George Galea · Marc Turner ·
Sharon Zahra · *Editors*

Essentials of Tissue and Cells Banking

Second Edition

Springer

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Editors
George Galea
National Blood Transfusion Service
St. Luke's Hospital
Pietà, Malta

Marc Turner
Scottish National Blood
Transfusion Service
Edinburgh, UK

Sharon Zahra
Scottish National Blood
Transfusion Service
Edinburgh, UK

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Preface

The 1st edition of this book has been very useful, in that it provided a concise and up-to-date information on the principles and scientific basis of tissue banking. Ten years have passed since its publication, and it was felt that an update was required.

Two important changes have been made to the previous edition. The first was a significant update on the all chapters. In some cases, the authors who had contributed before were asked to update their chapter to take into account of developments that have taken place in the intervening 10 years. In other instances, chapters have been written by different authors, who are recognized current experts in the field about which they wrote. All authors were given the same remit—to write succinctly and clearly on their chosen topic and to focus on current practices.

The second important change was a limited expansion into cell therapy. Many tissue establishments have developed processes that allow them to perform limited cellular therapy work. It was therefore felt that along with cord blood banking, haematopoietic stem cell processing should also be included. These are minimally manipulated cells that are now routinely banked.

It was very tempting to include a range of cellular therapies which are increasingly being handled; however, the techniques used in these areas such as culturing methodologies, measuring various cell markers, modifying them through gene therapy and producing advanced therapeutic medicinal products (ATMPs) are very different processes from the essentials of tissue banking that this book is meant to cover. Moreover, the target audiences of tissue and cellular banking are still broadly different although some convergence is beginning to take place, in that cells that are minimally manipulated are frequently handled by tissue establishments.

It may be that there will be more convergence in the future between tissue banking and cellular therapy and advanced therapeutic modalities. If that happens, it would make sense to combine the scientific principles covering all the available techniques into one book. I do not believe we are there yet, and the remit of this work would then diverge significantly from its original purpose. However, it was considered wise to add a chapter on the development of human embryonic cell banking. This was deliberately chosen to exemplify the complexities that banking such cells can provide and to illustrate the real differences between tissue banking and complexly manipulated cells.

I am grateful to two colleagues of mine Dr. Sharon Zahra and Prof. Marc Turner (both from the Scottish National Blood Transfusion Service and the University of Edinburgh) who helped me with identifying new experts to contribute to this edition and for supporting me in finalising this book. They were also very helpful in numerous discussions that took place throughout this book's gestation. I am also very grateful to all the authors who willingly gave their time and put much effort into this endeavour. Without exception, they wrote an up-to-date chapter in their area of expertise in a succinct and clear way.

There are now a number of universities and professional bodies that provide specific courses leading to a diploma or degree in tissue and cell banking. I believe that scientists, professional and medical staff working and wanting to develop their career in this field would find this book very useful. I believe it will also have a lot of relevance to clinical staff who use many of the products described in this book.

I believe therefore that this book should find a place on the shelf of many tissue establishments and who knows, in future years, there may be a third and future editions! I hope that the readers will find this book useful and as enjoyable as it was for me, editing and contributing to it.

Pietà/Msida, Malta

Dr. George Galea
Consultant, National Blood Transfusion Service
St. Luke's Hospital
Pietà, Malta

Senior Lecturer, Department of Pathology
University of Malta
Msida, Malta
george.galea@um.edu.mt
george.m.galea@gov.mt

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Live Donors of Tissue

1

Akila Chandrasekar

Abstract

Tissue transplantation is one of the recognized treatment strategies for replacing or repairing tissue damage resulting from disease or trauma. A living donor undergoing a surgical procedure can donate the surgical residues, such as femoral head, or placenta for amniotic membrane. Patients undoing solid organ cardiac transplantation can donate their own hearts removed for heart valve banking. Femoral head donated by patients undergoing primary hip replacement is the most widely and commonly banked tissue from living donors and is used in orthopaedic (spine and joint surgery) and oral/maxilliofacial surgery. Tissue donation to transplantation is a multistep process each of which is crucial to ensure safety and quality of the graft. There are many advantages of obtaining tissues from a living donor from tissue banking perspective. From donor selection perspective, the potential for self-exclusion and direct interview with the donor themselves to gather medical and behavioral history enhances the quality of information obtained. The blood tests can be performed on fresh, good quality samples rather than post-mortem samples as is often the case with deceased donors. Tissue is procured in a clean operating theatre environment under aseptic conditions, reducing the risk of contamination. The hospitals play key role in providing donations as well as users transplanting the tissues thereby living donor programme is a collaborative process between a tissue bank and these hospitals.

A. Chandrasekar (✉)
NHS Blood & Transplant, 14 Estuary Bank, Speke, Liverpool L24 8RB, UK
e-mail: akila.chandrasekar@nhsbt.nhs.uk

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1.1 Introduction

Human tissues and cells used in transplantation can be obtained from living or deceased donors. Some tissues and cells such as Haematopoietic Progenitor Cells (HPC) can only be obtained from living donors. Depending on the clinical circumstances, living donors donate tissues and cells for autologous use, where donation is collected for the patient's own treatment, or for allogeneic use, where tissues and cells are donated by a related or unrelated individual for the treatment of another person. HPC donations including Peripheral Blood Stem Cells, Bone Marrow and Cord Blood donations, and tissues consented and donated by living tissue donors for research and non-clinical use are excluded from the remit of this chapter.

A living donor undergoing a surgical procedure can donate the surgical residues, such as femoral head, or placenta for amniotic membrane, to be processed and stored as tissue grafts for allogeneic use. Patients undergoing solid organ cardiac transplantation can donate their own heart valves, derived from the heart that is removed during the procedure, to a heart valve bank if there are no medical contraindications to donation. In some cases, tissues from a patient can be processed and stored for autologous transplantation in the future, for example calvarial flaps removed during a decompression craniotomy. This chapter provides overview of altruistic tissue donation from voluntary unrelated living donors. Types of living tissue donations are summarised in Table 1.1.

Femoral head bone allograft is the most widely and commonly banked tissue from living donors and is used in mainly in orthopaedic (spine and joint surgery) and also in oral/maxilliofacial surgery. Amniotic membrane is used in the UK mostly in ophthalmic surgery as a biological dressing [1] but is sometimes used elsewhere as a skin substitute for management of burn wounds (as a temporary or permanent wound dressing) or to treat skin lesions such as vascular ulcers, epidermolysis bullosa or radiation burns.

1.2 Regulation

In the UK, the primary legislation covering tissue donation is the Human Tissue Act (2004) in England, Wales and Northern Ireland and Human Tissue Act (2006) in Scotland. The European Union Tissues and Cells Directives (EUTCD) comprising of the parent directive (2004/23/EC) [2] and the two associated technical directives were fully implemented into UK law via Human Tissue Safety and Quality (for human application) Regulations 2007. The regulatory body, the Human Tissue Authority (HTA), has the legal responsibility to license and inspect all establishments procuring and storing human tissue.

Table 1.1 Tissues donated by living donors

Tissue	Donor source	Operation	Donor interview staff	Procurement staff
Bone	Patients with osteo arthritis of the hip requiring hip joint replacement	Hip replacement with a prosthetic joint	• Tissue establishment staff • Hospital staff from the surgical team under a formal contract with the tissue establishment	Surgeons in operating theatre
Amniotic membrane	Mothers at the time of delivery	Elective caesarean section	• Tissue establishment staff Hospital staff based on a formal agreement • Between the tissue facility and hospital staff	Midwifery and obstetric staff
Heart valve	Patients undergoing heart lung block transplantation for cystic fibrosis where the heart may be unaffected	Explanted heart may be used as a source of heart valves	Tissue establishment staff Under contract with surgical team according to a clearly defined specification	Heart, heart/lung • Procured at time of explanation prior to heart lung transplant

1.3 Donor Identification

The banking of tissues for therapeutic use is a multi-step process, each of which is crucial to ensure safety and quality of the graft. The first step in that process involves identification and consent of medically suitable donors.

1.3.1 Femoral Head Donation

In the case of femoral head donations, potential donors may be identified in the out-patient department when they attend for surgical and medical assessment prior to their surgery. For those hospitals which run preoperative assessment clinics, this may be an ideal time to approach potential donors, when they are counselled about their forthcoming operation. They can be asked whether they are interested in donating the femoral head which otherwise would be discarded. At this time they will not be receiving pre-operative medication or receiving post-operative analgesia and therefore they should be able to make an informed decision, unclouded by

medication. Donors should be provided with information leaflets including information on medical contraindications, testing for microbiological markers, and the potential clinical use so that donors can self-exclude from donating if they have any risk factors or high risk behaviours that are contraindications to tissue donation. They should be reassured that their decision would not influence their surgical procedure or its timing.

1.3.2 Amniotic Membrane Donation

In the case of amniotic membrane, the information leaflets can be made available at the antenatal clinics to provide information on the process from donor selection, medical and behavioural history exclusion, the need of microbiological testing and the information about use of the donation. Information about potential donors may be obtained from the hospital through the obstetric lists of elective caesarean section deliveries to allow time to have a pre-consent discussion with the donor by trained healthcare professionals before commencement of labour, and for consent for collection to be given or declined before delivery.

1.4 Consent

The Human Tissue Act makes consent (authorisation in Scotland) the fundamental guiding principle covering the use of donated tissue for any purpose, and provides for financial and custodial penalties for breaches of its requirements. The HTA provides guidance and direction, through the publication of Codes of Practice, the first of which deals specifically with consent [3].

Donation of tissues must take place only after the person concerned has given free, informed and specific consent. For consent to be considered valid, it is important, that the person giving consent is fully informed with regard to all aspects of the donation process, including how, where and when the donation will take place, how much tissue will be removed, and the purposes for which the tissue may be used. With an adult living donor, the consent is taken generally from the donor themselves. Consent should be recorded and/or documented in the donor/patient's record. Informed consent must be discussed with the donor or their legal representative in a language and using terms they can understand. Records should confirm that the prospective donor and, where appropriate their legal representatives, has understood the information given, had the opportunity to ask questions and received satisfactory answers, before confirming their position regarding donation.

There are many models for obtaining consent from living tissue donors. They may be interviewed face-to-face by tissue establishment staff or by trained hospital staff working according to specified procedures. Hospital staff may be contracted to the tissue establishment and trained to use formal questionnaires, agreed in advance

and regularly updated using a document control system within a quality management system. The potential donors may be interviewed by telephone, by arrangement and with their prior consent, using the same questionnaire where the interview is documented using digital recording and where the telephone discussion becomes part of their donor record. The advantages of the latter model are considerable: the interview can be at a convenient time chosen by the donor in advance, the recording can be audited and used as a training tool, and not only is the consent recorded, but also the prior information provided to the donor. However, such a system does require resource and infrastructure to support it. All methodologies which are employed must ensure confidentiality, and the timing of the interview must consider donor competency issues, avoid the risk that the donor may be under the effects of medication, and consider any language comprehension issues.

Patients need to be informed that, if they consent to make a bone/amnion donation, it will require that they give permission to be tested for microbiology markers for infection which, in the UK, includes hepatitis B and C, HIV, HTLV1 &II and syphilis, and that where the results are pertinent to their health that they will be informed of those results and be advised how they should be managed. There may be additional discretionary microbiological tests such as for malaria or Chagas' disease if the donor's travel history indicates the need for this. Patients also need to be told that, in those tissue establishments where Nucleic Acid Testing (NAT) is not used to screen HIV/HBV/HCV on the blood sample taken at the time of donation, they would need to provide another follow up blood sample 6 months after the donation as well as one at the time of donation or very near to their operation [4]. Potential donors need to know that a comprehensive medical and behavioural history is needed to assess the suitability of the individual as a donor. The history taking is relatively time consuming and individuals should ideally be informed that all the questions are asked of every potential donor. They also need to be informed that donations unsuitable for therapeutic use may still be suitable for research and development. Research may be in the public sector where the outcome of research using donations of tissue may be published. Development work within the tissue establishment can lead to improvements in processing or other aspects of tissue bank procedures. Research may also be possible in the commercial domain, including for drug discovery work, although this would not benefit the donor financially. Donors should be also given the option to donate tissues for clinical use but decline consent for non-clinical use, with the understanding that if their donation is unsuitable for clinical use it will be discarded if they have not consented for research. For those donors who agree to allow donation a blood sample is commonly taken at the time of anaesthesia prior to the hip replacement operation. If a follow up sample is required (where the first sample is not NAT tested), this may be taken by the community doctor (primary care practitioner) 6 months post donation, then posted back to the testing laboratory or tissue facility. Patients who make donations need to be informed that the process of donor selection and testing mean that not all donations can be used therapeutically.

1.5 Donor Selection: Evidence Base and General Considerations

Transmissions of disease by tissue transplant, though rare, is well documented [5, 6] and includes examples of both infectious disease transmission and non-infectious disease transmission such as malignancy. The probability of blood-borne virus in tissue donors in the US has been documented [7] and although prevalence rates were lower than in the general population, they were higher than among first time blood donors. Brant found that the prevalence of infection is low among English tissue donors, but the risk was higher than that among new blood donors [8]. Safety measures for avoiding disease transmission to the tissue or cell recipient include careful selection of the donor, testing the donor for blood-borne infections, preventing contamination of the donation at procurement, processing [9] or storage and, where possible, disinfection or sterilization of the donated tissue prior to transplantation. This chapter mainly addresses aspects of donor selection.

The EUCTD [10] requires that adverse events and reactions are reported to the respective Member States' Competent Authorities. In addition to these vigilance and surveillance programmes, there are other useful resources such as the Notify Library [11] which is a publicly accessible database holding a collection and review of information from published literature. There are published reports of transmission of HTLV [12], HIV [13] and HCV [14] via frozen femoral heads from living bone donors. These donors were not tested at the time of donation as they donated prior to routine screening being introduced.

Considering tissues donated are stored for long periods before transplantation, the implications of changes to the donor selection criteria for tissue and cells to those grafts already in inventory, selected according to obsolete donor selection and testing, needs to be considered by tissue establishments in their policies concerning review of stock inventory. Such policies can usefully include a risk assessment-based approach on how to handle changes in selection criteria, and their impact on donations already in inventory.

1.6 Generic Contraindications

The main objective of donor selection procedures for living surgical tissue donors is to protect recipients without compromising the quality and sufficiency of the grafts to meet clinical demand. The major donor exclusion criteria for living tissue donors are:

1.6.1 Transmissible Infections

Potential donors with clinical or laboratory evidence of (have tested positive for) HIV, HCV, HBV or HTLV I/II infection at the time of donation must be excluded from tissue donation [4]. The tissue establishment should develop donor selection policies after considering epidemiological data on transmissible blood borne infections, the sensitivity and specificity of screening tests used for detecting these infections and the risk of undetected infection (window period) transmissions. Nucleic acid testing (NAT) can detect infection at a much earlier stage than the equivalent antibody test thereby reducing the "window period" where undetected infectious agents could potentially be transmitted. Many international guidelines recommend an interval of at least twice the window period since the last "at risk behaviour" for the length of deferral before donation. The deferral period would be longer if NAT is not done on donation sample [15].

Tissue donors are closely questioned about their behaviour to assess the risk of carriage of these blood borne viruses. Questions include whether an individual has had exposure through lifestyles which are associated with a higher incidence of certain infections than other sectors of the community. The risk associated with Men who have had Sex with Men (known as MSM) is higher with regards to blood-borne viruses like HIV than the general population [16]. Lifestyle risks include non-prescription drug taking, having sex in exchange for money or drugs, and, tattooing and body piercing done in settings not approved by the authorities. There are many countries which require some type of registration of tattoo parlours or acupuncture facilities and/or personnel and that makes it easier to exclude potential donors who have had procedures in non-registered facilities where the chance of re-use of needles or tattoo ink cannot be excluded.

It is a regulatory requirement that the antibody screening for HIV, HCV and HBV to be repeated in a second blood sample obtained from living tissue donors at least six months after the donation if NAT is not done on the donation sample when tissues are stored for long periods. The repeat negative result will ensure that there was no seroconversion and the first negative result on the donation sample was not in the window period.

If NAT is done on all donations, 3 months deferral of donors after potential exposure, for example if the donor is a sexual partner of a high risk individual, is acceptable [17]. Habitual drug users have additional risk of other infections that are not screened for and hence a longer period of exclusion of minimum 12 months is necessary for these donors [17]. The UK Blood Services Live Tissue Donor Selection Guidelines [18] and Guidelines for the UK Blood Transfusion Services [19] are available from the Joint Professional Advisory Committee for all tissue banks including non-blood service tissue banks.

1.6.2 Acute Infections

To minimize the risk of donor derived infections, donors with evidence of infection at the time of donation must be deferred and efforts should be taken to reduce the risk of contamination during processing. For amnion donors, maternal puerperal pyrexia or prolonged rupture of the membranes may point to a risk of bacterial infection, as might infants with possible congenital bacterial infection associated with amnionitis.

1.6.3 Risk of Transmission of Prion Diseases

It is unlikely people diagnosed with Creutzfeldt–Jakob disease (CJD), or variant Creutzfeldt-Jacob disease (vCJD) or those with a history of rapid progressive dementia or degenerative neurological disease would be considered for an elective surgery or considered fit to donate tissues or cells. In addition, the following risks must be considered [18].

(a) Living donors with a family history of non-iatrogenic CJD
(b) Recipients of hormones derived from the human pituitary gland (such as growth hormones) and recipients of grafts of cornea, sclera and dura mater, and persons that have undergone neurosurgery in the past (where dura mater allograft may have been used, but not documented).

For vCJD, further precautionary measures recommended in the UK include exclusion of blood transfusion recipients and individuals who have been told that they may be at increased risk (because a recipient of blood or tissues that they have donated has developed a prion related disorder) from donating blood and tissues.

1.6.4 Malignancy

Whilst transmission of malignancy is well documented for organs, the risk of transmission of malignant disease from tissues is very low, with no reported transmission of malignancy through bone allografts. The Commission Directive, 2006/17/EC [4] requires that malignancy, with a few exceptions, be considered a contraindication to donation. A survey published in 2006 [20] showed that the commonest exclusions from femoral head donors are reported to be pre-existing bone or joint conditions and malignancy. The reported exclusions of donors of femoral heads for "malignancy" represent active malignant disease and non-metastatic disease or individuals in remission for some years. On the other hand, some donors with no reported history of malignancy may have occult malignant disease, sometimes detectable in donated bone. Cases of low grade lymphoma were revealed in retrieved bone allografts [21] and evidence of malignant lymphoma and low grade chondrosarcoma were found in femoral heads

otherwise considered suitable for donation [22]. However, it can be argued that donor acceptance and deferral criteria should be risk assessed based on evidence of transmissibility. Processing of tissues to remove donor cells may be a valid means of accepting donations from individuals with a history of malignancy not affecting the specific tissue to be retrieved. Donor and recipient histo-incompatibility, plus recipient immune competence further reduce the chance of survival of malignant clones in the recipient and, together with the removal of oncogenic viruses, means that deferral for malignancy for bone donation could be reviewed on the basis of these combined factors. A firm evidence base is needed if standards for exclusion are to be effective in ensuring quality and safety of donated tissue, whilst preventing arbitrary exclusion of some tissue donors.

