in this application.
<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time limit to retrieval</strong></td>
<td>&lt; 48 hrs</td>
<td>&lt; 72 hrs</td>
<td>&lt; 48 hrs</td>
<td>&lt; 48 hrs</td>
<td>Same as 4.</td>
<td>&lt; 48 hrs</td>
<td>Paediatric: &lt; 12 hrs Adults: multi organ donors</td>
<td>&lt; 24 hrs</td>
</tr>
<tr>
<td><strong>Transport solution</strong></td>
<td>Buffered isotonic solution</td>
<td>Isotonic solution</td>
<td>N/A -- transported dry, wrapped in Hydrex-soaked gauze</td>
<td>Hartmans</td>
<td>Same as 4.</td>
<td>Saline</td>
<td>N/A (Dissected at multi organ donation)</td>
<td>Ringers solution</td>
</tr>
<tr>
<td><strong>Time limit to dissection</strong></td>
<td>&lt; 72 hrs</td>
<td>&lt; 72 hrs</td>
<td>24 hrs after removal</td>
<td>&lt; 72 hrs</td>
<td>Same as 4.</td>
<td>24 hrs after removal</td>
<td>Dissected in theatre at multi organ donation</td>
<td>&lt;40 hrs</td>
</tr>
<tr>
<td><strong>Rinse solution during dissection</strong></td>
<td>Buffered isotonic solution</td>
<td>Isotonic solution</td>
<td>Buffered isotonic solution</td>
<td>Hartmans</td>
<td>Same as 4.</td>
<td>Tested from original solution</td>
<td>N/A</td>
<td>Medium 199</td>
</tr>
</tbody>
</table>

Comparison of Procurement, Transport & Dissection of Cardiovascular Tissue
<table>
<thead>
<tr>
<th>Bank</th>
<th>Do you do a Transport Fluid qualitative test?</th>
<th>Do you do a pre disinfection test on tissue segment?</th>
<th>Do you do a post disinfection test on tissue segment?</th>
<th>Do you do Cryoprotection test on unfrozen tissue sample?</th>
<th>Do you routinely keep an archive sample of tissue?</th>
<th>Do you do a routine histology test on tissue segment?</th>
<th>Do you do any other tests with tissue?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>No</td>
<td>no</td>
</tr>
<tr>
<td>9</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>Yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

Types & Sampling Intervals of Microbial testing of cardiovascular tissue
<table>
<thead>
<tr>
<th>Stored in</th>
<th>Bank 1</th>
<th>Bank 2</th>
<th>Bank 3</th>
<th>Bank 4 &amp; 5</th>
<th>Bank 6</th>
<th>Bank 7</th>
<th>Bank 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid nitrogen vapour phase @ approx -170°C Or Electric freezer @ -80°C</td>
<td>Electric freezer @ -130 +/- 10°C</td>
<td>Vapour phase nitrogen</td>
<td>Liquid nitrogen vapour phase @ -170°C approx</td>
<td>Liquid nitrogen vapour phase @ approx -170°C</td>
<td>Liquid nitrogen vapour phase @ approx -170°C approx</td>
<td>Liquid nitrogen vapour phase @ approx -170°C approx</td>
<td></td>
</tr>
<tr>
<td>Maximum storage time</td>
<td>5 yrs</td>
<td>10 yrs in nitrogen storage</td>
<td>5 yrs</td>
<td>5 yrs</td>
<td>5 yrs</td>
<td>5 yrs</td>
<td>5 yrs</td>
</tr>
<tr>
<td>Or 3 months @ -80°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Packaging for transport</td>
<td>Dry ice</td>
<td>Dry ice</td>
<td>Dry Ice</td>
<td>Dry shipper within local area</td>
<td>Dry shipper</td>
<td>Dry ice</td>
<td></td>
</tr>
<tr>
<td>Or Thawed and stored @ 4°C (for own theatre)</td>
<td></td>
<td></td>
<td></td>
<td>Outside area in dry ice</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Preservation & Despatch procedures for cardiovascular tissue*
<table>
<thead>
<tr>
<th>Bank</th>
<th>Decontamination cocktail used</th>
<th>Incubation Temperature</th>
<th>Time of Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ticarcillin, Polymixin B Sulphate, Cefuroxime, Amikacin, Vancomycin, Nystatin, made up in Hepes buffered Medium 199</td>
<td>25°C</td>
<td>18 hours</td>
</tr>
<tr>
<td>2</td>
<td>Cefuroxime, Gentamycin, Ciprofloxacin, Colistin, Vancomycin, Amphotericin made up in Medium 199</td>
<td>Room Temperature</td>
<td>24 hours</td>
</tr>
<tr>
<td>3</td>
<td>Imipenem, Gentamycin, Polymixin B Sulphate, Vancomycin, Nystatin made up in Hepes buffered Medium 199 (Commercially manufactured media)</td>
<td>4°C</td>
<td>24 hours</td>
</tr>
<tr>
<td>4</td>
<td>Imipenem, Gentamycin, Polymixin B Sulphate, Vancomycin, Nystatin made up in Hepes buffered Medium 199 (Commercially manufactured media)</td>
<td>22°C</td>
<td>21-23 hours</td>
</tr>
<tr>
<td>5</td>
<td>Imipenem, Gentamycin, Polymixin B Sulphate, Vancomycin, Nystatin made up in Hepes buffered Medium 199 (Commercially manufactured media)</td>
<td>22°C</td>
<td>21-23 hours</td>
</tr>
<tr>
<td>6</td>
<td>Imipenem, Gentamycin, Polymixin B sulphate, Vancomycin, Nystatin made up in Hepes buffered Medium 199 (Commercially manufactured media)</td>
<td>37°C</td>
<td>24 hours</td>
</tr>
<tr>
<td>7</td>
<td>Metranidazole, Gentamycin and Fluocaxicillen made up in normal saline</td>
<td>4°C</td>
<td>Minimum 12 hours</td>
</tr>
<tr>
<td>8</td>
<td>Metranidazole, Gentamycin, Fluocaxicillen made up in normal saline</td>
<td>0-10°C</td>
<td>18-24 hours</td>
</tr>
</tbody>
</table>