1.7 Retrieval/Procurement

Once suitable donors are consented, the next step is to retrieve or procure the donation. In living tissue donors this is done at the time of hip replacement surgery, for bone donation, or at the time of delivery for amnion.

In the operating theatres a member of staff trained in the procurement of bone will have responsibility for holding and maintaining a stock of sterile packaged containers, supplied by the tissue establishment, to be used to contain the donated femoral head after it is removed by the surgeon. The surgeon may also remove an analyte validated to represent the donation, commonly bone chips from the femoral head, and place these into enrichment broth for aerobic and anaerobic bacteriology culture according to the requirements of the individual tissue establishment. All the documentation, bone containers and associated testing analytes, including bone chips for culture and blood samples for microbiology testing, must be labelled with a unique identifier to allow tracking from the donor, via processing and testing, to the recipient. Ideally the labelling technology used will be compatible with testing laboratory equipment so that manual transcription of information about the donor is avoided and computer traceability is ensured.

In the case of femoral head donation there is a need for extensive interaction between the tissue establishment and outpatient and theatre orthopaedic staff, with a need for training and audit, and updates to staff training.

Donation of amniotic membrane is in principle a very similar process. The amnion is collected at the delivery from mothers undergoing elective caesarian section, whereby the placenta is removed surgically under aseptic conditions, exposing it to less risk of contamination than a vaginal delivery. The whole placenta is collected and sent to the tissue establishment for further processing. Again there is a need for close co-operation between midwifery, obstetric out-patient and in-patient delivery suite staff.

1.8 Summary

Obtaining tissue from living donors has many advantages from a tissue banking perspective. Firstly, it is procured in a clean operating theatre environment by surgeons. As the tissue is taken from living donors under aseptic conditions, the risk that it may be contaminated is greatly reduced. From donor selection perspective, the potential for self-exclusion and direct interview with the donor themselves to gather medical and behavioural history enhances the quality of information obtained. Another advantage is that blood tests may be performed on fresh, good quality samples rather than post-mortem samples as is often the case with deceased donors.

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Deceased Tissue Donors

2

Sharon Zahra

Abstract

Tissue donation after death includes several different products which can be life-enhancing (such as tendons) or life-saving (such as heart valves), with an individual tissue donor being able to potentially help many different patients. Different to organ donation, tissue donation can take place up to several hours after death; this means that the number of potential tissue donors is much higher than that of organ donors, although careful attention to body cooling after death is required to ensure that the quality and safety of the tissue products is maintained. It is important to ensure that appropriate consent is in place prior to proceeding with tissue retrieval. Further, due to the nature of deceased donation, information provided at the time of donation as to the medical, behavioural and travel history of the donor is necessarily second hand, making it very important that all available information on the donor is reviewed and assessed (including speaking to the donor's family and family doctor, reviewing medical records and post-mortem as appropriate) to ensure a full risk–benefit assessment is carried out prior to releasing the donation for clinical use. Tissue retrieval requires well trained staff to ensure that the products are not damaged during the retrieval process; and finally due care and attention is required when taking blood samples for donor testing of mandatory markers of infection to ensure that the blood samples taken are truly representative of the donor's infectious disease status.

S. Zahra (✉)
Scottish National Blood Transfusion Service, Jack Copland centre, Edinburgh EH14 4BE, Scotland
e-mail: Sharon.Zahra@nhs.scot

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2.1 Introduction

An individual deceased tissue donor can improve and even save the life of many different patients. While tissue donations are essential for several routinely available surgical procedures e.g. cruciate ligament repair following knee trauma or repair of congenital heart defects, tissue donation is much less well recognised and understood than organ donation, the latter having a much higher profile with the general public and even medical professionals. This despite the fact that the number of potential tissue donors is likely to be far higher than the number of organ donors, and that a single tissue donor may be able to help numerous recipients.

The potential for tissue donation after death is ever increasing and currently includes the potential to donate corneas, tendons, bone, pericardium, blood vessels, cartilage, heart valves and skin. Tissue donors can also be organ donors; indeed some donors are able to donate both organs and tissues, and some donors who start off on the organ donation pathway but end up not being able to donate organs for any reason (e.g. time from cessation of supportive care to confirmation of circulatory death being too long for successful organ donation) may yet be able to donate some or all tissue products.

Organ versus Tissue Donation

While organ donation and transplantation tends to be immediately life-saving e.g. liver transplant in the setting of acute liver failure, most tissue products tend to be life-enhancing (although some tissue donations, e.g. heart valves, may be life saving). In view of this the risk/benefit assessment carried out when deciding on the suitability or otherwise of a potential tissue donor is different to that carried out when deciding on the suitability or otherwise of a potential organ donor; for example higher risk donors (e.g. evidence of chaotic lifestyle, illicit drug taking) are usually considered unsuitable for tissue donation.

Despite this the number of suitable potential tissue donors is higher than that of suitable organ donors due to the need to maintain metabolic function in organ donation as different to tissue donation. Indeed for organ donation to be possible the potential donor needs to die in hospital, most often in an intensive care unit, with retrieval surgeons able to mobilise for retrieval as soon as possible after the confirmation of death to ensure that the retrieved organs retain their metabolic function. On the other hand, compared to organ donation, tissue donation can take place at a later timepoint from the time of death. While it is important to maintain the structural integrity and sterility of tissue donations, there is no need to maintain metabolic function of living cells in most tissue donations; this means that tissue donation is possible in a larger number of deaths.

A further important difference between organ and tissue donations is that at present, donated organs need to be transplanted as soon as possible with no current possibility of prolonged storage. This is not the case with tissue donation, where donations have a “shelf-life” i.e. they can be stored for prolonged periods of time—at the longest up to 10 years for cryopreserved heart valves, with variable storage

periods for other tissue donations [1]. This ability to store donations before clinical use makes it possible to carry out a more detailed risk/benefit assessment before releasing tissue donations for clinical use, as compared to organ donations.

2.2 Deceased Tissue Donor Identification

A successful deceased tissue donation programme requires an established system for referral of potential deceased donors from the place of death to the retrieval service.

It is important to engage effectively and regularly with all areas where deceased tissue donors may be referred from. Given tissue donation can take place several hours after death, potential tissue donors can be referred from any place where a potential donor has died, including clinical areas within hospitals, hospices, funeral homes and even the potential donor's own family home.

Different to blood donation, where the volunteer healthy donor makes a personal decision to attend for donation on a given day, tissue donation after death can only take place if there is a well established referral system in place so that the potential of donation is routinely considered as part of the end of life care of an individual. With good engagement with clinical areas, it is possible to set up a system where a formal decision is taken at each and every death as to whether tissue donation is possible or not, and thus the potential donor referred on to the tissue donation service where appropriate.

Clinical areas are often very busy, such that facilitating donation after an individual's death may be seen as an extra responsibility that is getting in the way of managing living patients. However the management of some of these patients is dependent on tissue donation. Having a well established system such that the possibility of tissue donation is considered at each and every death is important to avoid missing donation potential. Providing the clinical areas with teaching about the potential of tissue donation and an easy to refer to list of the main reasons that would make an individual unsuitable for tissue donation (e.g. presence of certain infections) would allow effective decision making at the time of a patient's death on whether to refer a potential donor or not.

It is equally important to raise awareness about the potential of tissue donation with the general public. Given many organ donors can also be tissue donors, and vice versa, it is important to provide the general public with information about the potential of both organ and tissue donation after death. By regularly discussing the possibility of donation, and providing information as to why such donations are important, tissue (and organ) donations become the norm within a society, something that can be openly discussed from a young age. This means that the general public is already aware of the possibility of tissue donation, making the conversation at the time of a loved one's untimely death much easier to approach and also increasing the chance of a positive response from the family as tissue donation would be a subject that they are already aware of [2, 3].

In order to help ensure the safety of tissue donation it is important to continue to promote tissue donation from volunteer unpaid donors with no monetary incentive offered to their next of kin. It is equally important to protect the donors themselves by ensuring anonymity of the donor at all times to protect their personal privacy, even after their death.

2.3 The Law and the Role of the Family

Before tissue donation can be progressed from an individual it is important to confirm that legal consent is in place. Different countries manage consent for tissue (and organ) donation in different ways. Some countries, e.g. the United States of America, use an Opt-In system whereby individuals can make a decision in life as to whether they would like to be a tissue (and/or organ) donor at the time of their death. By deciding that they would like to be a tissue (and/or organ) donor they Opt-In to donation i.e. they register in life their decision that they would wish to be a donor at the time of their death. This decision can be registered in various ways: by adding one's name to a national Organ Donor Register, by carrying an Organ Donor Card, or by telling one's family about their decision or including it in a will.

On the other hand, other countries e.g. Spain operate an Opt-Out system for donation i.e. all individuals are considered to be potential donors at the time of their death unless an individual has registered a decision contrary to this i.e. not to be a donor. Again the decision to Opt-Out can be done in various ways including registering one's decision not to be a donor on a national register.

Both Opt-In and Opt-Out can be "hard" or "soft". Hard Opt-In/Out systems are ones where the individual's decision is final i.e. at the time of death this decision cannot be over-turned. A soft Opt-In/Out system on the other hand allows for the input of the next of kin to be taken into consideration at the time of an individual's death.

Both systems have their pros and cons. While a "hard" system would mean that the family would not be able to over-turn an individual's decision thus potentially increasing the number of successful donations, it is important to keep in mind that the family have an important role to play in deceased tissue donation. The family may have information that indicates that the potential donor had recently changed their decision regarding donation but had not had the opportunity to formally register their new decision. Further in deceased tissue donation the information required to assess the safety of the donation is by necessity second hand, usually from family members of the individuals who knew the potential donor well. It is therefore very important that the family are engaged with the process at the time of donation to ensure that they provide all information that is required to assess the safety of the donation. Further while it is very important that tissue donation potential is maximised to be able to meet the clinical demand for such donations it is equally important to respect the grief of acutely bereaved families and to also avoid any potentially damaging media attention that may result if tissue donation

were to be progressed in the face of family opposition in cases where the individual had taken the decision in life that they would have liked to be a donor at the time of their death.

Further a family's refusal to agree to donation may be a warning of potential high-risk behaviour—some families may prefer to say they do not wish donation to take place rather than providing highly personal and sometime embarrassing information about the donor in response to the sometimes probing questions that are asked of all donor families. It is therefore important to sensitively engage with the family; in the face of refusal, proceeding at all costs is unlikely to be sensible and should be discouraged. Trying to understand the family's reason for refusal would be important to reach the right decision for that donor and their family both on a personal level and to ensure the safety of the donation [4, 5].

2.4 Age Cut-Offs

The types of tissue donation progressed from an individual donor varies according to the donor age. Some of the age limits applied for tissue donation are relatively empirical, and indeed different tissue banks apply different age cut offs for the same type of tissue donation. The age limits recommended for a number of tissue types donated quoted in this section are based on the JPAC UK guidelines [6], which provide generic guidance on the recommended age limits for the different types of tissue donation. These age limits can of course be varied if a tissue bank can demonstrate that a particular tissue remains suitable for transplantation at older age limits.

When considering heart valve donation it is important to note that most heart valve donors are adults while most heart valve recipients are young children. When deciding to use a particular valve for an individual patient important considerations include the diameter of the valve as well as the length of vessel retained with the valve. The UK guidelines recommend heart valve donation from as young as 32 weeks gestation, up to 70 years of age. As individuals get older the effect of hypertension/cardiovascular disease are most pronounced on the aortic valve with a much lesser effect on the pulmonary valve. Indeed a significant proportion of aortic valves from donors above 60 years of age are found to be unsuitable for clinical use due to the presence of calcification or other age-related changes while pulmonary valves are often still in good condition up to and including 70 years of age.

The UK guidelines recommend that tendon donation can be progressed from donors between 17 and 60 years of age, the upper age limit to minimise the risk of wear and tear in the tendon from increasing age.

There is no upper age limit for skin donation—as long as the skin appears normal and not too thin (a consideration in older donors) then skin donation can be progressed. It is important to ensure that the donated skin is free of skin lesions and tattoos. The UK guidelines recommend an upper age limit of 70 years similar to heart valves, mainly as it not cost-effective to progress skin only donation in older donors. When considering progressing skin donation a consideration is the size of

the donor—the yield of skin from smaller donors is unlikely to be sufficient to be clinically effective.

The current UK age guidelines for cornea donation are 2–95 years of age. In reality if the cornea is clear cornea donation is likely to be suitable.

For bone donation the current recommended age limits are 17 to 50 years for structural bone donation, while there is no upper age limit for non-structural bone donations.

2.5 Medical, Behavioural and Social Assessment

All tissue donors are tested for the potential presence of a number of blood borne infections; mandatory testing within Europe at present includes serological testing for the presence of HIV, hepatitis B, hepatitis C and syphilis, and where indicated HTLV [7]. However, despite this mandatory testing for the main transplant transmitted infections, donor assessment to assess the risk of transmission of infection remains important, partly due to the possibility of false negative results in any assay carried out and partly due to the possibility of new emergent infections, the risk of which may (although not necessarily so) be reduced by deferring potential donors who are identified as showing high-risk behaviours. Further there are some current infections, namely prion related conditions, that cannot be screened for.

Donor assessment is also important to assess the risk of transmitting diseases other than infection, the main condition being the risk of transmitting cancer from donors with undiagnosed cancer at the time of their death.

While not ideal, and considered by most a blunt tool, donor medical, travel and behavioural questionnaires looking for the potential presence of infection or disease that could negatively affect the suitability of tissue donation therefore remain an important tool in ensuring the safety of tissue donations. In the setting of tissue donation after death the information obtained is always second hand from the next of kin (who knew the potential donor best), to the best of their knowledge. In view of this the number of questions used to elicit the medical, travel and behavioural history are numerous to try and gather as complete a picture as possible. When reviewing the information provided, it is important to be mindful that the information provided is second hand and may therefore be inaccurate, so that caution needs to be exercised when deciding whether to release a donation for transplant or not particularly in the setting of incomplete information being available or when contradictory information has been provided. It is also important that the person who is best placed to answer the different questions is approached—for example in the case of an adult donor, while the available next of kin may be the donor's parents, the parents may not be best placed to answer social and behavioural questions if the donor also had a partner or friends who were better placed to answer questions about the potential of drug taking or sexual contact for example.

It is important to ensure that all relevant information about a particular potential donor is obtained and reviewed, including the details of their last hospital admission (if relevant) prior to donation. This will involve reviewing the medical notes, in

particular looking for findings that would suggest untreated infection being present or red flag symptoms that may suggest the presence of an undiagnosed condition at the time of the donor's death such as symptoms suggestive of prion disease or underlying malignancy.

Prior to proceeding with tissue (or organ) donation an external body examination must be carried out as a further donor check looking specifically for findings that may suggest underlying infection or significant disease that could adversely impact on the safety of the donated tissue, in particular looking for the potential of track marks, fresh tattoos or body piercings, unexplained rashes, evidence of malignancy, evidence of infection etc. (refer to Annex IV of Commission Directive 2006/17/EC: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:038:0040:0052:EN:PDF [7]). This external body check provides further support for the safety of the proposed donation. Any unexpected findings need to be investigated as necessary to ensure there are no conditions in the donor that would adversely impact the safety of their donation.

If the tissue donor is also an organ donor, organ retrieval surgeons will carry out a visual inspection of all internal organs at the time of retrieval. Their findings must be documented in the donor's medical notes and any unexpected findings, e.g. previously unidentified lesions that could be cancerous, communicated with any tissue banks who are planning on retrieving tissue from the donor as this may impact the suitability or otherwise of the proposed donation. In fact in the case of organ donors the examination at the time of organ retrieval is akin to a post-mortem examination and may lead to important findings that could impact the safety of donation.

Some tissue donors will also have a post-mortem carried out after donation—usually in the case of donors who die unexpectedly or in suspicious circumstances. In such cases it is important that the tissue bank reviews the findings of the post-mortem prior to releasing any of the donations for transplant as such post-mortems may again identify a previously unidentified condition that may mean donation is not safe from that donor e.g. evidence of malignancy or infection.

Due to the importance of ensuring as complete a picture as possible about the medical and behavioural history of the deceased donor it is also important to ask for information from the donor's family doctor who may have information that the next of kin may not be aware of; example if the donor was only recently attending for investigation of new symptoms or had previously required treatment for infections that the donor may not have told his next of kin about.

When deciding on the suitability (or otherwise) of tissue donation from a particular tissue donor a risk/benefit assessment needs to be carried out in each and every case, taking into consideration all the information gathered from the various different avenues i.e. next of kin, hospital notes, family doctor, donor examination prior to tissue retrieval (and/or at organ retrieval) and post-mortem (if carried out). Caution needs to be taken when the information appears incomplete or contradictory. Due to the fact that donated tissue can be stored for extended periods of time there is sufficient time to obtain detailed information from a number of sources as described to ensure the safety of the donation.

The EU Directive [7] on the quality and safety of tissue donation provides broad guidance about the conditions that need to be excluded to ensure the safety of donation (specifically refer to Annex I of Commission Directive 2006/17/EC: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:038:0040:0052:EN:PDF). This Directive has been incorporated into national law in the various EU member states. Locally available national guidelines are available in most countries translating the EU Directive into professional guidance on what conditions would raise concern about the safety of donation and vice versa. In the UK deceased donor selection guidelines can be found on the publicly available website of the Joint United Kingdom Blood Transfusion and Tissue Transplantation Services [8]. These detailed guidelines aim to provide generic guidance to help ensure that tissue donor assessment is carried out consistently.

It is worth noting that the risk of disease transmission through cornea donation is different to that of other types of tissue donation, due to the fact that the cornea is avascular. Indeed while a history of malignancy is considered a contraindication for most types of tissue donation, the presence of most types of solid cancers (either current or historical) would not necessarily be a contraindication to cornea donation, although haematological cancers and cancers of the eye would still lead be a contraindication to cornea donation. The UK guidelines [8] indeed provide separate guidance on the suitability of cornea donation as different to other vascular tissues.

2.6 Blood Sampling and Haemodilution

The EU Directive on the quality and safety of tissue donation [7] (specifically refer to Annex II of Commission Directive 2006/17/EC: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:038:0040:0052:EN:PDF) defines the mandatory minimum testing that needs to be carried out on each tissue donor to minimise the risk of transplant transmitted infection. For deceased donors the mandatory testing for blood borne infection includes serological testing for HIV, hepatitis B, hepatitis C and syphilis. HTLV testing is also indicated if the donor, their sexual partner or their parents lived, or originated from, high-HTLV incidence areas.

Individual tissue banks often carry out more extensive testing than the minimum testing required by law. Most European tissue banks also do NAT testing for HIV, hepatitis B and hepatitis C. The UK also tests all donors for HTLV irrespective of their country of origin and has recently also started to do HEV NAT testing.

Additional testing for the possibility of the presence of other infections may also be indicated based on the donor's history, in particular their travel history—for example testing for West Nile Virus, malaria and other tropical viruses depending on the destination and timing of any travel.

When taking blood samples for blood borne infection testing it is important to consider the timing of blood sampling and the possibility of haemodilution . Due to the changes that happen in the blood after death it is preferable for blood sampling

to take place as close to the time of death as possible, and if possible before circulation ceases, to ensure reliable results. If the latter is not possible then blood samples must be taken within 24 h of death to minimise the risk of both false positive and false negative results [7].

In donors who have had blood loss and transfusion/infusion of colloids or crystalloids it is also important to consider the potential of haemodilution leading to false negative results particularly in donors who may have low levels of antibodies. The risk of missing HIV, hepatitis B and hepatitis C infection is reduced if also carrying out NAT testing, however before accepting the testing results it is important to consider the potential impact of haemodilution. As per the EU Directive [7] tissue establishments must validate the testing procedures used if wishing to accept blood samples (for blood borne infection testing) that are more than 50% haemodilute. The decision making process and the haemodilution algorithm currently used in the UK is shown below in Fig. 2.1a, b.

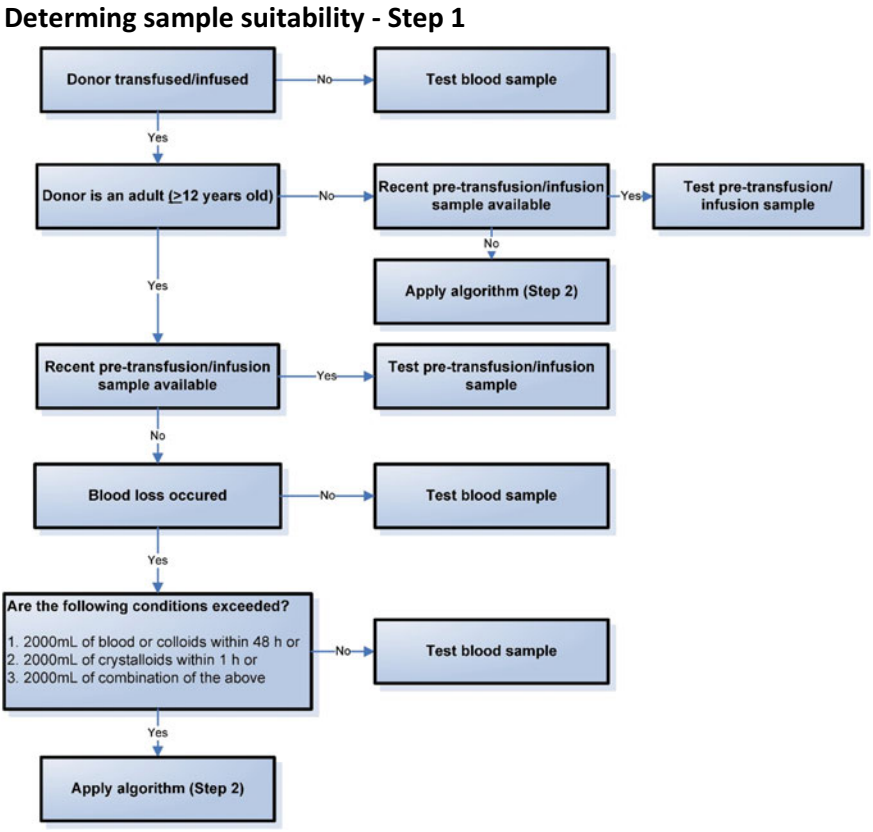

Fig. 2.1 **a** Flow chart to follow to determine whether a haemodilution calculation is required or not. **b** Haemodilution calculation currently in use in the UK

Determing sample suitability - Step 2

Donor ID:		Date & Time blood sample taken:	

Step 2A

Calculate plasma volume	Donor weight (kg) $\frac{\_\_\_\_\_}{0.025}$	_____mL
Calculate blood volume	Donor weight (kg) $\frac{\_\_\_\_\_}{0.015}$	_____mL
A) Record total volume of blood transfused in the **48 h** prior to death or sample collection (whichever comes first).	_____mL of RBC transfused/48 h _____mL of whole blood transfused/48 h _____mL of reconstituted blood/48 h	Sum A: _____mL
B) Record total volume of colloid infused in the **48 h** prior to death or sample collection (whichever comes first)	_____mL plasma/48 h _____mL platelets/48 h _____mL albumin/48 h _____mL HES or other colloids/48 h	Sum B: _____mL
C) Record total volume of crystalloid infused in the **1 h** prior to death or sample collection (whichever comes first)	_____mL	Sum C: _____mL

Step 2B

Calculated Plasma Volume	_____mL	Sum B + Sum C	_____mL	
Calculated Blood Volume	_____mL	Sum A + Sum B + Sum C	_____mL	
Calculate plasma dilution	Is Sum B + Sum C > plasma volume?		No	Yes
Calculate blood dilution	Is Sum A + Sum B + Sum C > blood volume?		No	Yes

If the answers to **both** questions are 'No', the post-transfusion/infusion sample is acceptable

If the answer to **either** of the questions is 'Yes' use a pre-transfusion/infusion sample. If a suitable sample is not available, seek expert advice and inform transplant centre, testing laboratory, tissue bank as necessary.

RBC = red blood cells; HES = hydroxyethyl starch

Fig. 2.1 (continued)

2.7 Timing and Venue of Retrieval

Different to organ donation, tissue donation can take place several hours after death. As previously mentioned in tissue donation there is no requirement to maintain metabolic function; instead the concerns with tissue donation is the structural integrity of the donation as well as minimising the risk of contamination from

endogenous micro-organisms due to the possibility of transmigration of micro-organisms from the gastro-intestinal tract and also from micro-organisms that may have been present in the donor in the final hours prior to death as a result of colonisation or infection.

The time cut-offs for tissue donation, with the aim of minimising tissue contamination, vary in different countries and for different types of tissue donated. In the UK tissue donation can take place up to 48 h after death as long as the donor has been cooled (in the mortuary refrigerator) within 6 h of death, except for cornea donation which needs to take place within 24 h of death. If the donor isn't cooled within 6 h of death then tissue donation needs to take place within 12 h of the time of death [1].

On the other hand when referring to the AATB guidance in the USA, the time limits used for tissue donation are different: if the donor body is cooled within 12 h, then tissue donation can take place up to 24 h after asystole; while if the donor body is not cooled then tissue donation must take place within 15 h of asystole [9].

Other countries (personal communication) apply varying time cut offs e.g. some tissue banks limit tissue retrieval to the first 6 h after death, while others will accept tissue retrieval up to 24 h after death as long as the potential donor was cooled within 4 h of death.

Due to the fact that tissue retrieval can take place several hours after death, retrieval does not need to take place in an operating theatre. Indeed tissue retrieval can occur in various other different venues too, including mortuaries, dedicated tissue retrieval suites and funeral homes. Whatever the venue used, care needs to be taken to ensure that tissue contamination is not introduced from the environment at retrieval. Using a dedicated tissue retrieval suite is ideal as tissue retrieval would take place in a controlled environment. If use of a dedicated tissue retrieval suite is not possible then a risk assessment of the environment should be carried out prior to proceeding with retrieval. If retrieving tissue in a mortuary it is important to ensure that no post-mortems are being conducted at the same time as tissue retrieval to minimise the risk of tissue contamination from the environment. Environmental monitoring during the retrieval process provides valuable information that would help the overall assessment of the donated tissue prior to release for clinical use.

2.8 Retrieval Process

Several tissue banks employ medical staff to retrieve tissues; there are however also successful tissue retrieval programme utilising non-medical staff who have been trained and competency assessed in the retrieval of the different types of tissue allowing a more cost-effective delivery of the retrieval process.

Whether medical staff or non-medical staff are responsible for tissue retrieval, the process to follow should be clearly defined in written standard operating procedures to ensure uniform practice. Whatever the venue used for tissue retrieval, the importance of minimising the risk of introducing contamination is paramount. The staff carrying out the retrieval must wear personal protective equipment similar to that used during surgical operations, both to protect themselves during the tissue

retrieval process and also to minimise the risk of introducing contamination. The donor skin needs to be decontaminated and prepared similar to the skin preparation carried out prior to a surgical procedure and a sterile field maintained at all times during tissue retrieval. Culture samples need to be taken from the different tissue donations, and environmental monitoring carried out, to ensure that any contamination that may be present is identified.

2.9 Conclusion

Tissue donation after death includes several different types of tissue. A single tissue donor can enhance and/or save the life of several patients. Such tissue donations are used routinely to treat several different conditions.

As in all aspects of medical and surgical treatment, ensuring the safety of tissue donation is very important and multi-factorial. All aspects of tissue donation can impact on the safety of the donated tissue and need to be managed carefully, from the initial referral right through to processing and storing of the donations.

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Banking and Use of Umbilical Cord Blood

3

Sergio Querol, Susana G. Gómez, Daniel Gibson, and Alejandro Madrigal

Abstract

Umbilical Cord Blood (UCB) is an alternative source of hematopoietic stem cells for transplantation. A UCB bank is a multidisciplinary structure that is responsible for the recruitment, collection, processing, testing, cryopreservation, storage, listing, reservation, release, and distribution of units for administration. Also, UCB can be transferred for life-saving research. Before labor, adequate information and consent, including possibility of repurposing donation shall be performed. After delivery and using appropriate techniques placental blood can be collected and processed for therapeutic purposes. Cell processing normally consists of volume reduction to achieve a reasonable reduce of storage area. Automatic procedures have some advantages on reproducibility. A banking project starts with an establishment phase where banks should have a rapid growth to achieve their operational size. Then a maintenance phase follows, once the optimum inventory is achieved where UCB banks should focus in increasing quality and diversity. As consequence a decrease in numbers of newly added units are expected. The fact that less than 20% of collected units have more than 150x107 total nucleated cells, estimated to be the threshold for efficient inventories, challenge ethics of donation programs. Therefore, UCB banks need to research on alternative uses to add value to the surplus units and the available inventory. Finally, integration with other cell therapy services will reduce fix costs of operation and contribute sustainability.

S. Querol (✉) · S. G. Gómez
Banc Sang I Teixits, Barcelona, Spain
e-mail: Squerol@bst.cat

D. Gibson · A. Madrigal
Anthony Nolan, London, UK

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3.1 Introduction

Umbilical Cord Blood (UCB) transplantation is an alternative source of hematopoietic stem cells for transplantation. After the first procedure performed in Paris by Dr Gluckman team, more than 40,000 procedures have been performed up to 2020 [1]. According to the World Marrow Donor Association (WMDA), more than 750,000 units are registered in Bone Marrow Donor Worldwide searchable for any patient in need [2].

The use of UCB cells has several advantages including absent risk for the donor, to be an off-the-shelf medicinal product, and, clinical benefits like low incidence of graft-versus-host disease which result in an increase of the donor pool availability [3]. Presence of UCB banks have therefore allowed to decrease substantially the unmet needs and facilitate the universal access to the therapy for patients searching for an adequate stem cell donor. However, the number of stem cells is relatively low delaying engraftment, and there is no possibility to use a donor lymphocyte infusion after transplantation [4]. There are interesting approaches to improve outcomes including the use of very high cellular units, and protocols for progenitor cell ex vivo expansion that is being successful. Most used protocol in older patients uses reduced intensity conditioning and the double graft approach [5]. Recently, immune active properties like an enhanced graft-versus leukemia effect has been proposed and reconstitution of the immune system is the current research area for achieve an improvement of UCB transplant methods [6].

What is a UCB Bank?

A UCB bank is a multidisciplinary structure that is responsible for the recruitment and subsequent management of maternal donors as well as the collection, processing, testing, cryopreservation, storage, listing, reservation, release, and distribution of units for administration [7]. According their purpose and organization banks can be public, familiar or hybrid:

- Public UCB banks: They are dedicated to the collection and storage of UCB after donation for unrelated use to patients who require a stem cell transplant and do not find an HLA-matched donor within their own family. The mother signs the informed consent and is the source material for the mandatory Infectious Disease Marker (IDM) testing. Units in public UCB banks often have stringent quality criteria (high volume and cell counts, absence of contamination). Units that do not meet these criteria may be used for research purposes if a proper consent is in place. In this model, HLA typing is typically performed after cell processing. Then, UCB unit information gets confidentially uploaded to a registry that transfers to different search engines networks.
- Family UCB banks: “Private” banks are for-profit companies that facilitate the collection and storage of UCB for a possible future use of the donor or by a member of the child’s immediate family. Here, the family has the right to use the UCB unit for their own anticipated need or for future use where the UCB unit is

perceived as "biologic insurance". There is a cost associated with private banking. Often private banks do not have the same stringent quality criteria that are in place in public UCB banks and then mother donor may dispose of the unit based on the wishes of the family. The units are not normally HLA typed. Information about privately stored UCB units is not uploaded onto search registries or match programs.

– Hybrid UCB banks: The hybrid approach consists of collecting units both for private storage and for public banking that will be searchable and available for unrelated transplant. This model allows the advantages of private banking to those who wish to use the service but also funding collection of UCB for public sector.

Regulatory Framework

The regulatory framework of UCB collection and processing to make it available to patients in need has evolved considerably over the past two and a half decades. What began as an effort to broaden the available transplant options has evolved into an expensive industry with robust regulation. UCB has become the first FDA licensed tissue product in the United States. Before licensure, concerns about safety and efficacy led the industry to develop voluntary standards, mainly crafted by NetCord-FACT and AABB (international reference on standards) [7, 8]. These accreditation programs established international standards. This is particularly important as there is significant exchange of UCB across national boundaries that has increased over time. Regulations establish standards for safety, quality, identity, purity, and potency from a given point in time going forward. Questions can remain regarding units banked before implementation of the regulations. Some form of regulatory guidance is in place in virtually every country with a UCB bank [9]. Accreditation and regulation has instilled confidence in clinicians, allowing them to select a UCB unit from across a wide range of banks in many different countries.

In this chapter we will focus in public UCB banks and review UCB donation programs, processing and storage of therapeutic products, their clinical use for HPC transplantation, following what is required in the international standards, and finally will discuss challenges that UCB banks confront for their future maintenance.

3.2 Donation Programmes

After delivery and the preceptive cord clamping, a residual amount of newborn blood is left in the placenta. Using appropriate techniques this blood can be collected and processed with therapeutic purposes. As this medicinal product of human origin has been extensively regulated, recycling of this blood shall be done fulfilling the necessary steps of information and consent under an ethical environment. This has required the design of donation programs that will be described below.

3.2.1 Collection Sites

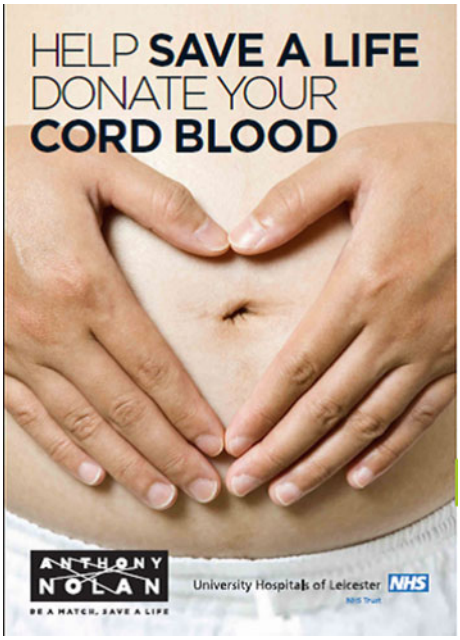
Collection sites are places were a UCB unit is collected. A collection site should have an agreement with the UCB bank to delimitate their responsibilities. Commonly, they are defined as fixed and non-fixed collection sites:

- Fixed UCB Collection Site: A fixed collection site requires an agreement between collection site and the bank for collecting UCB units. This agreement shall describe the interaction between the site and the bank for all aspects of the collection including, at a minimum, personnel training, record keeping, collection, storage, and transportation or shipping of UCB units. Staff for collecting units is usually part of the maternity. There are banks hiring their own staff and the agreement allows them to work on the hospital premises.
- Non-fixed UCB Collection Site: A non-fixed collection site is one with an agreement/contract between a licensed health care professional and UCB bank, to perform the collection and has been trained to cover every aspect of the collection process. This is normally done case by case when the collection is programmed.

3.2.2 Recruitment and Consent

Donor recruitment could be done using different models; although usually starts during the antenatal period, with information given by woman's health care provider, it may also occur as late as at admission into the labour. Programme often provide information leaflets or brochures to inform the mothers-to-be about banking. Pictures below show leaflets used in our banks. Topics described are about defining the therapeutic product, the importance of UCB banking for public use, the collection method, the risks and benefits, and the steps required to donate. For future uses, it is important to include possibility of re-purposing donations. It is during this period that the expectant mothers are encouraged to gather information and ask questions about procedure either directly or through a phone call or a website.

Motivated personnel at collection sites are important to approach potential donors. Training on this step is vital to make sure information provided to these mothers is real and all their questions can be answered. Another aspect to have in mind when designing a UCB program is the targeted population. It's important to understand the ethnicities, their diversity, and how to cover all patients that can benefit from this UCB program.



Educational leaflet used in Anthony Nolan UCB programme (UK)

Educational leaflet used in the Programa concordia banc sang i teixits (Spain)

After information is given, we need to obtain the informed consent of the mother donor. On this consent all aspects related with donation must be written and mothers must sign. Normally, there are questions about performing Infectious Disease Marker (IDM) tests, contacting maternal donor in case an IDM test is positive, using units for research, checking medical notes, etc. all these aspects might be covered and make sure that mothers have given their consent to them. Below, we described some ways of obtaining consent:

- Pre consent: It is preferable to obtain consent in the antenatal period of the mother's pregnancy. This allows her the opportunity to obtain information about UCB banking, to ask questions and make an informed decision regarding her decision to donate. Consent for the collection may be obtained during this period by the mother's health care provider/clinic staff or by bank staff.
- Full Consent: For mothers to give the full informed consent, they must be provided with information regarding all the procedures for collection, processing, testing, storage and use of the UCB donation. It is important that they be informed that their personal and medical information will be kept confidential. Mothers also should be counselled about how potential abnormal test results will be handled and disclosed to them and the importance for mothers to contact the UCB bank in the event that their child develops a serious illness.

Informed consent is not recommended being achieved during active labour since they can be distracted with physical and emotional stress. An alternative option has been proposed if mother arrives to the hospital in labour with no consent and it's not possible to get the consent at this time. A two-step procedure where the information given is basic, as a sort of verbal pre consent that could be signed by the health care personnel as witness, and after delivery, when mother is calm and she can understand and make questions about UCB, obtain the full consent for donation [10].

For private banks, informed consent is wider, as it's a contract between parents and bank. In this case all aspect about donation, UCB unit maintenance and future uses might be thoroughly explained there. Each bank will have their model and contract; however, aspects about informed consent might be similar than for a public bank, as most of the regulatory bodies enforce this step.

3.2.3 Donor Elegibility

After donor recruitment, a trained personnel, needs to determine donor eligibility. It's important to ensure that donation is safe for future patients. Maternal and infant donor eligibility shall be determined based upon results of screening and testing in accordance with each country regulations. To assess donor eligibility a donor medical history interview, that includes assessment for high-risk behaviors, shall be conducted identifying risk factors for transmissible and genetic diseases. The mother will be asked to provide personal and family medical details. There shall be

written criteria for maternal and infant donor evaluation and management. Results of this evaluation shall be documented and reviewed by trained personnel.