Comparison of disinfection Media of Cardiovascular Tissue
**Thawing, Removal & Dilution of Cryoprotectant procedures**

<table>
<thead>
<tr>
<th>Bank 1</th>
<th>Bank 2</th>
<th>Bank 3</th>
<th>Bank 4 &amp; 5</th>
<th>Bank 6</th>
<th>Bank 7</th>
<th>Bank 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. unopened in water bath @ 37°C until 10 mins</td>
<td>1. unopened in water bath @ 37°C until pack is soft.</td>
<td>1. unopened in water bath @ 37°C until pack is soft.</td>
<td>1. unopened in water bath @ 37°C until pack is soft.</td>
<td>1. unopened in water bath @ 37°C until pack is soft.</td>
<td>1. unopened in water bath @ 37°C until pack is soft.</td>
<td></td>
</tr>
<tr>
<td>3. add further 200mls saline for 5 mins</td>
<td>3. Empty contents into sterile bowl. Add 100ml sterile saline. Leave for 5 mins.</td>
<td>3. Empty contents into sterile bowl. Add 100ml sterile saline. Leave for 5 mins.</td>
<td>3. Empty contents into sterile bowl. Add 100ml sterile saline. Leave for 5 mins.</td>
<td>3. Empty contents into sterile bowl. Add 100ml sterile saline. Leave for 5 mins.</td>
<td>3. Empty contents into sterile bowl. Add 100ml sterile saline. Leave for 5 mins.</td>
<td></td>
</tr>
<tr>
<td>4. add further 400mls saline for 5 mins</td>
<td>4. Add a further 200mls sterile saline. Leave for 5 mins.</td>
<td>4. Add a further 200mls sterile saline. Leave for 5 mins.</td>
<td>4. Add a further 200mls sterile saline. Leave for 5 mins.</td>
<td>4. Add a further 200mls sterile saline. Leave for 5 mins.</td>
<td>4. Add a further 200mls sterile saline. Leave for 5 mins.</td>
<td></td>
</tr>
<tr>
<td>5. transfer to new container</td>
<td>5. Transfer to a fresh bowl and rinse in further saline.</td>
<td>5. Transfer to a fresh bowl and rinse in further saline.</td>
<td>5. Transfer to a fresh bowl and rinse in further saline.</td>
<td>5. Transfer to a fresh bowl and rinse in further saline.</td>
<td>5. Transfer to a fresh bowl and rinse in further saline.</td>
<td></td>
</tr>
<tr>
<td>7. If graft is not used immediately, cover basin in a sterile drape and cool to 4°C. Keep moist until implanted. Use within 1 hour.</td>
<td>6. Remove valve after further 3 minutes</td>
<td>6. Remove valve after further 3 minutes</td>
<td>6. Remove valve after further 3 minutes</td>
<td>6. Remove valve after further 3 minutes</td>
<td>6. Remove valve after further 3 minutes</td>
<td></td>
</tr>
</tbody>
</table>

**Thawing, Removal & Dilution of Cryoprotectant**

- **Protocol** (from dry ice)
  1. Bank 1: unopened in water bath @ 37°C until pack is soft.
  3. Bank 3: Empty contents into sterile bowl. Add 100ml sterile saline. Leave for 5 mins.
  5. Bank 6: Transfer to a fresh bowl and rinse in further saline.
  6. Bank 7: If graft is not used immediately, cover basin in a sterile drape and cool to 4°C. Keep moist until implanted. Use within 1 hour.
  7. Bank 8: Remove valve after further 3 minutes.

---

**Thawing, Removal & Dilution of Cryoprotectant**

- **Protocol** (from dry ice)
  1. Bank 1: unopened in water bath @ 37°C until pack is soft.
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  7. Bank 8: Remove valve after further 3 minutes.