The medical history review shall be obtained while mother is able to concentrate on the questionnaire and is not distracted by aspects of labour. Language used must be understood by her. It's not recommended that neither family nor friends serve as interpreters or translators. Confidentiality shall be preserved. If responses generate medical concern the collection should be rejected or cancelled. The mother's travel history to endemic areas shall be obtained and documented and eligibility determined according to national regulations. Screening for human transmissible spongiform encephalopathy, including Creutzfeldt-Jakob disease, shall be documented. Also, for any infectious risks that could occur seasonally or in epidemics. If history for communicable disease risk was obtained in advance of the maternal donor's presentation for delivery, the history shall be updated to include information up to the time of delivery. In the case of a surrogate mother who gives birth to an infant donor not genetically hers, a communicable disease risk history of the surrogate mother shall be obtained. The questionnaire shall include questions to obtain at a minimum genetic history, malignant disease, and inherited disorders that may be transmissible to the recipient.

In addition, IDM tests, performed to maternal blood samples will be obtained within seven days before or after collection of the UCB unit. These samples will be tested for evidence of infection of HIV 1, HIV 2, Hepatitis B, Hepatitis C, HTLV I, HTLV II, Syphilis, and any additional markers according to local regulations [11]. Assays used for testing must be validated for use in volunteer blood or tissue donations. In some countries there is a requirement for a second testing for IDM after six months of donation. In Europe this is not mandatory if a determination using nucleic acid tests (NAT) at time of delivery is performed to the maternal sample.

3.2.4 Collection Techniques

A successful UCB collection should have a high collection volume, a high total nucleated cell count, be non-contaminated and have the proper documentation. These factors are necessary to produce a unit that can be used for transplantation. A UCB banking program's continued viability depends on its ability to maximize the proportion of the units that it collects that are suitable for banking and transplantation.

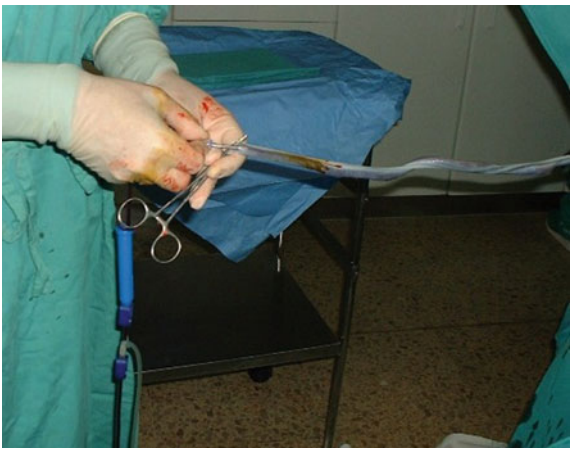
A UCB collection typically involves the following steps:

- The umbilical cord is clamped as distal from the placenta as possible. No interference with labor shall occur. Nowadays, many obstetrical medical associations recommend delay clamping [12]. Evidences suggest that an acceptable time of 1–2 min is compatible with public UCB banking [13].
- A section of the cord is cleaned with alcohol or another disinfectant.

– A needle that is attached to the collection bag is venipunctured into the umbilical cord vein.
– The collection bag is filled via gravity until the cord looks "white" and the placenta and umbilical cord are drained of the UCB bag.
– The collection bag should be labeled appropriately.

There are two main techniques to collect UCB from the cord vein: before the placenta is delivered (in-utero) or after the placenta is delivered (ex-utero). Both collection techniques have their own unique advantages and disadvantages, but both techniques require that the individuals performing the collections be adequately trained. With either technique, the collection is done in a collection bag that contains an anti-coagulant to avoid clotting:

In-Utero Collections: Collection is performed in the delivery room after the baby has been delivered and the placenta is still inside. After the baby has been assessed and the umbilical cord has been clamped and cut, the UCB is collected by venepuncture after the area of the needle insertion has been disinfected. The umbilical cord is cleaned by wiping a large area around the insertion site to clean up excess blood and then scrubbed a topical antiseptic. The UCB unit is collected by gravity, during which time the collection bag should be gently rocked to mix the blood with the anticoagulant in the bag. The collection is complete when blood stops entering the bag. The total time required for an in-utero collection by a health care provider (i.e., physician or nurse/midwife) is less than 5 min. A benefit of in-utero collection is lower collection cost, because the collection is performed by physicians and midwives rather than dedicated UCB bank personnel, and could result in higher volume collected [14].



In utero UCB collection (courtesy of programa concordia banc sang i teixits, Spain)

Ex-Utero Collections: Normally an ex-utero collection is performed by dedicated, trained staff in a separate clean collection room as soon as possible after the placenta has been delivered. This method has the benefit of not interfering with the birthing process. In the delivery room, immediately following the delivery, the physician, nurse or midwife clamps and cuts the cord before passing the placenta and cord in a recipient to the collection staff. The placenta is usually suspended on a stand or frame, allowing UCB to be collected by gravity. The cord is disinfected with an aseptic solution and the needle from the collection bag is inserted into the vein. The collection of the UCB unit can take 5–10 min and should not stop until the cord is "white" or the flow stops. Some of the benefits of ex-utero collections could include less clotting, less bacterial contamination and fewer labelling errors due to dedicated and trained collection staff, but also in less volume collected. However, the added collection staff in the hospitals adds increased expenses to a UCB bank.

Ex utero collection (courtesy of Anthony Nolan UCB bank, pictures taken by Alex Griffiths)

After collection, typically health care provider in charge will complete a report describing labour and completing variables that could be useful to release the unit like presence of fever, complications, type of delivery, etc. If there are any observation of severe adverse reactions need to be communicated according what regulatory requirement established. This should be done through the biovigilance competent authorities in Europe.

3.2.5 Donor Follow up

The UCB bank shall have a policy for follow-up of donors and for management of donation-associated adverse events. In the UCB donation, there are 2 donors, maternal and infant donor, so follow up should be done in both.

Health Questionnaire: All congenital diseases originating from bone marrow derived cells are transmissible on a hematopoietic transplant. Transmission of genetic diseases by UCB units has a higher risk than stem cells from peripheral or bone marrow donation since the disease might not be easily recognized at birth or even for some time later. It is possible that some genetic diseases will be missed as they might not be manifested until six months of life or later. So, ideally UCB bank should request information on the health status of the newborn to be provided by the family even sometime after the donation and prior to the listing of the unit. Mothers shall be provided with information to contact the UCBB if the infant donor later develops a serious disease. This second health questionnaire should be performed usually within at six months after donation but it is not generally mandatory.

Counselling: Mechanisms to inform back the family should be in place in the event of a positive test result for any IDM (other than CMV), an abnormal hemoglobinopathy screening or any other abnormal test found. Any attempt should be made to notify the mother and/or her physician. The UCB Bank shall have policies for handling specific cases.

3.2.6 Non-Frozen Transportation

After collection, UCB units shall be shipped or transported to the processing facility and sometimes, these facilities are far away from the collection sites. A validated procedure for transportation between these two facilities is needed to demonstrate a reliable method. Standard procedures shall be in place to describe current methods.

Time and temperature of storage. After collection, an appropriate conservation will be in place to preserve cell viability and potency as well as to protect the health and safety of the collection unit personnel. A validated method, which assesses maximum time for this transportation shall be in place, to assure that UCB units won't lose their viability and potency, and that they arrive at the bank in an acceptable condition. Standards require that an unrelated UCB unit is frozen before 48 h after collection.

Transportation from the Collection Site to the UCB Bank: UCB units will be transported at an appropriate temperature which will keep the integrity and safety of the cells. As happens with transportation time, a validation shall be done to have the most appropriate procedure regarding the transport conditions. For this validation, transport boxes/containers will be used and it will be performed in a standard and worst case scenarios to make sure all possible conditions are contemplated. UCB units will be transported with a validated and trained courier, following standard procedures written by the UCB Bank.

UCB collection bag will be identified with the following labels/paperwork:

- Unique UCB unit number and maternal blood
- Product name
- Collection site name
- Date/time of collection
- Name and volume/concentration of anticoagulants
- Recommended storage temperature
- Biohazard sign and/or other warning labels, following Regulatory Authorities of the country.

This collection bag shall be placed in a sealed secondary container which avoids any leakage. This secondary container shall be placed in an outer container validated to keep recommended temperatures during transportation to the processing laboratory. There shall be in place a procedure to document that the recommended temperature is kept or a continuous electronic monitor will be used. The outer container will be labeled with the product name and made using a material that avoids any leakage, pressure changes, shocks or other incident or deviation which could affect the UCB unit integrity.

All transportation records shall allow tracking back to the UCB unit from the collection site to the UCB Bank and any deviation reported. Transportation records shall identify the personnel at the collection site responsible for the transportation, date/time of transportation, identity of the trained courier and date/time of reception at the bank's processing lab. Upon receipt, the integrity of the UCB units and their containers will be checked and any deviation will be recorded within the processing records.

3.3 Umbilical Cord Blood Processing and Storage

A UCB bank must have appropriate facilities and personnel for the receipt, processing, testing and storage of UCB and maternal blood. All processes should be performed in compliance with the Regulatory Authorities of the country where the bank is located. Where aspects of processing, testing or storage are performed by an external party to the UCBB, there must be a written agreement in place between the bank and the external party providing the service. Below we describe processing aspects in place in our UCB banks.

3.3.1 Banking Structure and Resources

Processing Laboratory

The UCB Processing laboratory needs to be secure and have adequate space to perform all activities in a safe and sanitary manner. There must be a well-documented process for cleaning and sanitation. Relevant environmental conditions, such as temperature and humidity, need to be defined by the bank and monitored. Environmental monitoring including colony growth and particle counts should be carried out on a regular basis to ensure the facility maintains within the acceptable limits that have been set within the standards. The data on environmental monitoring should be recorded and trended over time to identify early any rising risk to the processing facility.

Cryogenic Storage Area

UCB units are stored in either liquid or vapor phase liquid nitrogen at −150 °C or colder. In order to maintain long term stability all refrigerators, freezers and cryostorage tanks used for storage of UCB units, associated reference samples, and maternal samples, have a system to continuously monitor or regularly record the temperature. In addition, there use alarm system in place 24 h a day in order for staff to be notified immediately, and with adequate time to respond, if a problem occurs with rising temperatures or liquid nitrogen levels. Finally, the processing laboratory needs additional storage devices of appropriate temperature in the event that a primary storage device fails.

Cryogenics area in Anthony Nolan cell therapy centre-UK (Picture taken by Stephen Pennells)

Long-term storage device in the clean room of the barcelona UCB bank (Banc Sang i Teixits, Spain)

Testing Laboratories

The UCB Processing laboratory has testing control procedures in place to support the processing and characterization of UCB units. This is vital to ensure the integrity and quality of the UCB unit in terms of how well the unit will engraft post-transplant, but also to ensure the safety of the recipient with respect to transmission of disease and microbial contamination. Where a specific test is not performed by the UCB processing laboratory per se, the UCB Bank needs to have agreements in place with the external parties who perform these tests on behalf of the Bank. Testing should be undertaken in accordance with that required by the Regulatory Authorities of the country where the bank is located. Testing control procedures need to ensure the use of established and validated appropriate assays, standards, and test procedures for the evaluation of the UCB unit, with appropriate identification, linkage and handling of all reference samples.

3.3.2 Reception and Process Acquisition

Reception

The decision as to whether a collected UCB unit will be acceptable for processing and banking will be made based on the acceptance criteria specified by the bank. The acceptance criteria will include parameters such as UCB volume, total nucleated cell (TNC) count, factors identified by maternal and family history, transport conditions and cell viability. UCB unit acceptance criteria adopted by each bank should be based on rationale and justified, usually based on quality and safety of the end product. However, many banks have further refined their acceptance criteria based on economics and the desire to build an international inventory of UCB units with very high TNC or percentage of ethnic minorities. UCB units of high TNC are

highly desirable for adult transplants, where there is a positive correlation between number of TNC infused/kg and successful transplant outcome. Many UCB banks are now committed to processing and storing only those UCB units with high TNC (ex. > 120 × 10^7 TNC), based on the greater likelihood of these units being used and balanced against the cost of processing and storage of UCB units with lower TNC that have a low likelihood of being requested for transplant [15].

Triage

The collected UCB unit should be received at the processing laboratory in a validated, secure, temperature-monitored transport container. On receipt of a shipment a series of checks need to be performed on the collected UCB, associated samples, and documentation to verify contents and determine if the specific acceptance criteria are met.

Initial triage of a collected UCB unit and determination of its acceptance to continue to processing will be based upon parameters such as volume, TNC content, correct documentation and labeling, signed maternal donor consent, appropriate transport temperature, absence of large/multiple clots and acceptable time in transit from collection center to processing laboratory. For NetCord-FACT accredited UCBBs, the unrelated UCB unit must arrive at the processing laboratory in time to allow initiation of cryopreservation within 48 h of collection (this time is extended to 72 h for related or directed UCB donations).

Acquisition to Process

Once a UCB unit meets the initial acceptance criteria, as described above, it will continue on to be processed. The processing of UCB units is an expensive and time-intensive exercise and the establishment of appropriate initial acceptance criteria by a bank will ensure that only those UCB units of the highest quality are processed.

3.3.3 Volume Reduction

Volume reduction of UCB is considered essential to the provision of a high-quality product and cost-effective UCB banking. The final product volume and cellular characteristics are dependent on the collected starting product as well as the processing/separation methodology.

Rationale

Reducing the volume of the final product increases the stem cell yield available for transplantation. It allows for storage efficiency in terms of space and cost, and, most importantly, reduces the risk of ABO incompatibility and DMSO toxicity to the potential recipient [16]. Despite some loss of cells, volume reduction has additional practical and clinical benefits; the process yields RBC and plasma components as waste products that can be used for immediate or future testing, thereby minimizing the loss of the actual stem cell product for testing purposes.

Methods

Early attempts at volume reduction resulted in unacceptably high loss of haematopoietic progenitor cells (HPC). Over the years a variety of techniques have been explored, including density gradients, RBC lysis and differential sedimentation; however, most were unsuitable for large-scale banking as they were manual, labor intensive and employed open systems.

Over the past decade there have been three major methods used in large-scale banking which produce reproducible results that could be standardized (reviewed by Armitage [17]). These include the manual hydroxyethyl starch (HES) method, the semi-automated bottom and top method—BAT process, and the newer fully automated and programmable closed systems—Sepax (Biosafe SA, Eysins, Switzerland) and AXP™ AutoXpress™ (ThermoGenesis Corporation, Rancho Cordova, CA, USA) and more recently the SynGenX—1000 system (SynGen, CA, USA) which were designed specifically for the UCB banking market, although the latter is now discontinued for UCB processing. More recently, other UCB processing platforms have entered the market, such a Prepacyte-UCB, which utilizes a proprietary reagent for UCB processing and is being utilized by some banks. Additional platforms being assessed by those in the field include novel filtration methods and double extraction techniques.

Expected Results

Several groups have performed or reviewed in-depth comparative analyses of the various UCB processing platforms being utilized by UCBB around the world. These studies have evaluated TNC and $CD34^+$ recovery as well RBC reduction and colony forming unit (CFU) assays to evaluate engraftment potential. Each system has its advantages and disadvantages, with no one system at this stage being clearly superior to the other [18].

Whichever platform is employed by the bank for UCB processing, it is essential that the equipment and reagents used do not adversely affect the viability of the UCB units and that the process does not allow the introduction of adventitious agents or the transmission of communicable disease.

Acceptable end points for processing of UCB should be defined by the bank, based on documented rationale and validation. Examples of desirable end-points are:

- A minimum threshold for post-processing TNC recovery. Based on the literature, ideally this would be 70% or greater.
- A target range for RBC depletion
- A target limit for final volume after processing
- A target limit for viability.

3.3.4 Cryopreservation

The selection of a suitable protocol for cryopreservation of UCB for use in transplantation is critical to optimize the recovery of functionally viable progenitor cells, most of which lie within the CD34+ compartment. Some important considerations that are potential sources of cell damage include the type and concentration of cryoprotectant, the cell concentration, and the cooling and warming rates.

SOPs related to cryopreservation should specify that the following information is recorded for each unit:

- TNC concentration within a defined range
- The cryoprotectant, its final concentration, and the duration of the cell exposure prior to freezing
- Method of freezing and end-point temperature of cooling
- Cooling rate within a defined range
- Freezing curve parameter within a defined range
- Storage temperature.

UCB units must be stored in freezing bags designed and approved for the cryopreservation of human cells and placed into metal canisters to afford protection during freezing, storage, transportation and shipping. It is important that after filling, each freezing bag is visually examined for possible leaking and breakage of seals.

Cryoprotectant

Dimethyl sulphoxide (DMSO), a membrane permeating molecule, has been used for over 30 years as the cryoprotectant for freezing HPC, almost exclusively in a final concentration of 10%, although there are some reports to indicate that a 5% concentration is also appropriate. In general, a concentration of 10% DMSO is considered optimal for UCB.

Dextran-40 is a neutral polysaccharide of high molecular weight that does not easily permeate white blood cells and maintains a favourable osmotic environment. When used in conjunction with DMSO, Dextran-40 enhances the cryoprotective effect by allowing stabilization of the cell membrane.

While alternatives have been proposed, it is generally considered that a concentration of 10% DMSO and 10% Dextran-40 results in the best recovery rates for TNC, CD34 + and colony-forming units (CFU) and is therefore the ideal cryoprotectant [19].

Prolonged exposure of cells to DMSO can result in damage to cells. It is therefore essential that the duration from addition of cryoprotectant to initiation of freezing is minimized and the time allowed is validated by the cell processing lab.

Controlled Rate Freezers

Ideally, UCB units should be cryopreserved using a controlled rate freezer with a validated freezing program. Most, but not all, banks use cooling rates of 1–5 °C/min in order to allow the cells to slowly dehydrate as the ice phase progresses and the extracellular solute concentration increases. Traditionally liquid nitrogen-based

controlled rate freezers have been used, but newer technologies now use electric based engines to freeze down the cells in a controlled manner. This would allow for UCB banks without a strong liquid nitrogen delivery system to freeze UCB without compromising quality.

Where controlled rate freezing is not performed, an equivalent procedure, such as "dump freezing", may be used. If an equivalent procedure is used, it must be validated to maintain equivalent recovery and viability of nucleated cells. Although this is a suitable method, controlled rate freezers can compensate for the latent heat released when the UCB freezes below a certain temperature, which reduces ice formation within the cell. Ice build-up during freezing can have detrimental effects at the point of thawing to stem cell viability. The controlled rate freezing method for UCB is usually considered standard.

Cryo-freezing bag (courtesy of Anthony Nolan UUCB bank; picture taken by Stephen Pennells)

3.3.5 Testing and Product Conformity

Quality Assessment

UCB banks aim to provide a high-quality product capable of reconstituting a patient's immune system by preserving the ability for the stored cells to home to the bone marrow and repopulate the haematopoietic system in a timely fashion. As there are numerous different methodologies to collect, process and store UCB, each being a potential source of variability, reproducible manufacturing methods need to be adopted to ensure process consistency, reliability and more importantly predictability. Critical quality attributes (CQAs) are employed to select and compare whether the product will achieve this. The complexity of identifying characteristics of a cell population that guarantee its function is argued by some to be unachievable with current technology due to the complexity and number of potential dimensions in the data sets, and limited knowledge of mechanism of action.

We may have quasi-CQAs such as TNC count, CD34 expression, and the colony forming potential to monitor the effectiveness of the processes employed and to determine the impact, allowing assessment techniques to quantify variability inherent to the repertoire of processes and donor variability.

UCB users rely on the rigorous assessment carried out in banks to avoid poor engraftment post thaw and that cellular enumeration is used as a surrogate of graft potency in the absence of a better marker such as CD34+ , CFU or cellular viabilities due to ease of standardisation across all banks. As is accepted in bone marrow transplant (BMT) hematopoietic potential is proportional to the TNC and thus correlates to transplantation endpoints. Evaluating transplant data and by observation of UCB usage it is apparent that TNC is the greatest attribute (after HLA disparity) transplant Centre's (TC) consider during unit selection. As a result registries will facilitate algorithms that put the greatest emphasis on TNC dose above any other cellular attribute. TNC is of critical importance in predicting graft success alongside HLA disparity and that increasing TNC dose can mitigate the disadvantages of a greater HLA disparity at 5/6 level match. Other factors such as the colony forming unit (CFU) assay and markers such as CD34+ have been shown to correlate to engraftment however these factors are not weighted highly in selection algorithms.

A UCB bank must be able to confirm testing and product conformity in order to be able to release a UCB unit for transplant. All tests performed must use established and validated relevant assays and, if required, comply with that mandated by local Regulatory Authorities.

Safety

In order to provide a safe UCB product for release, it is essential that UCB is screened for those infectious diseases which can be transmitted via blood. Maternal blood obtained within 7 days before or after the collection of the unit is used as a surrogate test for IDM, and is strongly reflective of the infectious status of the UCB units due to the shared circulation during gestation. Testing the UCB unit for IDM provides an additional degree of safety. At a minimum, prior to release for administration, the maternal donor of each UCB unit must be tested for evidence of infection by at least the following communicable disease agents using licensed donor screening tests when available according to national regulations (below a list of virus to test is presented as required by Netcord-FACT standards):

- Human immunodeficiency virus, type 1
- Human immunodeficiency virus, type 2
- Hepatitis B virus
- Hepatitis C virus
- Human T cell lymphotropic virus, type I
- Human T cell lymphotropic virus, type II
- Treponema pallidum (syphilis)
- Any additional agents required by national regulations.

UCB units for unrelated use must be shown to be free of microbial contamination. Microbial testing must be performed using a system validated for the growth of aerobic and anaerobic bacteria and fungi.

Prior to release for administration, each UCB unit must have undergone hemoglobinopathy screening. Abnormal red blood cell diseases are carried by populations previously considered unable to be affected by them, and therefore hemoglobinopathy testing must be performed regardless of the family's ethnic background or history.

Identity

An error in UCB unit identity could be catastrophic to a transplant recipient and it is therefore imperative that all identity checks are performed on a UCB unit prior to release for administration [20].

Human leukocyte antigen (HLA) typing must be performed on a reference sample from each UCB unit. HLA-A, B and DRB1 loci must be determined using DNA-based methods and result included when listing a UCB unit on the search registries. Many transplant centers also use HLA-C and DQB1 matching in their UCB unit selection algorithm, and therefore the UCB Bank may also wish to determine and report HLA-C and DQB1 typing. At a minimum, DNA high resolution molecular typing must be performed for Class II DRB1 typing prior to release for administration. It is recommended that HLA typing is performed in an accredited laboratory.

Prior to release of a UCB unit for administration it is imperative that HLA-identity of the UCB unit to be shipped is confirmed to match that of the UCB unit requested for transplant. This is known as confirmatory HLA typing. Ideally, confirmatory typing will be performed on a sample taken from a contiguous segment of the UCB unit. However, this is often not possible in older UCB units, prior to the requirement for contiguous segments, and therefore processes must be in place to ensure linkage between the reference sample used for HLA typing and the UCB unit. The use of a contiguous segment to verify UCB unit identity through HLA typing of a UCB unit is considered critical to patient safety.

HLA typing on maternal blood may also be performed prior to release of a UCB unit. Haplotype matching between maternal donor and infant donor confirm linkage between the two and serves as a secondary confirmation of identity. Furthermore, many transplant centers now consider non-inherited maternal allele (NIMA) matching as part of their analysis of transplant outcome, which is not possible if maternal HLA typing is not performed.

ABO blood group and Rh type must be reported prior to listing a UCB unit for search and confirmed, if possible. Nowadays using NGS approached all these genetic tests can be done once with very few sample requirement (i.e. attached segments of the freezing bag) [21].

Purity

The purity or cellular content of a UCB unit is often an important factor in selection of a UCB unit for transplant. It is now well-established that a minimum of 2.5–

3×10^7 TNC/kg is required to ensure the best chance of engraftment and favorable outcome post UCB transplant. Since the number of TNC infused per kg will be dependent upon the size of the recipient, it is highly desirable the UCB units contain a high number of TNC (ideally greater than 120×10^7).

The total nucleated red blood cell count (nRBC) must be reported. This is in order to allow determination of the contribution of nRBC to the nucleated cell population for the Transplant Center to facilitate an informed UCB unit selection.

CD34 is a surface glycoprotein present on stem and progenitor cells, and the number of CD34+ cells in a UCB unit provides an indication of the number of stem / progenitor cells available for transplant. The total number of CD34+ cells is key to be reported since there are one the most important predictor of engraftment [22].

In order to screen for any hematological abnormalities the infant donor may have, a cell blood count with differential should be performed, with parameters for neutrophils, lymphocytes, monocytes and platelets defined.

Potency

Potency testing to determine the growth potential and viability of progenitor cells in a UCB unit should be performed post-processing (prior to cryopreservation), in addition to being performed on a thawed sample prior to release for administration.

Viability and/or potency can be assessed measuring viable CD34+ cells in the UCB unit using flow cytometry and/or by colony forming unit—assay (CFU) to enumerate clonogenic hematapoietic progenitor cell (HPC) growth and differentiation in semi-solid media. Potency assays should be performed both post-processing (prior to cryopreservation) and post-thaw, as the cryopreservation and thawing process inevitably leads to some loss of viability of cells; studies have shown that the post-thaw viability or potency of HPC are a more accurate indicator of how a UCB unit will perform post-transplant than the post-processing cell viability/potency results [23].

3.3.6 Inventory Management

The storage of UCB units and associated reference samples must be in a secure storage device, stored in a secure location. The storage device and/or area must be locked when the area is not occupied by Lab staff.

There must be an inventory management system to ensure that each UCB unit and its associated reference samples, maternal samples, and records can be located in a timely manner. This inventory management system should be designed in such a way to prevent mix-ups, contamination of the UCB units during storage, and the improper release of quarantined UCB units.

The inventory management system must be designed in a way to address the duration of the storage for cryopreserved UCB units. The UCB Bank, or facility storing the UCB units, needs to validate the duration and conditions of storage, with particular attention paid to the effects of long-term storage on UCB unit viability, function, and/or stability.

Quarantine

There must be procedures defined and maintained to minimize the risk of microbial cross-contamination of UCB units.

Each UCB Bank needs to have an SOP to define when a UCB unit can be released from quarantine. Each UCB unit must remain in quarantine storage until the UCB Bank Director or delegate has approved the release of the UCB unit from quarantine status based upon satisfactory testing and screening results pertinent to that UCB unit, and as required by the relevant Regulatory Authorities. Quarantine may be temporal, physical, or a designation within the UCB unit record.

Supplies, Reagents, and Equipment

The inventory management system must include a system to document receipt, inspection, verification, acceptance and storage of all critical supplies and reagents used for UCB processing.

It is important that refrigerators and freezers used for the storage of UCB units and all associated reference samples, and reagents used in UCB unit collection, processing or cryopreservation should not be used for any other purpose, in order to minimize the risk of cross-contamination.

Transient Warming Events

UCB units must be stored at −150 °C or colder. There is the potential for significant warmings events to occur when the UCB unit temperature rises above −123 °C [24]. Each UCB Bank needs to assess the potential risk within their processes for transient warming events to occur, such as when a UCB unit is outside of its proper storage temperature for extended periods of time. Examples of these opportunities include transfer of UCB units from the controlled rate freezer to the cryostorage tank, removal of segments for confirmatory testing and storage of UCB units in vapor vessels that may exhibit unstable temperatures when open. Each of the relevant scenarios pertinent to a bank's operations should be validated to show that at the end of processing and all accumulated transfers, the viability of the UCB unit has not been compromised.

Any warming events that may occur after the process of storage must be minimized in order to prevent the occurrence of transient warming events.

3.4 Use of Umbilical Cord Blood for Transplantation

Once a UCB unit is produced and validated, it should release for listing to a stem cell registry that made them available for any patient in need. Physicians on behalf their patient requiring bone marrow transplant procedures might search in these inventories to find the UCB unit that better match patient requirements.

UCB unit are made ready for listing in reference stem cell registries after comprehensively reviewing characteristics for each UCB unit, including maternal donor selection and maternal and infant donor evaluation for the specific medical requirements and testing and product conformity for the minimal product requirements. It is the bank responsibility to be able to ensure that the information provided to the Transplant Center is correct and complete.

3.4.1 Selection Principles

UCB unit selection is performed by transplant physicians usually with the help of transplant coordinators and the advice of HLA experts. They must provide full information on the patient including:

- age, sex, weight
- Diagnosis, stage of the disease, urgency of the transplant
- ABO and Rh blood group
- Infectious diseases marker results
- High resolution HLA typing for −A, −B, −C, −DRB1.

Transplant centers can send their search request to different organizations according to national regulations and registry policies.

Selection criteria may vary between transplant centers but there is a consensus provided by different organizations. For instance, in Europe, the Eurocord group edited a recommendation of how a UCB unit needs to be evaluated to benefit different types of patients [25]. This was also recently published in USA by National Marrow Donor Program (NMDP) and Center for International Blood and Marrow Transplant Research (CIBMTR) [26].

3.4.2 Request for Shipping

The bank must receive a formal request from a transplant center before the work up will start. Return of unrelated UCB units is generally not permitted by international standards. Next to reviewing the list of characteristics of a UCB unit, the bank must perform some required tests before the UCB unit will leave the bank premises.

Verifying UCB unit identity: UCB unit identity can be verified by performing HLA typing using a segment which is still connected to the freezing bag containing cryopreserved UCB cells. The UCB bank should have a policy in place for the cases where there are no remaining attached segments.

Verifying UCB potency: It is required to assess the functional capacity of the UCB unit prior to release to the TC. CFUs are grown from functionally viable cells and increase confidence in UCB unit quality and ability to engraft. Therefore, it is recommended to perform CFU from a frozen segment (or a UCB unit sample based on UCBB alternative policy) prior to release for administration.

Confirming safety if required: IDM testing of the maternal samples is understood to be a surrogate test, and strongly reflective of the infectious status of the UCB unit since the circulation is shared during gestation. Prior to release for administration the results of maternal donor screening tests (HIV1/2, HBV, HCV, HTLV-I/II and Syphilis) should be available. Because of differing requirements by various National regulations, transplant centers may require additional IDM test results.

3.4.3 Frozen Transportation

Cryopreserved UCB unit shall be transported or shipped in a liquid nitrogen-cooled dry shipper to maintain a temperature of −150 °C or colder for at least 48 h beyond the expected time of arrival at the receiving facility. It is mandatory to measure and to document the temperature in the dry shipper throughout the period of transportation or shipment. UCB banks need to arrange how the transport container will be returned to the bank.

The courier should be educated on how to handle the dry shipper and how to take care of the UCB unit. A plan for an alternative transportation or shipping in an emergency should be available. The identity of courier must be known and documented. Tracking and tracing of the transportation of the UCB unit in the dry shipper from the UCB bank to its final destination as well as information about the date and time of packaging and leaving the bank should be documented.

Information about date and time of receipt of the dry shipper and the UCB unit. The use of a reception form to note this information is preferred, including the questions about integrity and internal temperature of the dry shipper and integrity of the unit arriving at the final destination. The problems during transportation include temperature of cryoshipper, condition of UCB unit freezing bag when received at transplant center and post-thaw viability issues. When a problem is detected, this may generate delays in transplantation. It is important to notice that UCB unit shall be received at transplant center before starting any conditioning procedure.

3.4.4 Thawing and Infusion

It is important to ensure the TC receives information on how to handle and use the UCB UNIT. Handling includes thawing and washing of the UCB UNIT. Providing information about indications, contraindications and cautions is the responsibility of the UCBB. A jointly prepared document called Circular of Information for the Use of Cellular Therapy Products is available (www.ISCT.org or www.factwebsite.org). Next to this Circular, banks should be able to provide instructions for a validated thawing process of their UCB unit and the results of the validation. In general, there are three ways to administer the UCB product: direct infusion; 1:1 volume dilution with a hyperosmolar buffer (generally dextran and albumin mixture); or in cases where the infusing volume is too high or the product contains an excess of RBC using an additional washing step after dilution [27].

3.4.5 Transplantation Outcomes

UCB banks need to get information on transplant outcomes in order to fulfill requirements for accreditation as a control of quality of the unit. This information can be provided either by the transplant centers or through outcomes registries such as Eurocord or NMDP-CIBMTR. WMDA also collects severe adverse effects.

UCB banks must provide information on UCB unit characteristics (UCB ID, TNC and CD34 cell counts, HLA typing, ABO, infectious disease markers, date of collection and details on cell processing, cryopreservation, and shipping) to the registry of the transplant center. The transplant center registry will contact the transplant centers and collect information on transplant characteristics and outcomes. A specific form is sent to the center each time a UCB unit is released. Data are typically collected at 3, 6, and 12 months after transplant then annually after that.

The information required may include:

- Patient and disease characteristics
- Conditioning regimen and graft versus host disease (GVHD) prevention
- Numaternal blooder of cells infused
- Early adverse events
- Date of neutrophil and platelet engraftment, chimerism
- Acute and chronic GVHD
- Infectious complications and toxicity
- Relapse
- Transplant related mortality at 3, 6, 12 months and then annually.
- Overall survival and disease free survival.

Outcome data can be sent to the cord banks on request. Overall results are published every year by Eurocord-EBMT, NMDP-CIBMTR, WMDA and other organizations.

Collection of outcome data provides important guidelines on donor selection, indications, role of HLA, prognostic factors and comparison with other stem cell sources [28].

3.5 Future Challenges for Umbilical Cord Blood Banking

A banking project can be divided into two distinct developmental phases. First, the establishment phase where banks should have a rapid growth to achieve their operational size. For that, large collection programs are required to select and process the best donations. This phase normally requires public or charitable funding to compensate the decrease on effective rate of use during inventory growth. Second, the maintenance phase once the optimum inventory is achieved where UCB banks should focus in increasing quality and diversity. As consequence

a decrease in numbers of newly added units are expected. The fact that less than 20% of collected units have more than 150×10^7 total nucleated cells (TNC), estimated to be the threshold for efficient inventories, challenge the ethics of donation programs. More efficient programs are required to ensure collected UCB units meet clinical requirements. The maintenance phase requires very different banking structures for sustainability. Integration with other cell therapy services will reduce fix costs of operation. Finally, UCB banks need to research alternative uses of UCB to add value to the available inventory. We recently reviewed these vision in order to propose this evolution for future developments of UCB banks.

UCB Bank 2.0

A new public UCB bank project should be re-formulated to solve these inefficiencies by improving HSC registry service, reducing numbers of discarded donations, and developing a multidisciplinary cell therapy platform, in structures with low operational costs. Thus, the integration in bigger institutions like blood banks or cell therapy laboratories makes sense. UCB banks should focus efforts in developing new applications of innovative UCB-derived products and services within the following areas [29]:

(1) Improving donor provision service:

In the general inventory, many UCB units have very limited information on their specifications. This generates a number of uncertainties about the final characteristics of these units and a time delay in the donor selection process. Availability of NGS technology allows the upfront characterization of the best UCB unit to minimize releasing times. Developing an off-the-shelf, ready-to-use, high quality inventory of pre-released UCB units will help clinicians to fasten their decisions.

(2) Researching new cellular sources for advanced therapy medicinal products (ATMPs)

Developing a well characterized UCB panel will facilitate the use of these units for other applications. There are proposals formulated in the scientific literature covering areas of cellular immunotherapy and regenerative medicine that need further development. Third-party donor banks of Tregs, NK cells, antigen-specific T cells and iPS have been proposed. Availability of a large pool of GMP-compliant products could be used as starting materials to develop new ATMPs.

(3) Developing blood hemocomponents

High proportions of routinely and aseptically collected units of biological material donated by carefully qualified donors screened for transmissible diseases, whose data are registered in fully traceable electronic data sets, offer the opportunity for novel products development. Some innovative products developed from UCB

blood components have been described like cell and tissue culture media derived from UCB plasma and platelets, platelet gel for wound healing, plasma and serum eye drops, and even red blood cells for transfusion to preterm infants.

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Ocular Tissue Banking

4

W. John Armitage

Abstract

Disease and trauma can cause loss of corneal transparency or distortion of corneal shape, resulting in severe visual impairment. For many of these patients, the only option to restore vision is a corneal transplant where clear, healthy tissue from a deceased eye donor is used to replace the diseased tissue. Corneal transplantation is one of the oldest and most frequently performed transplant operations with at least 180 000 procedures annually worldwide. Hypothermic storage allows corneas to be kept for up to two weeks. Alternatively, organ culture at 28–37 °C, allows up to four weeks of storage. For the great majority of transplants, a healthy corneal endothelium is required. These cells do not readily proliferate in situ and there is a gradual decline in endothelial cell density throughout life. The endothelial cell density is therefore one of the principal quality assessment criteria used by eye banks. Full-thickness corneal transplantation (penetrating keratoplasty) has in many cases been superseded by techniques where only the diseased part of the cornea is replaced. Endothelial keratoplasty, where the graft comprises endothelium supported only on its basement membrane with or without a thin layer of corneal stroma, is now the method of choice for endothelial deficiency. Stromal opacities can be treated with transplants of stromal tissue without the endothelium, retaining the patient's

W. J. Armitage (✉)
Head of Research and Development for Ocular Tissue, Tissue and Eye Services, NHS Blood and Transplant, Bristol, UK
e-mail: w.j.armitage@bristol.ac.uk

W. J. Armitage
Translational Health Sciences, University onf Bristol, Bristol, UK

W. J. Armitage
Bristol Eye Hospital, Lower Maudlin Street, Bristol BS1 2LX, UK

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own healthy endothelium. Sclera and limbal tissue are used, respectively, for reconstructive surgery and for treating ocular surface disease.