---

**Thawing, Removal & Dilution of Cryoprotectant**

- **Protocol** (from dry ice)
  1. Bank 1: unopened in water bath @ 37°C until pack is soft.
  3. Bank 3: Empty contents into sterile bowl. Add 100ml sterile saline. Leave for 5 mins.
  5. Bank 6: Transfer to a fresh bowl and rinse in further saline.
  6. Bank 7: If graft is not used immediately, cover basin in a sterile drape and cool to 4°C. Keep moist until implanted. Use within 1 hour.
  7. Bank 8: Remove valve after further 3 minutes.
<table>
<thead>
<tr>
<th>Bank 1</th>
<th>Bank 2</th>
<th>Bank 3</th>
<th>Bank 4 &amp; 5</th>
<th>Bank 6</th>
<th>Bank 7</th>
<th>Bank 8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Max storage after thawing</strong></td>
<td>Use within 4 hrs @ 4°C</td>
<td>Use within 24 hrs @ 4°C</td>
<td>Use within 1 hr @ 4°C</td>
<td>Use within 4 hrs @ 4°C</td>
<td>Within 2 hrs @ 4°C</td>
<td>Use within 4-6 hrs @ 4°C</td>
</tr>
<tr>
<td><strong>Max storage if tissue is still frozen</strong></td>
<td>In -80°C freezer, use within 3 months</td>
<td>In Standard freezer, use within 1 week</td>
<td>In -80°C freezer, use within 6 months</td>
<td>In -130°C freezer or in liquid nitrogen vapour phase, use within 5 years</td>
<td>-80°C until date of expiry on label.</td>
<td>In -80°C freezer, use within 3 months</td>
</tr>
<tr>
<td><strong>Are returns accepted</strong></td>
<td>Rarely accepted but ok if prearranged with bank and security tagged packaging or data loggers used or dry shipper.</td>
<td>No returns except local area</td>
<td>No returns except local hospital</td>
<td>Yes if data logger used</td>
<td>Yes in dry shipper</td>
<td>No</td>
</tr>
<tr>
<td><strong>Is tissue ever put back into storage @ &lt; -130°C after transport in dry ice</strong></td>
<td>Yes or into -80°C Freezer and use within 3 months</td>
<td>Yes</td>
<td>No.</td>
<td>Yes</td>
<td>No</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Storage & Return of Cardiovascular tissue Comparison
transplantation |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reviews</td>
<td>Microbiology</td>
<td>Light microscopy</td>
<td>Electron microscopy</td>
<td>Membrane transport</td>
<td>Tissue culture</td>
</tr>
<tr>
<td>1. QA check (external &amp; internal) at dissection</td>
<td>Competency test</td>
<td>histology and when required</td>
<td>only in old rejection protocols</td>
<td>Unusual attached lesions reported in OAI NHH cardiac surgery articles</td>
<td>OAI NHH cardiac surgery articles</td>
</tr>
<tr>
<td>2. QA check (external &amp; internal) at dissection</td>
<td>Competency test</td>
<td>histology and when required</td>
<td>only in old rejection protocols</td>
<td>Unusual attached lesions reported in student theses</td>
<td>only early (30 day) pre-op follow up &amp; adverse incident reports</td>
</tr>
<tr>
<td>3. QA check (external &amp; internal) at dissection</td>
<td>Competency test</td>
<td>histology and when required</td>
<td>only in old rejection protocols</td>
<td>Unusual attached lesions reported in student theses</td>
<td>only early (30 day) pre-op follow up &amp; adverse incident reports</td>
</tr>
<tr>
<td>4. QA check (external &amp; internal) at dissection</td>
<td>Competency test</td>
<td>histology and when required</td>
<td>only in old rejection protocols</td>
<td>Unusual attached lesions reported in student theses</td>
<td>only early (30 day) pre-op follow up &amp; adverse incident reports</td>
</tr>
<tr>
<td>5. QA check (external &amp; internal) at dissection</td>
<td>Competency test</td>
<td>histology and when required</td>
<td>only in old rejection protocols</td>
<td>Unusual attached lesions reported in student theses</td>
<td>only early (30 day) pre-op follow up &amp; adverse incident reports</td>
</tr>
<tr>
<td>6. QA check (external &amp; internal) at dissection</td>
<td>Competency test</td>
<td>histology and when required</td>
<td>only in old rejection protocols</td>
<td>Unusual attached lesions reported in student theses</td>
<td>only early (30 day) pre-op follow up &amp; adverse incident reports</td>
</tr>
<tr>
<td>7. QA check (external &amp; internal) at dissection</td>
<td>Competency test</td>
<td>histology and when required</td>
<td>only in old rejection protocols</td>
<td>Unusual attached lesions reported in student theses</td>
<td>only early (30 day) pre-op follow up &amp; adverse incident reports</td>
</tr>
<tr>
<td>8. QA check (external &amp; internal) at dissection</td>
<td>Competency test</td>
<td>histology on well segments</td>
<td>only in old rejection protocols</td>
<td>Unusual attached lesions reported in student theses</td>
<td>only early (30 day) pre-op follow up &amp; adverse incident reports</td>
</tr>
<tr>
<td>9. QA check (external &amp; internal) at dissection</td>
<td>Competency test</td>
<td>histology on well segments</td>
<td>only in old rejection protocols</td>
<td>Unusual attached lesions reported in student theses</td>
<td>only early (30 day) pre-op follow up &amp; adverse incident reports</td>
</tr>
</tbody>
</table>

QA & Validation Procedures performed in cardiovascular tissue banks
<table>
<thead>
<tr>
<th>Cryoprotection, Packaging &amp; Cooling</th>
<th>Bank 1</th>
<th>Bank 2</th>
<th>Bank 3</th>
<th>Bank 4 &amp; 5</th>
<th>Bank 6</th>
<th>Bank 7</th>
<th>Bank 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time limit for cryopreservation</td>
<td>&lt; 96 hrs after death</td>
<td>1-2 weeks</td>
<td>24 hrs after disinfection</td>
<td>&lt; 96 hrs</td>
<td>24 hrs after disinfection</td>
<td>&lt;72 hrs</td>
<td>&lt;48 hrs</td>
</tr>
<tr>
<td>Solution for media Rinse</td>
<td>n/a</td>
<td>n/a</td>
<td>Buffered isotonic solution</td>
<td>Sigma rinse solution</td>
<td>Saline</td>
<td>RPMI</td>
<td>N/a</td>
</tr>
<tr>
<td>Cryo media</td>
<td>Hanks and DMSO (10%/w/w)</td>
<td>DMSO (10%/w/w) or glycerol when DMSO not available</td>
<td>10% DMSO in Hanks</td>
<td>DMSO &amp; Hanks (Sigma)</td>
<td>10% DMSO in RPMI 16/40</td>
<td>10% DMSO in RPMI</td>
<td>DMSO 10%/v/v</td>
</tr>
<tr>
<td>Method of adding cryoprotectant</td>
<td>Drop by drop addition (in 4 mins) to 16% and then 2nd stage one step dilution to 10% DMSO</td>
<td>One stage</td>
<td>One stage</td>
<td>Two stages, 50ml total volume, pour out solution</td>
<td>One stage</td>
<td>Open inner bag, drop straight into 100mls</td>
<td>One stage</td>
</tr>
<tr>
<td>Total amount of cryo media added</td>
<td>Total 100ml (Valve and cryomedia)</td>
<td>No cryomedia added</td>
<td>100ml</td>
<td>50 ml cryomedia</td>
<td>Depends on size of valve</td>
<td>Approx 100mls</td>
<td>Total 90 ml (Valve and cryomedia)</td>
</tr>
<tr>
<td>Type of packaging</td>
<td>Two Teflon pouch</td>
<td>Gauze tray, nylon tube, tri aluminate sachet</td>
<td>Fresenius Hemofreeze bags (Kapton/Teflon)</td>
<td>Nylon pouch, foil pouch</td>
<td>Nylon inner – trialuminate outer &amp; eva bag</td>
<td>2x teflon bags</td>
<td>Inner and outer pouch</td>
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<tr>
<td>Controlled rate freezer program</td>
<td>One program</td>
<td>One program</td>
<td>Details sent by email</td>
<td>-1°C @ 100 mins</td>
<td>One program</td>
<td>One Program</td>
<td>One program</td>
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<tr>
<td>Sammy sachet cool?</td>
<td>During each run</td>
<td>Yes, every 6/12</td>
<td>During each run</td>
<td>During each run</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Temp of program</td>
<td>-150°C</td>
<td>-65°C</td>
<td>-150°C</td>
<td>-110°C</td>
<td>-150°C</td>
<td>-180°C</td>
<td>-190°C</td>
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<tr>
<td>Rate of cooling</td>
<td>Approx. -1°C per minute</td>
<td>Approx. -1°C per minute</td>
<td>Approx. -1°C per minute</td>
<td>Approx. -1°C per minute</td>
<td>Approx. -1°C per minute</td>
<td>4°C over -180°C for 2 hrs 20mins</td>
<td>Approx. -1°C per minute</td>
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Cryoprotection, Packaging & Cooling procedures for cardiovascular tissue
## Cardiovascular tissue banking concerns

| Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns | Concerns |
|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| Is max. yr. storage @ 10°C after dispatch proven? | Is max. viable tissue to return @ 10°C after dispatch proven? | Should thawed valve be cryopreserved? | Is freezing to OPD optimised? | Is freezing to OPD consistent with current banking methods? | Is QA check at desalination consistent with UK? | Are surgical quality info sheets consistent with surgical cocktail safety? | Are your testing methods validated? |

**Summary of Concerns expressed by Cardiovascular tissue bankers regarding current Cardiovascular tissue banking methods, quality assessment and validation**
Storage of tissue for human application licences

updated 30 January 2009

This document provides an overview of licences issued to establishments undertaking storage of tissue for human application.

A licence can fall into one of four categories depending on its current status: "Licensed establishments", "Licence offer issued", "Short term licence", or "Licence application in progress".

- "Licensed establishments" denotes those establishments that are deemed licensed by the HTA.
- "Licence offer issued" indicates that an offer to the establishment for a final licence has been made.
- "Short term licence" means that the establishment has received a short term licence.
- "Licence application in progress" means that the establishment has submitted a licence application. The establishment’s compliance is currently being evaluated and a final decision about the licence will be made.

Details about a licence include the establishment, the address where the licensed activities are to take place, the Designated Individual (DI) and the Licence Holder (either corporate or personal). The field "establishment" in some instances does not contain details about the department in which the activities are to take place, which may be only a part of the establishment denoted in the field.
The activities field denotes what activities the establishment is licensed for i.e.

- Procurement
- Processing
- Testing
- Storage
- Distribution and/or
- Import/export

under the Human Tissue (Quality and Safety for Human Applications) Regulations 2007 and/or

- Storage for Scheduled Purpose (SfSP) e.g. research

under the Human Tissue Act 2004 (HT Act).

**Licensed establishments**

<table>
<thead>
<tr>
<th>Establishment</th>
<th>Address</th>
<th>DI</th>
<th>Licence Holder</th>
<th>Activities</th>
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<tbody>
<tr>
<td>Aberdeen and North East Scotland Blood Transfusion Centre</td>
<td>Foresterhill Road, Foresterhill, Aberdeen, AB25 2ZW</td>
<td>Dr Marc Turner</td>
<td>Scottish National Blood Transfusion Service</td>
<td>All</td>
</tr>
<tr>
<td>Addenbrookes Hospital</td>
<td>Hills Road, Cambridge, CB2 2QQ</td>
<td>Professor Andrew Bradley</td>
<td>Cambridge University Hospitals NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>Angel Biotechnology Holdings</td>
<td>Pentlands Science Park, Bush Loan, Penicuik, Edinburgh, EH26 0PZ</td>
<td>Ms Angela Scott</td>
<td>Angel Biotechnology Holdings</td>
<td>All</td>
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## Licensed establishments

(167 licences)

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<th>Address</th>
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<th>Licence Holder</th>
<th>Activities</th>
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<tr>
<td>Anthony Nolan Cord Blood Bank</td>
<td>Nottingham Trent University, Clifton, Nottingham, NG11 8NS</td>
<td>Dr Sergio Querol</td>
<td>The Anthony Nolan Trust</td>
<td>SISP Procurement Donor Testing Processing Storage Distribution</td>
</tr>
<tr>
<td>Basingstoke Hospital, Orthopaedic Department</td>
<td>Aldermaston Road, Basingstoke, Hampshire, RG24 9NA</td>
<td>Dr Carl Brookes</td>
<td>Basingstoke &amp; North Hants NHS Foundation Trust</td>
<td>All</td>
</tr>
<tr>
<td>BCH Stem Cell Bank</td>
<td>Department of Haematology, Belfast City Hospital, Lisburn Road, Belfast, BT9 7AB</td>
<td>Dr Francis Jones</td>
<td>Belfast Health and Social Care Trust</td>
<td>All</td>
</tr>
<tr>
<td>Belfast Cord Blood Bank</td>
<td>Northern Ireland Blood Transfusion Service, Belfast City Hospital Complex, Lisburn Road, BELFAST, BT9 7TS</td>
<td>Dr William McClelland</td>
<td>Northern Ireland Blood Transfusion Service (Special Agency)</td>
<td>All</td>
</tr>
<tr>
<td>Belfast Health and Social Care Trust</td>
<td>Royal Hospitals, Clinical Services Division, Grosvenor Road, Belfast, BT12 6BA</td>
<td>Mr Khalid Khan</td>
<td>Mrs Patricia Donnelly</td>
<td>All</td>
</tr>
<tr>
<td>BIOEDEN LIMITED</td>
<td>Suite G37, Daresbury Innovation Centre, Daresbury Science &amp; Innovation Campus, Keckwick Lane, Daresbury, Cheshire, WA4 4FS</td>
<td>Mrs Joanne Duffy</td>
<td>BIOEDEN LIMITED</td>
<td>All</td>
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<tr>
<td>BioHorizons UK</td>
<td>17 Wellington Business Park, Dukes Ride, Crowthorne, RG45 6LS</td>
<td>Mr Christopher Nethercliff</td>
<td>BioHorizons Implant Systems Inc</td>
<td>Storage Distribution Import/Export</td>
</tr>
<tr>
<td>BioVault</td>
<td>24 Brest Road, Derriford, Plymouth, PL6 5XP</td>
<td>Mr Paul Giles</td>
<td>BioVault Ltd</td>
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</tr>
</tbody>
</table>

Page 3 of 22
### Licensed establishments

(167 licences)

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<th>Establishment</th>
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<tbody>
<tr>
<td>Birmingham and Midland Eye Centre</td>
<td>Sandwell and West Birmingham Hospitals NHS Trust, City Hospital, Dudley Road, Birmingham, B18 7QU</td>
<td>Miss Saeeda Rauz Birmingham and Midland Eye Centre - Sandwell and West Birmingham Hospitals NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>Birmingham Children's Hospital</td>
<td>Steelhouse Lane, Birmingham, B4 6NH</td>
<td>Dr Alistair Cranston Birmingham Children's Hospital NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>Blackpool Fylde and Wyre Hospitals NHS Foundation Trust</td>
<td>Department of Haematology, Whinney Heys Road, Blackpool, FY3 8NR</td>
<td>Dr Paul Kelsey Blackpool Fylde and Wyre Hospitals NHS Foundation Trust</td>
<td>Procurement Distribution</td>
</tr>
<tr>
<td>Blond McIndoe Centre</td>
<td>Queen Victoria Hospital, Holtye Road, East Grinstead, Sussex, RH19 3DZ</td>
<td>Dr Nigel Jordan Blond McIndoe Research Foundation Ltd</td>
<td>All</td>
</tr>
<tr>
<td>BMI Bath Clinic</td>
<td>Claverton Down Road, Combe Down, Bath, BA2 7BR</td>
<td>Mr Kevin Walsh BMI HEALTHCARE</td>
<td>Procurement</td>
</tr>
<tr>
<td>BMI Bishops Wood Hospital</td>
<td>Rickmansworth Road, Northwood, HA6 2JW</td>
<td>Ms Karen Kousilla Persad-Blyth BMI Healthcare Ltd</td>
<td>Procurement Donor Testing Storage</td>
</tr>
<tr>
<td>BMI The Blackheath Hospital</td>
<td>40 - 42 Lee Terrace, Blackheath, London, SE3 9UD</td>
<td>Mrs Katherine Catchpole BMI The Blackheath Hospital</td>
<td>Procurement</td>
</tr>
<tr>
<td>BMI The Princess Margaret Hospital</td>
<td>Pathology Department, Osborne Road, Windsor, SL4 3ZSJ</td>
<td>Mr Barry Orton BMI The Princess Margaret Hospital</td>
<td>Procurement</td>
</tr>
<tr>
<td>BMI The Priory Hospital</td>
<td>Priory Road, Edgbaston, Birmingham, BS7UG</td>
<td>Mrs Patricia Kathleen Munday BMI Healthcare Ltd</td>
<td>Procurement Donor Testing Storage Distribution Import/Export</td>
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## Licensed establishments

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<tbody>
<tr>
<td>Bone Bank</td>
<td>New Cross Hospital, Wolverhampton, WV10 0QP</td>
<td>Mr Eric Isbister</td>
<td>Mr Eric Isbister</td>
<td>All</td>
</tr>
<tr>
<td>Bristol Tissue Bank</td>
<td>United Bristol Healthcare NHS Trust, Bristol Eye Hospital, Lower Maudlin Street, Bristol, BS1 2LX</td>
<td>Professor John Armitage</td>
<td>University of Bristol</td>
<td>All</td>
</tr>
<tr>
<td>Broomfield Hospital</td>
<td>Court Road, Broomfield, Chelmsford, Essex, CM1 7ET</td>
<td>Mr Peter Dziewulski</td>
<td>Mid Essex Hospital Services NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>C3 Hamwic Day Ward</td>
<td>Southampton General Hospital, Tremona Road, Southampton, SO16 6YD</td>
<td>Dr Deborah Richardson</td>
<td>Southampton University Hospitals NHS Trust</td>
<td>Procurement Donor Testing</td>
</tr>
<tr>
<td>Castle Hill Hospital</td>
<td>Cottingham, East Yorkshire, HU16 5JQ</td>
<td>Mr Christopher Shaw</td>
<td>Hull and East Yorkshire NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>Cells 4 Life Limited</td>
<td>Science Park Square, Falmer, Brighton, BN1 9SB</td>
<td>Miss Rebecca Rutter</td>
<td>Cells 4 Life Limited</td>
<td>All</td>
</tr>
<tr>
<td>Chapel Allerton Hospital</td>
<td>Chapeltown Road, Leeds, LS7 4SA</td>
<td>Mr Peter Bobak</td>
<td>Leeds Teaching Hospitals NHS Trust</td>
<td>SfSP Procurement Donor Testing Processing Storage</td>
</tr>
<tr>
<td>Chase Farm Hospital Bone Bank</td>
<td>Chase Farm Hospital, The Ridgeway, Enfield, Middx, EN2 8JL</td>
<td>Mr Howard Ware</td>
<td>Barnet &amp; Chase Farm Hospitals NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>Chelsea &amp; Westminster</td>
<td>Burns unit, Chelsea &amp; Westminster NHS Trust, 369 Fulham Road, London, SW10 9NH</td>
<td>Mrs Michelle Das</td>
<td>Chelsea and Westminster NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>Colchester General Hospital</td>
<td>Turner Road, Colchester, Essex, CO4 5JL</td>
<td>Dr Marion Wood</td>
<td>Essex Rivers Healthcare NHS Trust</td>
<td>All</td>
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### Licensed establishments