Keywords

Corneal transplantation · Penetrating keratoplasty · Descemet stripping Automated endothelial keratoplasty · Hypothermic storage · Organ culture · Limbal stem cells

Cornea is one of the oldest transplants of an anatomically and physiologically intact, viable tissue with the first successful full-thickness corneal allograft being attributed to Eduard Zirm in 1905 [1]. The patient was a farm labourer who had suffered bilateral lime burns, a condition that even today has a poor prognosis, which makes Zirm's success all the more remarkable. At that time, it was not only thought that tissue taken from a deceased donor would be harmful to the recipient but there was no satisfactory means of tissue preservation. The corneal graft was therefore taken from a child's eye that had just been enucleated following an injury and used immediately. By contrast, corneas are now stored routinely in eye banks for up to four weeks and distributed nationally and internationally for elective transplant surgery. A worldwide annual total of at least 180,000 transplants was estimated from a survey of 2010 data [2]. In 2016, more than 45,000 corneal transplants were carried out in the USA with perhaps a further 30,000 each year in Europe. While cornea is by far the most frequently transplanted ocular tissue, sclera and limbal tissue are also used therapeutically for, respectively, reconstructive surgery and the treatment of ocular surface disease. Cryopreserved or freeze-dried amniotic membrane is also used to treat ocular surface disease [3–5].

4.1 Eye Donation

Eyes are typically retrieved up to 24 h after death. In the UK, the whole eye is enucleated with sterile, single-use instruments; but in many countries an in situ excision of the cornea with a 2–4 mm rim of sclera (corneoscleral disc) is performed, leaving the rest of the eye intact in the donor. Restoration of the donor's appearance after eye or corneal retrieval is an important final step of the eye donation procedure.

Some donor selection criteria for corneal transplantation differ from other types of tissue transplant owing mainly to the avascularity of the cornea. It is accepted, therefore, that cancers, except for haematological or ocular malignancies, do not necessarily preclude eye donation and use of the corneas for transplantation. Malignant cells from a cholangiocarcinoma have been found in the vascularized sclera but not in the avascular cornea of a donor's eye, which supports the exclusion of sclera and limbal tissue from donors with malignancies [6]. Moreover, malignant cells have been found in the peripheral cornea in a donor with malignant cutaneous

melanoma [7]. There is a single, well-documented report of transmission of a poorly-differentiated adenocarcinoma by corneal transplantation, which, on review, was thought to be a result of choroidal metastases in the donor's eye [8]. While the risk of metastatic spread of non-ocular tumours to the eye is considered to be extremely low, [9] these cases underline the importance of reviewing a donor's medical history and careful examination of donor eyes by slit lamp to exclude eyes with evidence of metastatic disease in the anterior chamber. Depending on the method of corneal storage, donors with infections and bacteraemia may also be accepted. Apart from these exceptions for cornea, which do not apply to limbal tissue or sclera since these are vascularized, the main exclusion criteria are similar to other tissues. Provided the corneal endothelium is examined to ensure an adequate number of cells, there is no need to set an upper age limit for cornea donation.

4.2 Corneal Transplantation

The cornea forms part of the outer coat of the eye, merging with the sclera at the corneal limbus (Fig. 4.1). It is tough, having both to withstand the intraocular pressure and protect the delicate internal structures of the eye. The human cornea is approximately 0.55 mm thick and is bounded on the outer anterior surface by a stratified epithelium 5–7 cells thick and on the inner posterior surface by a monolayer of closely apposed endothelial cells (Fig. 4.1). The bulk of the cornea comprises a collagenous stroma containing fibroblastic cells, the keratocytes.

The cornea is the major refracting element of the eye and this function critically depends on its transparency [10]. Accordingly, normal cornea allows transmission of between 86 and 94% of light in the visible spectrum. This remarkable degree of clarity is a result of the long-range ordering achieved by the highly structured organization of the uniformly-sized stromal collagen fibrils embedded in a proteoglycan matrix [11]. These fibrils run parallel to each other in sheets (lamellae) that stretch across the width of the cornea with successive lamellae being arranged orthogonally. The fixed negative charge on the proteoglycans draws water and solutes into the stroma from the aqueous humour. In the absence of blood vessels, this influx of solutes is essential for the nutrition of the keratocytes. The consequent influx of water is countered by the endothelium, which forms a passive, albeit leaky, barrier and actively pumps ions, including bicarbonate, from the stroma to the aqueous, which induces an efflux of water thereby controlling stromal hydration [12, 13].

Adult human endothelium has only a limited proliferative capacity and there is a slow, continuous decline in endothelial cell density throughout life from approximately 5000 cells/mm^2 at birth to approximately 2500 cells/mm^2 in 80-year olds [14]. However, disease or trauma exacerbates this decline resulting in endothelial failure, which causes stromal oedema and loss of transparency owing to disruption of the structural organization of the collagen fibrils.

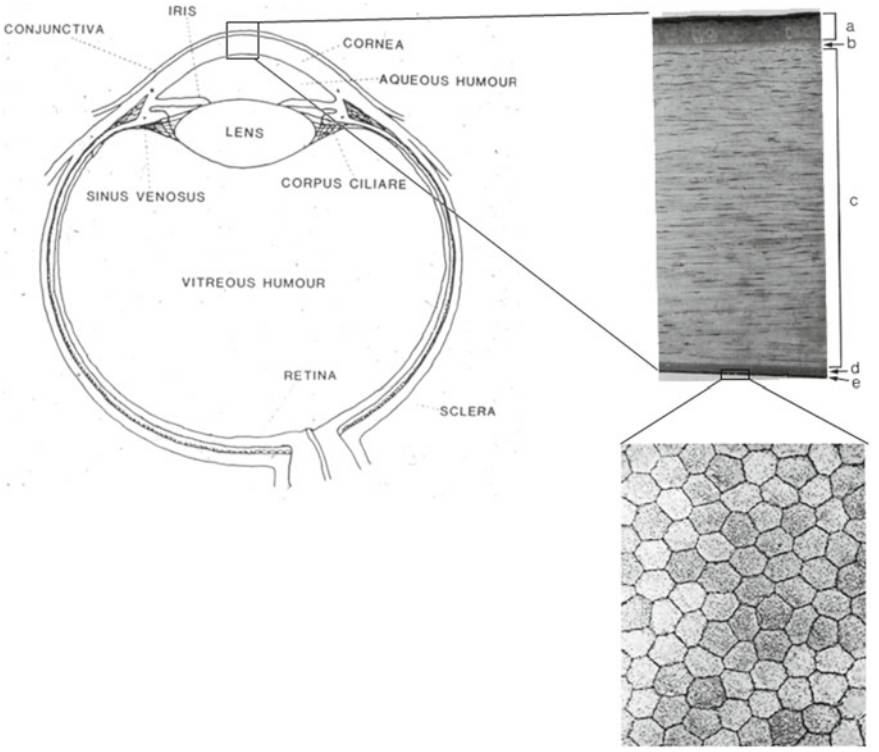

Fig. 4.1 Morphology of eye, cornea and corneal endothelium. Key to corneal section: **a** epithelium; **b** Bowman layer; **c** stroma; **d** Descemet membrane; **e** endothelium. The posterior surface of the endothelium shows a mosaic of closely apposed, mainly hexagonal cells. (Reproduced from Hogan et al. [65] with permission)

Loss of transparency through endothelial failure or from other causes can be treated by corneal transplantation (keratoplasty). More than 50% of corneal transplants in the UK are for the treatment of endothelial disease, principally Fuchs' endothelial dystrophy and bullous keratopathy—the latter being a complication of previous ocular surgery. Another major indication for transplantation is keratoconus, which results in severe visual impairment caused by a change in corneal shape. In this tissue matrix disease, the cornea progressively thins and its surface becomes conical instead of spherical. Collagen cross-linking can slow the progression of this disease, [15] and its application in recent years has led to a fall in the proportion of keratoplasties for keratoconus. Other indications for transplantation include infections, such as *Herpes simplex* keratitis, scarring, opacification and regraft for failed keratoplasties.

In full-thickness corneal grafts (penetrating keratoplasty, PK) a disc of diseased cornea, typically 7.5 mm in diameter, which includes epithelium, stroma and endothelium, is removed and replaced by a similarly sized graft of healthy corneal tissue from a donor (Fig. 4.2). Partial thickness grafts (lamellar keratoplasty) are also used where just the diseased part of the cornea, rather than all three layers, is replaced [16]. In endothelial keratoplasty (EK), dysfunctional endothelium is replaced by donor endothelium supported on either a thin layer of stroma (Descemet Stripping Automated Endothelial Keratoplasty, DSAEK), or even just endothelium on its basement membrane, Descemet membrane (Descemet Membrane Endothelial Keratoplasty, DMEK). The advantages of EK over PK are faster visual rehabilitation for the patient and less astigmatism, which is a common postoperative complication of PK, and the risk of allograft rejection appears to be rather lower for DMEK than for DSAEK and PK [17]. Because of these advantages, EK has increasingly become the preferred treatment option for endothelial failure. Both PK and EK critically depend on an allograft with an intact, functioning endothelial cell layer. Assessment of the endothelium is therefore important to ensure an adequate number of cells and good morphology of this cell monolayer for these types of allograft.

A cell therapy approach to the treatment of endothelial disease is being actively researched and is currently the subject of early clinical trials in Japan. Human corneal endothelial cells are expanded ex vivo and injected directly into the anterior chamber [18]. Effective methods for stimulating endothelial cells to proliferate have been developed and proof of principle demonstrated in animal experiments. If

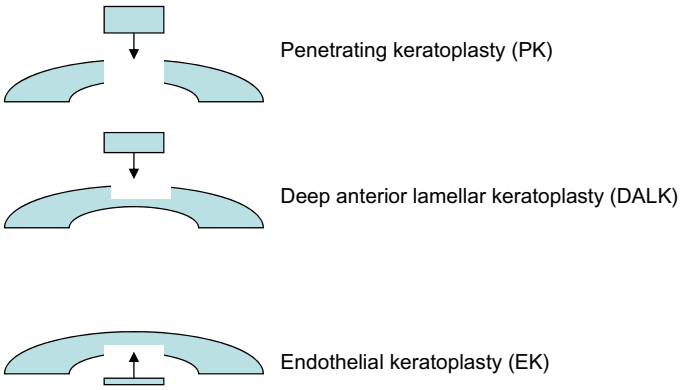

Fig. 4.2 Types of corneal transplant. Penetrating keratoplasty is a full-thickness graft where a central disc of host cornea (typically 7.5 mm) is removed and replaced with tissue from a donor cornea. Where the host endothelium is healthy but the stroma is misshapen and thinned, a partial thickness graft (DALK) leaves the host endothelium intact. For endothelial disease, replacement with healthy endothelium on a thin layer of stroma or just on Descemet membrane is an option (EK)

successful, it may be possible to treat many patients with endothelial cells expanded from just a single donor.

Deep anterior lamellar keratoplasty (DALK) is an alternative to PK for treating keratoconus with the purported advantage that the recipient's own healthy endothelium is not replaced but remains intact. Other anterior lamellar grafts can be used to replace scarred or opacified stromal tissue. Clearly, there is no requirement for the corneas used for these types of allografts to have an intact endothelium. However, corneas for DALK are often supplied with a good endothelium in case the surgeon needs to switch to PK during the operation owing to accidental perforation and breach of the anterior chamber during the lamellar dissection of the patient's cornea.

4.3 Corneal Storage

Hypothermia and organ culture are the two principal methods used for storing corneas. Cryopreservation has been used to a very limited extent in the past but is currently rarely used and then only for emergency grafts to save an eye rather than to improve vision [19].

4.4 Hypothermia

It was not until the 1930s that Filatov pioneered the use of corneas from deceased donors and the storage of eyes in pots (moist chambers) in ice for several days [20]. This method continued in use until the early 1970s when removal of the corneoscleral disc from the eye and storage in tissue culture medium containing 5% dextran (McCarey-Kaufman medium, M–K medium) was introduced [21]. The corneas were stored refrigerated at 4 °C for 2–4 days compared with the 24–48 h storage time for whole eyes in moist chambers.

The principle underpinning hypothermic storage is the reduction in rates of chemical reactions with falling temperature. For biological reactions there is a two-threefold reduction in rates for every 10 °C fall in temperature, which means that the energy demands of cells are markedly lower at 4 °C than at normothermia. There are, however, several limitations to the maximum storage period [22]. In particular, metabolism is reduced but not completely suppressed, which means that there is still a demand for high energy compounds such as ATP. The ability of cells to generate ATP at these low temperatures is compromised, which leads to an overall loss of high energy compounds. Ion pumps are also suppressed, leading to altered ionic balance and cellular oedema and cellular acidosis. There are also possible phase changes in membrane lipids and generation of reactive oxygen species (ROS) by the Fenton reaction.

The hypothermic storage medium currently most widely used is Optisol-GS [23, 24]. This solution contains both dextran and chondroitin sulphate to help control stromal hydration and provides up to 14 days of storage; however, most eye banks do not store beyond 7–10 days owing to poor epithelial preservation. There are other media available that aim to support the reduced cellular metabolism, counter damage from ROS and support cell membrane repair [25–27].

4.5 Organ Culture

Organ culture of corneas was introduced in the USA in the early 1970s and was based on culture techniques that had been developed for skin [28, 29]. Owing to its perceived complexity, the occurrence of stromal oedema during storage and potential problems of bacterial and fungal contamination, North American eye banks favoured hypothermic storage in M–K medium. However, the technique was further developed in Denmark and became the method of choice for many eye banks in Europe [30–33].

A key element to the method is the cleaning of the ocular surface before excision of the corneoscleral disc by rinsing in sterile saline and immersion of the eye in Povidone-iodine (PVP-I) solution [34]. An additional rinse with chlorhexidine increases the effectiveness of the disinfection process [35]. The corneoscleral disc is then suspended in organ culture medium for up to four weeks. In the UK, corneas are kept in Eagle's minimum essential medium with Earle's salts containing 26 mM sodium bicarbonate, HEPES buffer, 2% fetal bovine serum and antibiotics (penicillin and streptomycin) and an antimycotic (amphotericin B) [36]. This medium is not changed during the organ culture period and is sufficient to maintain the integrity of the epithelium and endothelium [37]. Alternatively, some eye banks do change the medium every one or two weeks and there have even been reports of successful corneal transplants with tissue stored for up to seven weeks with changes of medium [38]. A wide range of different media have been used for corneal organ culture with seemingly similar clinical outcomes. Recently, there have been attempts to remove serum and constituents of animal origin with encouraging results [39].

In the organ culture method as applied in the UK, a sample of the medium is taken after seven days of culture to test for bacterial and fungal contamination. Three days before the scheduled date of transplant, the corneal endothelium is examined by transmitted light microscopy after staining with trypan blue to identify dead or missing cells and a hypotonic sucrose solution that renders the cell borders visible. In the UK, a minimum endothelial cell density of 2200 cells/mm^2 is considered acceptable for corneas to be used for PK or EK (Fig. 4.3). Corneas are then transferred to organ culture medium containing 5% dextran to reverse the stromal oedema that occurs during organ culture storage. A further sample of medium is taken after 24 h for microbiological testing and then the cornea is despatched to the recipient hospital. Up to four days storage is permitted in the dextran medium.

Given the limited time available for this final microbiological testing, corneas are issued on the basis of a 'negative to date' test result. If microorganisms are subsequently detected, the surgeon is informed immediately and, as soon as the information is available, provided with the identity and antibiotic sensitivity of the microbes.

The donor's cause of death is the main factor influencing whether corneas are lost during organ culture through microbial contamination [36]. Virtually every eye coming to the eye bank will be carrying bacteria and/or fungi on the ocular surface consequent to the loss of tear film and blinking, but it would appear that this microbial load varies with the cause of death. It is not surprising, therefore, that corneas from donors who died with infections are at greater risk of becoming contaminated during organ culture. So far as the likelihood of the corneal endothelium meeting the minimum criteria for PK or EK is concerned, the major influencing factor is increasing donor age. Up to the age of 60 years, approximately 10% of corneas fail to meet the minimum endothelial cell density of 2200 cells/mm^2, but this rises to more than 30% for donors over the age of 80 years [36].

Five year graft survival for PK in the UK with organ cultured corneas is 70% (95% CI 68–72) [40]. The indication for transplantation has the largest influence on five-year survival, which ranges from 91% for keratoconus to less than 60% for bullous keratopathy. No donor or storage factors have been found to influence graft survival, which suggests that the donor selection criteria, storage time and endothelial assessment are all appropriate and these clinical follow-up data provide direct validation of the efficacy of the organ culture method [41].

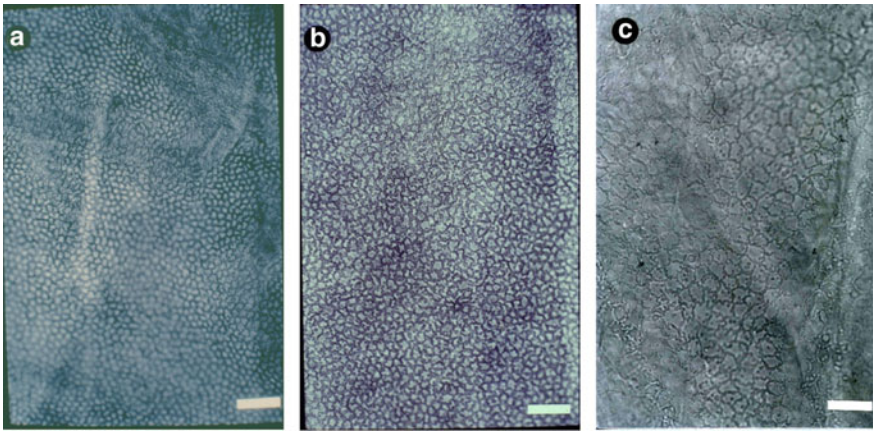

Fig. 4.3 Corneal endthelium after organ culture and staining with trypan blue and hypotonic sucrose (bar = 100 µm). **a** Cornea from an 18 year old donor after 23 days of storage, cell density >3000 cells/mm^2. **b** Cornea from a 75 year old donor after 29 days of storage, cell density >2500 cells/mm^2. **c** Cornea from an 84 year old donor after 26 days of storage, cell density <1000 cells/mm^2 (unsuitable for penetrating keratoplasty or endothelial keratoplasty). (Reproduced from Armitage [66] with permission)

4.6 Limbal Stem Cells

The corneal epithelium forms a protective barrier on the anterior surface of the cornea. In conjunction with the tear film, the epithelium provides a smooth anterior corneal surface, which is essential for the transmission of light and thus normal vision. Unlike the endothelium, the epithelium is constantly being renewed. The progeny of a population of slow-cycling stem cells residing in the basal limbal epithelium become transient amplifying cells (TAC) that migrate to the basal layer of the corneal epithelium where they divide and thus maintain the epithelial cell mass [42, 43]. As the TAC divide, their progeny move anteriorly becoming post-mitotic and, eventually, they form a superficial layer of terminally differentiated cells. Failure of the limbal stem cell population compromises the integrity of the epithelium, which results in ocular surface disease that can be painful and difficult to treat, causing severe visual impairment. Attempts to restore vision by corneal transplantation are almost certain to fail. This limbal stem cell deficiency (LSCD) can be treated by replacement of the stem cells by transplantation of limbal tissue or the application of epithelial cell sheets expanded in vitro from limbal tissue explants onto the ocular surface [44].