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<td>CryoLife Europa, Ltd.</td>
<td>Bramley House, The Guildway, Old Portsmouth Road, Guildford, Surrey, GU3 1LR</td>
<td>Dr David Nicholas Hollinworth</td>
<td>CryoLife Europa, Ltd.</td>
<td>Distribution Import/Export</td>
</tr>
<tr>
<td>Cryo-Store Ltd</td>
<td>2 Greenwich Centre Business Park, 53 Norman Road, London, SE10 9QF</td>
<td>Mr Malcolm Wilkinson</td>
<td>Mr John Heffernan</td>
<td>All</td>
</tr>
<tr>
<td>Cumberland Infirmary</td>
<td>Newtown Road, Carlisle, CA2 7HY</td>
<td>Sister Shirley Scott</td>
<td>North Cumbria Acute Hospitals NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>Department of Clinical Haematology</td>
<td>Leukaemia Laboratory, Manchester Royal Infirmary, Oxford Road, Manchester, M13 9WL</td>
<td>Professor John Liu Yin</td>
<td>Dr Kate Ryan</td>
<td>All</td>
</tr>
<tr>
<td>Department of Haematological Medicine, King's College London</td>
<td>Rayne Cell Therapy Suite, Rayne Institute, 123 Coldharbour Lane, London, SE5 9NU</td>
<td>Professor Farzin Farzaneh</td>
<td>King's College London</td>
<td>All</td>
</tr>
<tr>
<td>Droitwich Spa Hospital</td>
<td>St Andrew's Road, Droitwich Spa, Worcs, WR9 8DN</td>
<td>Mrs Shanti Jacqueline Anthony-Smith</td>
<td>General Healthcare Group</td>
<td>Procurement</td>
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<tr>
<td>DRWF Islet Isolation Facility</td>
<td>Oxford Centre for Diabetes, Endocrinology &amp; Metabolism, Churchill Hospital, Headington, Oxford, OX3 7LJ</td>
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<td>University of Oxford</td>
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<td>East of Scotland Blood Transfusion Centre</td>
<td>East of Scotland Blood Transfusion Service, Ninewells Hospital, Dundee, DD1 9SY</td>
<td>Dr Peter Rawlinson</td>
<td>Scottish National Blood Transfusion Service</td>
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<tr>
<td>ECACC</td>
<td>Centre for Emergency Preparedness &amp; Response, The Health Protection Agency, Salisbury, SP4 0LE</td>
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<tr>
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<td>Bretton Gate, Peterborough, PE3 9GZ</td>
<td>Mr John Randall</td>
<td>Peterborough and Stamford Hospitals NHS Foundation Trust</td>
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## Licensed establishments