If the disease is unilateral, the best chance of successful treatment is with an autograft taken from the healthy eye. A small limbal biopsy can then be used as a source of stem cells that are expanded in vitro on a support such as fibrin or amniotic membrane [44]. If the disease is bilateral, then one option is to use allograft limbal tissue or cells, but this requires immunosuppression in an attempt to prevent allograft rejection and, even so, there is little evidence that such allografted cells persist in the long term [45]. Alternatively, other sources of autologous cells, such as oral mucosa, are being explored [46].

Despite the therapeutic use of limbal tissue grafts, there is still no single marker for corneal epithelial stem cells that can be used specifically to identify and enrich this cell population, which forms just a few percent of the cells in the limbal basal epithelium [47]. However, combinations of several immunohistochemical markers do appear to localize to limbal stem cells, such as positive staining for transcription factor p63 and the ATP binding cassette transporter ABCG2, but negative staining for cytokeratins K3/K12 and for connexin 43, [43] Nonetheless, limbal epithelial cells can be successfully expanded in vitro and there is currently a focus on improving the culture technique, for example to avoid the need for 3T3 cell feeder layers, bovine serum and other medium constituents of animal origin [48, 49]. Alternatives to amniotic membrane and fibrin as supports for epithelial cell sheets are also being investigated [50].

4.7 Extending the Role of Eye Banks

During the development of lamellar techniques (DSAEK, DMEK) as alternatives to full-thickness corneal transplants, grafts were initially prepared by surgeons in the operating theatre. This was both time-consuming and risked cancellation of the operation owing to the tissue being damaged during the lamellar dissection. Eye banks now routinely prepare corneas for DSAEK and DMEK and ship the prepared grafts to surgeons [51]. This also permits improved quality control of the graft and, if the tissue is damaged during preparation, another cornea can readily be selected for processing. Grafts for DSAEK can be prepared by manual dissection or cut to a standard thickness either by a microkeratome or femtosecond laser. However, in some reports, the laser has been shown to result in a less satisfactory graft [52, 53]. For DMEK, the graft is prepared by peeling Descemet membrane with the endothelium from the stroma, leaving the graft attached either by a peripheral 'hinge' or centrally. This prevents the graft from rolling up and allows it to be laid back down on the posterior stroma for shipping. The surgeon then makes the final separation of the graft ready for insertion into the patient's anterior chamber. Several inserters have been developed for introducing these EK grafts into the anterior chamber, [54] and eye banks are also offering to ship the graft already in an inserter. There appears to be little or no difference in clinical outcomes between corneas prepared by eye banks or by surgeons in theatre [55].

Additional donor selection criteria have been introduced, in particular for DMEK. When Descemet membrane is peeled from the posterior stroma, it forms a roll with the endothelium outermost. If this roll is too tight, it can be very difficult for the surgeon to unroll the graft in the anterior chamber and attach it to the patient's posterior stroma. Typically, the younger the donor, the tighter the roll and corneas from donors aged over 60 years are therefore preferred [56]. There have also been reports that preparation of DMEK grafts from donors with diabetes mellitus are more prone to tearing during preparation, although this may be related to the severity of disease [57].

Transplantation of Bowman layer has been advocated for mid-stromal transplantation to stiffen and strengthen keratoconic corneas and delay disease progression [58, 59]. Another option is to decellularize stroma for use in patients with corneal infections, scars and other superficial opacities. This has been reported for porcine cornea but this technique could be applied to human cornea [60]. Pieces of cornea have also been irradiated and stored in albumin for up to 2 years for use in glaucoma surgery and other ocular procedures [61, 62]. Techniques such as these and the various lamellar techniques increase the chance that corneas unsuitable for PK may still be used clinically for different types of graft and even for tissue from one cornea to be used for more than one patient thereby helping to alleviate the shortages of corneas in many countries worldwide.

4.8 Other Cell-Based Therapies for Ocular Disease

Apart from limbal stem cell transplants for ocular surface disease and the prospect of treatments for endothelial dysfunction with endothelial cell suspensions, the development of cellular therapies for retinal disease, such as age-related macular degeneration, is perhaps the next major step forward [63, 64]. The regulatory requirements for the production of cellular therapies are typically more stringent than for 'traditional' eye banking. However, eye banks could certainly apply their considerable expertise to develop appropriate facilities or become integrated with bespoke laboratories specializing in cell-based therapies in order to be able to deliver such treatments when they become available.

In summary, full-thickness corneal transplants are increasingly being superseded by partial-thickness grafts, especially endothelial keratoplasty. The possibility of injecting endothelial cell suspensions expanded ex vivo to replace deficient endothelium and use of decellularized stromal tissue for scars and other stromal opacities are extending the treatment options available to surgeons for their patients. The treatment of ocular surface disease with limbal tissue or epithelial cell grafts is far less common and still the subject of continuing research into improving the cultivation of epithelial cell sheets and exploring other sources of cells that could be used for autologous grafts in patients with bilateral ocular surface disease. The role of eye banks is changing with more extensive processing and preparation of corneas for lamellar grafts and the application of cellular therapies for treating ocular surface disease and, potentially, endothelial failure and retinal disease.

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Banking of Heart Valves

5

Robert Parker

Abstract

Human heart valves have been used in transplant surgery for nearly sixty years and banking of valves has been performed for the majority of this time. Cardiac valves have been disinfected using chemical agents, radiation and in present times by antibiotics and stored freeze dried or in solution at +4 °C, solid carbon dioxide and nowadays in the vapour phase of liquid nitrogen refrigerators. Heart valve banks have a list of criteria that valves must meet with relation to age of donor, atheroma, fenestrations and absence of virological markers. Cardiac valves are normally tested for microbiological contamination at least twice during their processing. The main research topics of interest in the development of heart valve banking today concern ensuring that the mechanical properties of the valves are maintained, whether it is advantageous to decellularize the valves as this lowers immune response and if vitrification could be a storage method for the future as this could alleviate the need for low temperature during transportation. Consideration also needs to be made as to whether matching of valves improves results. In the early days of heart valve banking, most hospitals processed their own valves but in the twenty-first century most banking is performed by centralized units which may be companies, national blood services or banks that are a collaborative between hospitals.

R. Parker (✉)
Heart Valve Bank (Retired) Royal Brompton & Harefield NHS Foundation Trust, Sydney Street, London SW3 6NP, U.K.

R. Parker
37 Petters Road, Ashtead KT21 1NB, U.K.

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5.1 Introduction

The first aortic valve was implanted into a patient at Guys Hospital in London by Donald Ross in 1962 [1] and over the following 55 years major changes have occurred in the processing of heart valves. The first valves were preserved using chemical agents such as formalin, glutaraldehyde, beta propriolactone and in the latter years of the decade ethylene oxide. Prosthetic valves, mainly of the ball in the cage variety or tilting disc, were introduced in the previous decade to the allograft and in the sixties these were the two main types of valves that were used [2, 3]. Xenograft valves were introduced in the seventies being mainly the porcine aortic valve [4], with valves made from bovine pericardium following later in the decade [5]. Also around this time surgeons tried to manufacture heart valves from human tissue with autologous fascia lata [6] being the first material tried and later homologous dura mater [7], and both autologous and homologous pericardium being tried [8]. In the present millennium there are only really three types of valves regularly being implanted and these are bileaflet prosthetic valves, porcine and bovine xenografts (which can be stented or unstented) and allografts. Seventy five percent of the allografts are used nowadays in paediatric cases with a further 15% in adult congenital cardiac surgery and the final 10% in adult acquired surgery, particularly in redo operations and patients with bacterial or fungal endocarditis. The advantages and disadvantages of allografts are given in Table 5.1.

Table 5.1 Advantages and disadvantages of allografts

Advantages	Disadvantages
Blood flow through the valve orifice remains normal and anatomically correct (unstented xenografts give normal orifice but being from another species are not totally anatomically correct)	Unpredictable supply due to donation rate
No anticoagulation required and therefore less clinic appointments (particularly important in females who may become pregnant)	Greater risk of cross infection because of antibiotic disinfection rather than terminal sterilsation
No mechanical damage to blood cells that can occur with prosthetic valves	Cardiopulmonary bypass time during implantation is 50% greater than for prosthetic and stented xenograft valves
Gradual wear out of valve which is symptomatic, whereas prosthetic valves can fail shut suddenly or can lose disc or leaflet	Surgical technique is more complex
Low evidence of post operative endocarditis	Possibility of atheroma, fibrosis and calcium in valve due to donor age
Adaptable for paediatric and endocarditis surgery	More complex storage procedure and preparation at time of operation
Complete range of sizes. Prosthetic valves cannot be made below 15 mm diameter	Shorter valve life than prosthetic valve, though better patient survival

5.2 Selection Criteria

The donor criteria operated by the majority of heart valve banks allows age from new born to 65 years of age for male donors and 70 of age for female donors with no evidence of the standard virological markers such as HIV, Hepatitis B, Hepatitis C, HTLV or Treponema (syphilis). A previous medical history of diseases that affect the connective tissue of the valve cusps such as rheumatic fever are also contraindications, but cardiac conditions such as myocardial infarction and left ventricular hypertension are acceptable for donation. The removal of the heart needs to occur within 24 h of death (although a few banks will accept up to 48 h if the donor's cadaver has been refrigerated within 2 h of death) and dissection of the valves from the heart should occur as soon as possible after cardiectomy and within a maximum of 12 h (although a few banks allow up to 40 h under controlled conditions). The conditions of the area where the heart is removed from the donor should be as sterile as possible, so operating theatres or dedicated removal suites are preferable to post mortem rooms, and sterile instruments and receptacles should be used in all cases. In Europe depending on country between 33 and 50% of the donations are retrieved from heart beating donors undergoing multi-organ retrieval where the heart is either unsuitable for heart transplantation or cannot be placed, with about 4% of the donations being from hearts removed from patients undergoing heart transplantation and the remainder from non-heart beating donors, where some post mortem room retrieval will occur.

5.3 Processing and Disinfection of Heart Valves

The only valves that are routinely dissected nowadays are the aortic and pulmonary valves. Photographs of dissected aortic and pulmonary allografts are shown in Figs. 5.1 and 5.2. Mitral allografts [9] were used for a time in the 1990s and early 2000s, but problems arose with the chordae and myocardial connections and many became incompetent, so very few centres use this type of valve nowadays. There were also surgical and technical difficulties as mitral valves do not form a ring in the same way that aortic and pulmonary valves do. Processing needs to be performed in a Class A environment and most banks perform the dissection in a cabinet at this level in a Grade B or C background. All heart valve banks worldwide use antibiotic disinfection methods using a combination of broad spectrum antibiotics with or without antifungal agents. Most antifungal drugs are cytotoxic so will affect the cell viability and therefore some banks who are concerned with this parameter now have omitted the antifungal agents, particularly amphotericin B [10–12], but this does mean that they will have a higher discard rate disinfection. The majority of banks in United Kingdom use a disinfection solution containing Gentamicin 4000 mg/L, Vancomycin 50 mg/L, Polymixin B 200 mg/L, Imipenem 240 mg/L and Nystatin 50 mg/L, but the Nystatin has been replaced by Amphotericin B because of the non-availability of the former in sterile format in United Kingdom. The antibiotic

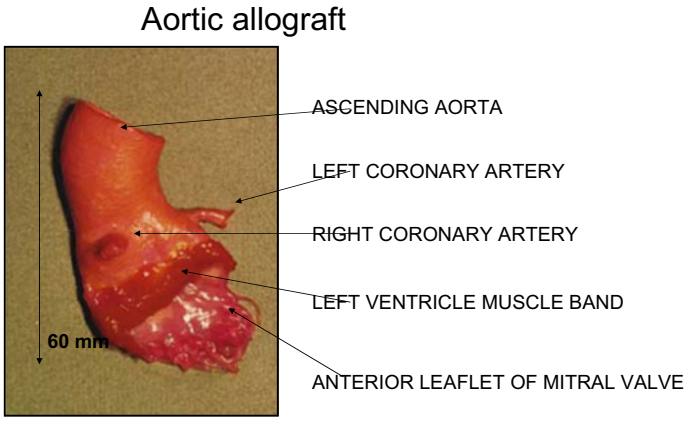

Fig. 5.1 Dissected aortic allograft

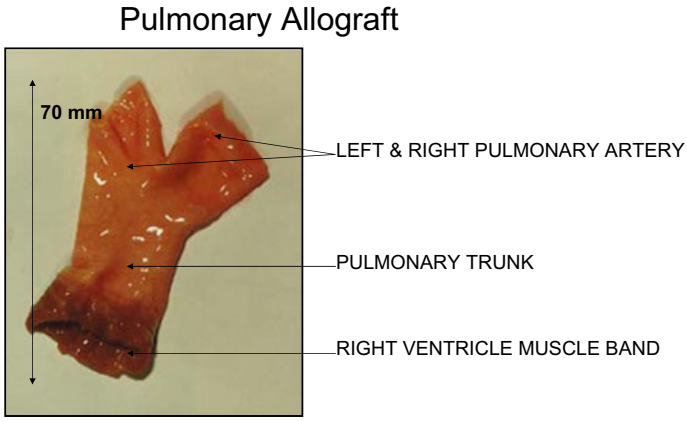

Fig. 5.2 Dissected pulmonary allograft

cocktails have been formulated by microbiologists [13–15] to cover as wide a range of bacteria and fungi as possible and the levels of antibiotics in most is 20–50 times the minimum inhibitory concentration, but can be up to one thousand times. Germain presented a systematic review in 2016 of the disinfection of human cardiac valve allografts where he compared results given in 22 investigations, including banks in Europe, Southeast Asia, Australia, South America, South Africa and North America. The greatest reduction in contamination rate (3.2%) was shown by Villalba in Spain using a cocktail composed of amikacin, vancomycin, metronidazole and amphotericin B The commonest disinfection temperature is +4 °C, but a number of banks use room temperature (22–25 °C) and a few in Europe and

Australia use 37 °C. The time in antibiotics used by banks varies from 6 to 24 hours with shorter times usually being used with the higher temperatures. It is considered by most workers that extension of the incubation time beyond 24 hours does not extend the effectiveness of the antibiotic treatment. Germain showed a greater reduction in bioburden at 37 °C compared to 4 °C. Macdonald has presented work at conferences using similar assays on the antibiotic solutions used at heart valve banks in United Kingdom and showed that all were most effective at 37 °C. The converse to this argument is that lysosomal activity is higher at this temperature and therefore more tissue degradation can be expected.

To compare the efficacy of the microbiology testing in cardiovascular tissue banks a quality round was organized (de By 2017) 20 banks (19 in Europe and 1 in Singapore) participated and each was sent heart valve tissue samples contaminated with known micro-organisms. 17 of the banks correctly identified the contaminating organisms before using their disinfection solution. After disinfection 44% of banks using temperatures of 4 °C, 50% of those using temperatures of 20–30 °C and 100% of those using temperatures of 36–37 °C had negative results. Because of the contaminating organisms (Escherichia coli, Enterococcus faecalis, coagulase negative Staphylococcus and Streptococcus anginosus) almost all the tissue banks would have rejected the tissue even if they were not present in the final result because of a list indicating that certain organisms even in the transport solution means the valve should be discarded. Quality rounds are important as surgeons may obtain their valves from several banks so they need to know that there is consistency in testing and they will also identify where methods are insufficient.

The microbiology test performed by most heart valve banks involves putting samples of valve tissue (normally the aortic or pulmonary wall) into bottles of aerobic broth, anaerobic broth, Sabourauds broth and incubating at 37 °C and placing another sample in a general purpose broth and retaining at room temperature. Samples of the broth are sub-cultured on to respective agar plates at day six and the plates are read at day nine or day twelve. A fifth sample is used for testing for mycobacteria, which is commonly performed by heart valve banks but not always by the bankers of other tissues.

Concern has been expressed about antibiotic carry over with heart valve allografts both with possible of negative sterility tests and adverse effects in predisposed subjects. Leeming [16] at a National Institute of Biological Control Meeting in showed the amounts of antibiotics remaining in the tissues and Gatto has proposed a procedure for the removal of decontaminating antibiotics. After decontamination they suggest a two-stage rinsing step using nutrient solution at 4 °C for 30 min each before addition of the cryoprotective medium. Gatto showed that heart valve tissues washed in this way showed no antibiotic carryover that inhibited plated seeded with Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermidis or Candida albicans.

There was evidence in the 1970s of transmission of TB with heart valve allografts and the advent of testing dates from this time [17]. In a paper Warwick and co-workers [18] have shown that in 38,413 donors in 24 heart valve banks tested, none were positive for Mycobacterium tuberculosis, but 24 were positive for

non-tuberculosis mycobacterium. The donor selection criteria for heart valves was not as strict in the 1970s and it is probable for this reason that no positive donors have been detected since. It has been questioned whether mycobacterial testing of heart valves should be continued, but it would seem important that because of the incidence of no-tuberculous mycobacteria which have also been transmitted with porcine valves and also as a contaminant at time of cardiac surgery that the testing is continued. The commonest reason for non-tuberculous mycobacteria is water borne and is probably contaminating the tissue at time of removal in the post mortem room.

After antibiotic disinfection, valves are transferred to na isotonic solution containing a cryoprotective agent with dimethyl sulphoxide (DMSO) being the commonest in use. The solution would normally contain 10–15% of DMSO and a time of up to an hour is used for the valve to absorb the solution before cryopreservation. The valve is frozen at the rate of 1 °C/min from +4 °C to at least −80 °C and is then stored either in ultra low temperature freezers at −140 °C or the vapour phase of liquid nitrogen refrigerator at −175 °C. Most banks have validated storage at this temperature for 5 years, but an increasing number are extending the validated period to 10 years and Mirabet has shown that it is possible to at least 13 years [19]. It is also important that the thawing of the valves is controlled as it has been shown by various groups that if valves are transferred directly from ultra-low temperatures to body temperature water baths, cracking can occur [20, 21]. Most banks now instruct that there is an intermediate stage either by allowing the valve to thaw at the lower temperatures in the air before transfer to the water bath or by placing the valve in solid carbon dioxide (cardice). The latter is the usual method of transfer from bank to operating theatre, so is being performed without realizing the significance of the stage.

Depending on the amount of washing when the valve is thawed up to 20% of the initial content of DMSO can still be detected in the valve. DMSO can cause hepato- and nephro-toxicity so needs to be removed. Gatto et al. proposes two sequential washings for a total of 25 minutes with nutrient medium after thawing. With this washing procedure the DMSO content can be reduced to less than 4% of the initial content.

5.4 Quality Assurance of Heart Valves

At the time of dissection valves are assessed for their quality. Many banks still use a fair, good, very good and excellent terminology, but the Heart Valve Bank in Beverwijk has tried to bring greater rationale to the system and have introduced a Quality Code List.