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<td>Exeter Bone Bank</td>
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<td>Royal Devon &amp; Exeter Foundation NHS Trust</td>
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<td>Fairfield General Hospital</td>
<td>Rochdale Old Road, Bury, BL9 7TD</td>
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<td>Pennine Acute Hospitals NHS Trust</td>
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<tr>
<td>Fisher BioServices</td>
<td>Woodside, Bishop's Stortford, Herts, CM23 5RG</td>
<td>Mr Colin Grant</td>
<td>Thermo Fisher Scientific</td>
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<td>Frenchay Hospital</td>
<td>Neurosurgery Theatres, Frenchay Park Road, Frenchay, Bristol, BS16 1LE</td>
<td>Mr Ian Pople</td>
<td>North Bristol NHS Trust</td>
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<tr>
<td>Future Health Technologies</td>
<td>Unit 1 &amp; Unit 10 Faraday Building, Nottingham Science &amp; Technology Park, Nottingham, NG7 2QP</td>
<td>Miss Beverley</td>
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<tr>
<td>Glasgow Royal Infirmary</td>
<td>Haematology Department, Castle Street, Glasgow, G4 0SF</td>
<td>Dr Michael Alcorn</td>
<td>NHS Greater Glasgow and Clyde</td>
<td>All</td>
</tr>
<tr>
<td>GOSH Cell therapy Laboratory (Immunology and Haematology)</td>
<td>Camelia Botnar Laboratories, Great Ormond Street Hospital, Great Ormond Street, London, WC1N 3JH</td>
<td>Dr Catherine Cale</td>
<td>Great Ormond Street Hospital for Children NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>Guy's and St. Thomas' Stem Cell Laboratory</td>
<td>Stem Cell Laboratory, 4th floor Thomas Guy House, Guy's Hospital, London Bridge, SE1 9RT</td>
<td>Ms Tamara Elston</td>
<td>Guy's and St. Thomas' Foundation Trust</td>
<td>All</td>
</tr>
<tr>
<td>Hampshire Clinic</td>
<td>Basing Road, Old Basing, Basingstoke, RG24 7AL</td>
<td>Mrs Julie Berry</td>
<td>Hampshire Clinic</td>
<td>Procurement</td>
</tr>
<tr>
<td>Heartlands Hospital</td>
<td>Haematology Department, Bordesley Green East, Birmingham, B9 5SS</td>
<td>Dr Richard Lovell</td>
<td>Dr Donald Milligan</td>
<td>Procurement</td>
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<tr>
<td>Hospital Innovations Limited</td>
<td>Willowbrook Technology Park, Llandogo Rd, St Mellons, Cardiff, CF3 0EF</td>
<td>Mr David Lound</td>
<td>Hospital Innovations Ltd</td>
<td>Storage Distribution Import/Export</td>
</tr>
<tr>
<td>Institute of Ophthalmology</td>
<td>Cells for Sight Tissue Bank, 11-43 Bath Street, London, EC1V 9EL</td>
<td>Dr Julie Daniels</td>
<td>Moorfields Eye Hospital NHS Foundation Trust</td>
<td>All</td>
</tr>
<tr>
<td>Intercytex</td>
<td>Innovation House, Crewe Road, Manchester, M23 9QR</td>
<td>Mr Gavin Nixon</td>
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<tr>
<td>Ipswich Hospital</td>
<td>South Theatres, Heath Road, Ipswich, IP4 5PD</td>
<td>Mr Derek Bartle</td>
<td>Ipswich Hospital</td>
<td>Procurement</td>
</tr>
<tr>
<td>Islet Isolation Laboratory</td>
<td>Leicester General Hospital, Gwendolen Road, Leicester, LE5 4PW</td>
<td>Mr Ashley Dennison</td>
<td>University Hospitals of Leicester NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>James Paget University Hospitals NHS Foundation Trust</td>
<td>Operating Theatre, Lowestoft Road, Gorleston, Great Yarmouth, NR31 6LA</td>
<td>Mrs Nicola Steggles</td>
<td>James Paget University Hospitals NHS Foundation Trust</td>
<td>SfSP Procurement Storage</td>
</tr>
<tr>
<td>John Radcliffe Hospital</td>
<td>Oxford Heart Valve Bank, Cardiac Unit, Level 0, Oxford, OX3 9DU</td>
<td>Dr James Hywel Morris</td>
<td>Oxford Radcliffe NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>King's Cell Isolation Unit</td>
<td>King's College Hospital, Denmark Hill, London, SE5 9RS</td>
<td>Professor Anil Dhawan</td>
<td>King's College Hospital NHS Foundation Trust</td>
<td>All</td>
</tr>
<tr>
<td>King's College Hospital</td>
<td>Bessemer Road, Denmark Hill, London, SE5 9RS</td>
<td>Dr Aleksandar Mijovic</td>
<td>King's College Hospital NHS Trust</td>
<td>All</td>
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<tbody>
<tr>
<td>Leicester Bone Bank, University Hospitals of Leicester NHS Trust</td>
<td>Glenfield Hospital, Groby Road, Leicester, LE3 9QP</td>
<td>Mr Richard Power</td>
<td>University Hospitals of Leicester NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>Lifeforce Immune System Bank Plc</td>
<td>Imperial House, Imperial Way, Newport, NP10 8UH</td>
<td>Professor Bradley Stringer</td>
<td>Lifeforce Immune System Bank Plc</td>
<td>All</td>
</tr>
<tr>
<td>London Bridge Hospital</td>
<td>27 - 29 Tooley Street, London, SE1 2PR</td>
<td>Miss Lynne Cleland</td>
<td>Mrs Shirley Edwards</td>
<td>All</td>
</tr>
<tr>
<td>London Centre for Aesthetic Surgery (LCAS)</td>
<td>15 Harley Street, London, W1G 9QQ</td>
<td>Dr Roberto Viel</td>
<td>London Centre for Aesthetic Surgery</td>
<td>All</td>
</tr>
<tr>
<td>Main Theatres</td>
<td>Gloucester Royal Hospital, Great Western Road, Gloucester, GL1 3NN</td>
<td>Mr John Norman Wells</td>
<td>Gloucestershire Hospitals NHS Foundation Trust</td>
<td>Storage</td>
</tr>
<tr>
<td>Manchester Eye Bank</td>
<td>Manchester Royal Eye Hospital, Oxford Road, M13 9WL</td>
<td>Dr Isaac Zambrano</td>
<td>Manchester Royal Eye Hospital, Central Manchester and Manchester Children's University Hospital, NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>Mayday University Hospital</td>
<td>530 London Road, Croydon, Surrey, CR7 7YE</td>
<td>Mr Robert Ian Swift</td>
<td>Mayday Healthcare NHS Trust</td>
<td>All</td>
</tr>
<tr>
<td>Microbiology, Northern General Hospital</td>
<td>Bone Bank, Microbiology Dept., Herries Road, Sheffield, S5 7AU</td>
<td>Mr Rob Eggington</td>
<td>Sheffield Teaching Hospitals NHS Foundation Trust</td>
<td>All</td>
</tr>
<tr>
<td>Moorfields Eye Hospital NHS Foundation Trust</td>
<td>162 City Road, London, EC1V 2PD</td>
<td>Ms Tracy Lloyd</td>
<td>Moorfields Eye Hospital NHS Foundation Trust</td>
<td>All</td>
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<td>Professor Harry Moore</td>
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<td>Dr Ann Hunter</td>
<td>University Hospitals of Leicester (UHL) NHS Trust - Leicester Royal Infirmary (LRI)</td>
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<td>Derby Hospitals NHS Foundation Trust</td>
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<td>Mr Simon Pleydell</td>
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<tr>
<td>The John Goldman Centre for Cellular Therapy</td>
<td>Stem Cell and Immunotherapy Laboratory - Catherine Lewis Centre, Hammersmith Hospital, DuCane Road, London, W12 OHS</td>
<td>Mr John Davis</td>
<td>Professor Jane Apperley</td>
<td>All</td>
</tr>
<tr>
<td>The London Clinic</td>
<td>20 Devonshire Place, London, W1G 6BW</td>
<td>Mr Paul Richard Apps</td>
<td>Trustees of the London Clinic Ltd</td>
<td>All</td>
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<tr>
<td>The Meriden Hospital</td>
<td>UHCW Hospital Site, Clifford Bridge Road, Coventry, CV2 2LQ</td>
<td>Mrs Maria Hardman</td>
<td>The Meriden Hospital, BMI Healthcare</td>
<td>Procurement</td>
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<tr>
<td>The Thornbury Hospital</td>
<td>312 Fulwood Road, Sheffield, S10 3BR</td>
<td>Mrs Janet Cooper</td>
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<td>The Wellington Hospital</td>
<td>Wellington Place, London, NW8 9LE</td>
<td>Mrs Andrea Costello</td>
<td>Mrs Kim Frances Handel</td>
<td>Storage</td>
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<tr>
<td>UK Stem Cell Bank</td>
<td>National Institute for Biological Standards</td>
<td>Dr Glyn Stacey</td>
<td>Dr Stephen Inglis</td>
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<td></td>
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<td>University Hospital of North</td>
<td>North Road, County Durham, DH1 5TW</td>
<td>Mrs Lesley Fowler</td>
<td>County Durham and Darlington NHS</td>
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<td>Durham</td>
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<td>Dr Nicholas Clowes</td>
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<tr>
<td>University Hospitals Bristol</td>
<td>Department of Haematology, Heath Park,</td>
<td>Dr Christopher Fegan</td>
<td>Cardiff and Vale NHS Trust</td>
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<td>Upper Maudlin Street, Bristol, BS2 8BJ</td>
<td>Dr Colin Steward</td>
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<td>Clifford Bridge Rd, Coventry, CV2 2DX</td>
<td>Dr Richard Kennedy</td>
<td>University Hospitals Coventry and</td>
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<tr>
<td>and Warwickshire NHS Trust</td>
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<td>Virgin Health Bank</td>
<td>24 Brest Road, Plymouth, PL6 5XP</td>
<td>Mr Paul Giles</td>
<td>Virgin Health Bank Limited</td>
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### Licensed establishments

(167 licences)

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<tr>
<td>Warwick Hospital</td>
<td>Lakin Road, Warwick, CV34 5BW</td>
<td>Dr Ian Chant</td>
<td>South Warwickshire General Hospitals NHS Trust</td>
<td>Procurement Distribution</td>
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<tr>
<td>Welsh Blood Service</td>
<td>Ely Valley Road, Talbot Green, Pontyclun, CF72 9WB</td>
<td>Mrs Tracey Rees</td>
<td>Velindre NHS Trust</td>
<td>Procurement Donor Testing Storage Distribution Import/Export</td>
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<tr>
<td>Weston General Hospital</td>
<td>Grange Road, Uphill, Weston-s-Mare, BS23 4TQ</td>
<td>Mr Michael Radford</td>
<td>Weston Area Health Trust</td>
<td>Procurement</td>
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<tr>
<td>Whiston Hospital</td>
<td>Warrington Road, Prescot, L35 5DR</td>
<td>Mr Kenneth Graham</td>
<td>St Helens and Knowsley (NHS) Hospitals Trust</td>
<td>All</td>
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<tr>
<td>William Harvey Hospital</td>
<td>Kennington Road, Ashford, Kent, TN 24 0LZ</td>
<td>Mr Helmut Zahn</td>
<td>East Kent Hospitals NHS Trust</td>
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<tr>
<td>Wolfson Cellular And Gene Therapy Unit</td>
<td>University College London Hospital, 51 Chenesies Mews, London, WC1E 6HX</td>
<td>Dr Michael Watts</td>
<td>University College London Hospitals NHS Foundation Trust</td>
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<tr>
<td>Wrexham Maelor Hospital</td>
<td>Trust Headquarters, PO Box 18, Wrexham, LL13 7ZH</td>
<td>Dr David Fletcher</td>
<td>North Wales NHS Trust</td>
<td>Procurement Distribution</td>
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<tr>
<td>Wrightington Hospital</td>
<td>Bone Bank, The Broc Centre, Hall Lane, Appley Bridge, Wigan, WN6 9EP</td>
<td>Mr Peter Kay</td>
<td>Wrightington, Wigan and Leigh NHS Trust</td>
<td>All</td>
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<tr>
<td>Wycombe Hospital</td>
<td>Queen Alexandra Road, High Wycombe, HP11 2TT</td>
<td>Mr Andrew Northeast</td>
<td>Buckinghamshire Hospitals NHS Trust</td>
<td>All</td>
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<tr>
<td>York Pharma (R&amp;D) Plc</td>
<td>Innovation Centre, 217 Portobello, Sheffield, S1 4DP</td>
<td>David Haddow</td>
<td>York Pharma (R&amp;D) Ltd</td>
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<td>Ysbyty Gwynedd</td>
<td>Penrhosgarnedd, Bangor, North Wales, LL57 2PW</td>
<td>Dr Mark Lord</td>
<td>North West Wales NHS Trust</td>
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<tr>
<td>Ysbyty Gwynedd Bangor</td>
<td>Penrhosgarnedd, Bangor, North Wales, LL57 2PW</td>
<td>Mr Robert Walters</td>
<td>Dr David Edwards</td>
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## Licence offer issued

(4 licences)

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<tr>
<td>BMI The Alexandra Hospital</td>
<td>Mill Lane, Cheadle, SK8 2PX</td>
<td>Mr Michael Stroud</td>
<td>BMI Healthcare Ltd</td>
<td>Procurement</td>
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<tr>
<td>City Hospital</td>
<td>Waverley Rd, St. Albans, AL3 5PN</td>
<td>Mr Neil Davies</td>
<td>West Herts Hospitals Trust</td>
<td>Procurement, Storage</td>
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<tr>
<td>Mid Yorkshire Hospitals NHS Trust</td>
<td>Pinderfields Hospital, Aberford Road, Wakefield, WF1 4DG</td>
<td>Dr John Ashcroft</td>
<td>Mid Yorkshire NHS Trust</td>
<td>Procurement</td>
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<tr>
<td>Royal Alexander Hospital</td>
<td>Corsebar Road, Paisley, PA2 9PN</td>
<td>Mr Sanjeev Lakshaman Chitnis</td>
<td>NHS Greater Glasgow &amp; Clyde</td>
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## Licence application in progress

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<tr>
<td>Gartnavel General Hospital</td>
<td>Haematology Department, 21 Shelley Road, Glasgow, G12 0XL</td>
<td>Dr. Michael Alcorn</td>
<td>NHS Greater Glasgow &amp; Clyde</td>
<td>Procurement, Donor Testing, Processing, Storage, Distribution, Import/Export</td>
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<tr>
<td>Nuffield Hospital Bristol</td>
<td>Uppre Byron Place, Clifton, Bristol, BS8 1JU</td>
<td>Mr Ashley Livesey</td>
<td>Mr Ashley Livesey</td>
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<tr>
<td>Spire Alexandra Hospital</td>
<td>Impton Lane, Walderslade, Chatham, Kent, ME5 9PG</td>
<td>Mrs Josephine Withrington</td>
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<tr>
<td>Spire Cheshire Hospital</td>
<td>Fir Tree Close, Stretton, Warrington, WA4 4LU</td>
<td>Mrs Elizabeth Tottle</td>
<td>Spire Cheshire Hospital</td>
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<tr>
<td>University Hospital North Staffordshire</td>
<td>Princes Rd, Hartshill, Stoke on Trent, ST4 7LN</td>
<td>Dr David Allotey</td>
<td>university hospital of north staffordshire nhs trust</td>
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OHVB TISSUE PROCESS FLOW & 3rd PARTIES

1. Donor Referral co-ordinators
   - Donor Referral
   - Family interview

2. Procurement staff
   - 3rd PARTIES

3. NTMRL serology lab
   - Critical goods Suppliers
   - ORH bacteriology lab

4. Medical Advisors
   - Medical Advisors

5. Transplanting Surgeons
   - Transplantation

6. Contingency planning

7. Couriers
   - Retrieval of Tissue
   - Transit to OHVB
   - Transit to surgical centre
   - Registration & inspection
   - Testing
   - Performance Review
   - Storage
   - Processing
   - Packing & Issue
   - Quality Assurance Review

8. Environmental monitoring team

WITHIN OHVB FACILITY