The list gives a Code to each valve depending on its abnormalities. The description of the Codes are as follows:

Acceptable for further processing[a]	
Code 01	No visible morphological abnormalities
Code 02	Minimal atheroma in basal attachment of leaflet
	Minimal fibrosis in (basal attachment of) leaflet/vascular wall Fenestration(s) in otherwise perfect graft
	Petechiae in otherwise perfect graft
Code 03	Minor atheroma in vascular wall (conduit)
	Atheroma in <1/3 of the basal attachment of the leaflet
	Fibrosis in <1/3 of (the basal attachment of) the leaflet Fenestrations
Code 04[b]	Atheroma in the vascular wall Atheroma in <2/3 of the basal attachment of the leaflet Fibrosis in <2/3 of (the basal attachment of) the leaflet Fenestrations
Code 05[b]	Discrete atheroma in the vascular wall or the basal attachmemt of the leaflet Discrete fibrosis in (basal attachment of) leaflets Minor adhesions of leaflets at the commisures Fenestrations
Not acceptable for further processing	
Code 06	Extensive fibrosis or atheroma and/or calcification in the leaflets, calcification of the vascular wall
Code 07	Damaged during removal of the heart
Code 08	Damaged during dissection of the heart
Code 09	Incompetent valve
Code 10	Other abnormalities (anatomical or procedural)
Code 11	Failure to meet one or more of the time limits adherence requirements needed by bank

Note: [a]Items are "and/or"
[b]Codes 04 and 05 are not acceptable for aortic valves from donors ≥ 56 years of age

It would be an excellent idea if banks who co-operated in the supply of heart valve allografts could adopt this nomenclature and could also adopt a standard method of sizing valves. At the present time there is no consistent measuring device for internal diameter of valves with some banks using obturators and other banks dilators and the position of the measurement with regard to cusp height is also variable. Likewise when it comes to length it is agreed that the starting point is at myocardium/wall junction but as aortic valve has a curved arch and pulmonary artery bifurcates within 3 cm of valve, there is not a consensus about the various lengths that need to be measured. As many surgeons obtain valves from more than one bank depending on supply it would be extremely helpful if all banks agreed a protocol for classification and sizing.

5.5 Current Issues in Heart Valve Banking

5.5.1 Mechanical Properties of Valves

Viability is not considered as important nowadays as it was when allografts were first introduced so the most important factor today in most scientists view is that the allograft valve should mimic the action of the native valve A number of workers have considered that the mechanical properties of the valve may be the best parameter to evaluate after preservation. Wright and Ng [22] devised a method in 1974 for measuring the elasticity of human aortic valve cusps and this was used by staff at National Heart Hospital to compare the elasticity of valves after storage in nutrient medium. The results of this was that there was a slow decline in elasticity and tensile strength over the first 3 weeks which became faster thereafter. Wassenaar [23] reported that cryopreservation resulted in a considerable reduction of contraction in response to potassium, but that the response to noradrenaline, endothelin-1 and prostaglandin F remained unchanged. Vesely [24] compared the mechanical behaviour of cryopreserved allograft leaflet to that of fresh tissue and xenografts by measuring their bending stiffness and their uniaxial tensile stress/strain and stress/relaxation behaviour. The results for the bending tests showed no significant difference between the pliability of cryopreserved allografts and fresh tissue but the xenograft material which had been treated with glutaraldehyde was significantly stiffer than both. The transition from a low to a high modulus on the stress/strain curves, a measure of extensibility was also similar for fresh and cryopreserved tissue but less for xenograft. In later work Vesely [24] reported that the mechanical differences between aortic and pulmonary valve tissue are minimal, but the pulmonary root will distend about 30% more than the aortic root when subjected to aortic pressures. This has significance in the Ross Procedure (pulmonary autograft) where the native pulmonary valve is implanted into the aortic position and could affect the function in the long term.

It can therefore be concluded that most cryopreservation methods are satisfactory and retain the physical structure and biochemical profile of the valve at similar levels to that at the time of freezing. It may therefore be important as Gall and O'Brien [25] have shown that it is important that heart valves are procured as soon as possible after cessation of heart beat and should be cryopreserved within 1 or 2 days if not clinically implanted. Scenke-Layland using histology, electron microscopy and multiphoton imaging concluded that cryopreservation causes some destruction of the extracellular matrix but Gerson et al. using two-photon laser scanning confocal microscopy has shown no statistically significant change in collagen and elastin laser transmission after cryopreservation indicating that the procedure does not detrimentally affect the structural integrity of valves., Yacoub [26] has shown that his homovital valves, which came either from heart transplant recipients or multi organ donors where the heart could not be used for transplantation and had very low level antibiotic disinfection and were implanted within 24 h produced better results than the cryopreserved valves.

5.6 Decellularization

Cryopreserved allografts contain donor cells that can initiate an adverse host response due to their antigens. Several groups have proposed that the removal of these cellular components would minimize the immune response and lessen the likelihood of calcification. Cryolife were the first processor to develop a process [27] and their Cryovalve SG™ is marketed today. Da Costa et al. [28] and Haverich's group in Germany [29, 30] have also been processing decellularized valves for ten years with reported success. An example of a decellularization method has been published by Kearney and Ingham's group Vafaee et al. [31]. The valves were washed with 10 mM Tris buffer, 0.1% EDTA, 10 KIU/l aprotinin for 24 h, then 0.1% sodium dodecyl sulphate in hypotonic Tris buffer for 24 h and then with three further washes of PBS plus 10KIU/l aprotinin (the first two washes for 15 min and the last for 24 h). The valves were then incubated twice at 37 °C in nuclease solution (10 U/ml Benzonase [Novagen]) in 50 mM Tris, 1 mM $MgCl_2$ buffered to pH 8.0–8.2 with stirring at 80 rpm for 3 h. The valves were then washed in PBS and disinfected in antibiotic solution. This decellularization method showed no evidence of cells or cell nuclei when stained with haematoxylin and eosin but showed well preserved histoarchitecture. The DNA content of the tissue was reduced to 3% of the level before decellularization. The process described by Elkins does not include a detergent wash and the da Costa method does not use nuclease. Haverich's method uses a harsher detergent treatment. In a later paper from the Ingham group [32] the authors have shown the biomechanical parameters for decellularized valves and cryopreserved valves were similar.

VeDepo and co-workers [33] consider that re-endothelialization of decellularised valves occurs across all valve surfaces, but interstitial recellularization is limited to the valve wall and sinus. As the leaflet is probably the most important part of the valve, an important challenge lies in finding a solution to the repopulating of the leaflet interstitium.

Early results for the Cryovalve SG™ have shown a superior freedom from significant insufficiency at intermediate follow up [34] but conversely Helder et al. [35] with results from two major centres in United States have shown that although early results were promising the freedom from re-operation over 10 years was 51% for decellularized valves compared to 80% for cryopreserved valves. Da Costa has reported [36] that decellularized and cryopreserved valves show similar results for freedom from allograft dysfunction, allograft reintervention, survival and event free survival over a five year period. Haverich's group [37] showed that after a decade of follow-up decellularized valves used for pulmonary valve replacement had reduced re-operation rates compared to cryopreserved allografts. However they note that there were a number of these valves that exhibited stenosis or regurgitation showing the susceptibility of decellularized valves, which are softer, to problems when suboptimally implanted. However d'Udekem [38] in analyzing the results points out that Saricouch did not match his groups or the areas in which they were operated and that the decellularized group had a average follow-up of 4.6 years and

therefore the results are unreliable beyond this point. It therefore would seem that like other valves in the past, there could be problems between 5 and 10 years. The results from Da Costa are mostly performed by one surgeon so there is less technical variability and more experience in implanting this type of valve. The jury therefore is still out on the long-term future of decellularized valves.

5.7 Vitrification

Cryopreserved valves need to be stored below −175 °C and need to be shipped preferably at this temperature in a dry shipper, which then needs to be returned to the processing bank. The use of low temperature storage and transport is expensive and one of the ways that this cost could be lowered would be the valves were vitrified. Vitrification is a method of ice-free cryopreservation using a higher molarity solution than that used for routine cryopreservation. The vitrification solution replaces 83% of the water in the tissue so that it remains unfrozen in a non-crystalline state and therefore prevents ice-crystal damage Fifteen years ago it was considered that viability of valves was important, but this parameter is no longer considered as important. Cell viability is lost during vitrification which reduces the immunogenicity of the valve. Brockbank et al. [39] proposed a method for vitrification of heart valve tissues. The vitrification solution is composed of hepes buffered Euro-Collins solution, propylene glycol, formamide and dimethyl sulphoxide. This solution may need to be added in increasing concentrations and the bag containing the tissue and vitrification solution needs to be shaken at room temperature for at least one hour before cooling. The bags can then be cooled for 10 min in a precooled bath of 2-methylbutane (<100 °C) and transferred to a −80 °C mechanical freezer or caddice for storage. When the valve is needed for use the bag needs to be submerged in a 37 °C waterbath, the outside of the bag sterilised and then opened with sterile scissors. The valve needs to be rinsed at least five times for 5 min duration with Euro-Collins solution or Lactated Ringers solution containg 5% dextrose. Brockbank showed well-preserved extracellular matrix structures with few CD3 + T-cells and improved haemodynamics when compared to cryopreserved valves.

Huber et al. [40] compared the haemo-compatability of valves stored by cryopreservation and vitrification and found little difference between the two. It was postulated that as endothelial cells are largely lost, detached from their substrate exposing the basement membrane (as would also be true for decellularised valves) these valves would have a higher susceptibility for thrombotic events but no evidence was found that this was so.

In Schenke-Layland's paper mentioned in the mechanical properties section earlier showed that vitrification causes less problems to the extracellular matrix than cryopreservation and demonstrated comparable morphologic features to fresh tissue.

5.8 Matching of Valves

During the period that heart valve allografts have been inserted, it has often been asked whether there are advantages in trying to match valves from donor to recipient. Boll [41] in a retrospective study looked at implantation data for 363 adult patients and found that there was no significant difference between gender matched and gender mismatched allografts regarding death, reoperation and echocardiographic allograft function during follow up. Vogt [42] using the same 363 patients looked at blood group compatible and blood group non-compatible allografts and found no difference between the groups for event free survival. Although most banks test the blood group of the donor and his/her sex, there is probably no necessity for the implanting surgeon to know this. With many of the methods like decellularisation and vitrification to be used in the future decreasing cell numbers and hence the tissue immunogenicity, the idea of matching of valves will become even less important.

5.9 Centralised Supply

A number of countries now use a national system for supply of heart valve allografts. In United Kingdom, the National Fulfilment System run by National Health Service Blood and Transplant holds information on the stock of heart valves at each bank in the country. When a surgeon requires a valve they telephone the National Fulfilment System who offer the 5 most suitable valves to their request. The surgeon can then choose the valve they want regardless of its location and the National Fulfilment Scheme arranges for the valve to be sent. Spacek et al. [43] has shown an organization model for co-operation of cryopreserved vascular grafts in Czech Republic where all the tissue banks co-operate. Some banks in Europe including the European Homograft Bank in Brussels collect valves from across National borders and supply likewise, whilst in USA most heart valve allografts pass through either Lifenet or Cryolife. Centralised supply systems make it easier for surgeons when they require valves and can equilibrate supply where hospitals are performing different types of surgery particularly with regard to paediatric and grown-up congenital heart cases.

5.10 Conclusions

Since the small beginnings in 1962 the number of heart valve banks worldwide is now constant at around 70. The number of banks in Europe and Australia has declined due to increased legislation and financial concerns, whereas a considerable number of new banks have opened in Asia [44–46]. More than sixty percent of the activity is in North America with 20% in Europe. In the United States, most of the

heart valve banking is performed by two large companies whilst in all other countries the banking is per formed either by hospitals or the national blood service of the country. In 2016, 3500 heart valves were processed in Europe with France, United Kingdom, Spain, Belgium, Sweden and Germany having the greatest throughput. The number of donations to the European Homograft Bank increased from 228 in 2001 to 298 in 2016 with an increase in implantations increasing from 217 to 257 over the same period. Allografts do not need anticoagulation so will also be the valve of choice in women of child bearing age who could become pregnant and children who because of the long term side effects should not have anticoagulation therapy for their entire lives. The allograft gives greater flexibility in insertion as it does not have a fixed sewing ring so allows the surgeon to shape the valve for complex congenital conditions or to replace diseased tissue in endocarditis cases. The non-stented xenograft which has been introduced over the last 20 years is also showing good results in these operations as well.

Allografts in Europe, North America and Australia account for about 5% of the aortic valve replacements performed in these countries and 80% of the pulmonary valve replacements.

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Cryopreservation and Banking of Haematopoietic Progenitor Cells

6

Claire Wiggins

Abstract

Haematopoietic progenitor cell processing within stem cell laboratories has steadily evolved over the last 25 years. The increased external regulation by agencies such as the Human Tissue Authority (HTA) and Joint Accreditation Committee ISCT/EBMT (JACIE) has played a key role in this evolution. This has subsequently led to improved quality and safety standards throughout the stem cell laboratories. Procedures within the stem cell laboratories are not universally standardised and the following chapter summaries the most commonly used approach. It discusses the journey of the haematopoietic progenitor cell product from collection, to receipt within the stem cell laboratory, processing, cryopreservation, storage and subsequent issue to the patient for transplantation. The various types of processing of haematopoietic progenitor cells both for autologous and allogeneic products are discussed. Also, within this chapter the procedure for quality control of each haematopoietic progenitor cell product the stem cell laboratory receives is described. This includes post cryopreservation quality control to ensure the stored product is safe and effective. Although there is some variability between different stem cell laboratories each laboratory must have documented validation on their own processes to ensure they are accurate and reproducible. They must continue to monitor and perform regular audits of procedures to ensure the methods produce the expected results and the products meet specification.

C. Wiggins (✉)
Retired. Previously, Stem Cell Laboratory, NHS Blood and Transplant, Southampton, UK

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6.1 Introduction

Hematopoietic cell (HPC) transplantation is an established procedure which can be used to treat an ever-increasing range of conditions. HPC transplantation can be either autologous or allogeneic using HPCs sourced from bone marrow, mobilised peripheral blood or cord blood. Autologous HPC transplantation is where the patient's own HPCs are used, and allogeneic transplantation is where the HPCs come from a family member or unrelated donor. Autologous HPCs are cryopreserved and stored prior to treatment and infusion whereas the majority of allogeneic HPCs are infused fresh although they can also be cryopreserved as required. Sometimes allogeneic HPCs require extra processing prior to transplant. This chapter discusses the processing, cryopreservation and storage of HPC in the stem cell laboratory. Stem cell laboratories must meet stringent standards and are regularly inspected by the Human Tissue Authority (HTA) and Joint Accreditation Committee ISCT/EBMT (JACIE).

6.2 Transportation of HPC Product from the Collection Site to Stem Cell Laboratory

The HPC product must be transported to the processing laboratory under controlled conditions in order to ensure the quality and safety. The primary container must be securely sealed and placed in a secondary container (double bagged) and sealed to prevent leakage. This double bagging procedure is to help protect the cells and the transport container.

The product must then be transported to the laboratory within a sealed transport container which has been validated for the required product temperature and the duration of the transport. The container must be labelled with both the collection site and destination details including contact details. The product must be tracked from the collection site to the processing laboratory and must be transported without delay.

6.3 Receipt and Initial Storage of HPC Products

On receipt of the HPC product from the collection site the product must be examined for integrity, labelling and quality of the product. The product must also be tracked from collection all the way through processing, cryopreservation, storage and subsequent infusion of the recipient. HPC product can be stored 2–8 °C for up to 72 h in a temperature monitored fridge however most laboratories will process the product within 24 h. All stem cell processing laboratories should specify storage conditions to maintain cell viability. There are several studies which have identified the effects of time and different storage temperatures prior to cryopreservation.

Higher CD45+, CD34+ cell and CFU recoveries were observed in samples stored at 4 °C compared to 20 °C [1]. The study by Seo et al. [2] also demonstrated that storage of products overnight at room temperature results in reduced viability and CD34 cell recovery. There is increasing centralisation of processing laboratories which means the collections sites are often some distance from the processing laboratories which has led to an increasing length of time from collection to cryopreservation for some laboratories. It is generally recommended that if the product requires storage >24 h at 2–8 °C that the maximum nucleated cell concentration should not exceed 2×10^8/ml, however there is limited data to support this. If required the HPC product can be diluted with autologous plasma, human albumin or another solution licensed for clinical application.

6.4 Preparation of HPC Products for Cryopreservation

It is essential that sterility of the HPC product is maintained during the processing and subsequent cryopreservation. The processing prior to cryopreservation can be performed either in a grade A environment within a cleanroom using sterile techniques or on the bench using a closed system and sterile docking devices. If a closed system is not used for processing the HPC product the product must be processed in a grade A environment. The grade of environment is defined by particle counts and microbial colony counts according to the current European Guide to Good Manufacturing Practice (GMP) annex 1 [3] and EU Directive 2003/94/EC [4]. The background environment must be at least equivalent to GMP grade D. Cleanrooms need to be regularly cleaned, monitored and maintained and high levels of personal hygiene and cleanliness are essential to work in a cleanroom. Dedicated cleanroom garments are worn which must be sterile and non-shedding.

HPC products can be processed on the laboratory bench by utilising a closed process using sterile consumables without the need for an expensive cleanroom suite. Sterile docking devices are used to cut and then weld tubing together which maintains sterility. The DMSO is supplied in a syringe with tubing which can be welded onto the sterile tubing.

Some laboratories dilute the HPC product so the nucleated cell count $<2 \times 10^8$/ml prior to cryopreservation even if processed the same day as collection. However, studies have shown that cryopreservation at higher cell concentrations does not impair the haematopoietic recovery after transplantation [5]. Laboratories need to validate their method of cryopreservation considering cell concentration, DMSO concentration and freezing profile.

6.5 Cryopreservation of HPC Products

Cryopreservation of products enables them to be stored long-term and then for patients to be transplanted at a later date. The objective of cryopreservation is to minimise the damage to the cells during low temperature freezing and storage. The extent of freezing damage depends on the amount of free water in the system and the ability of that water to crystallise during freezing. Water is the major component of all living cells and must be present for chemical reactions to occur within a cell. During freezing, most of the water changes to ice and cellular metabolism ceases. Ice formation initiates in the extracellular environment, resulting in increased salt concentrations as water is removed to form ice. This ice formation results in an osmotic imbalance. Water then leaves the cells by osmosis and cellular dehydration results. Excessive dehydration can be detrimental to cell recovery. In order to protect the HPCs during cryopreservation a cryoprotectant is added. The most common cryoprotectant used is dimethyl sulphoxide (DMSO). DMSO penetrates cells and binds water molecules in solution thereby preventing cellular dehydration. DMSO is diluted to a certain concentration, most commonly 20% before addition to HPCs. The addition of DMSO to HPCs causes an exothermic reaction, which can cause cell damage so the DMSO solution and the HPCs are often precooled. Studies have indicated however that reducing the final DMSO concentration from 10 to 7.5% and 5% does not influence haematopoietic recovery after autologous transplantation and may result in improved tolerance of stem cell infusion [6]. Although cryopreservation of HPCs is performed at many transplant centres there are very few publications about the standardisation of the procedure.

The steps for the preparation of HPC products for cryopreservation are essentially the same whichever processing environment is used.

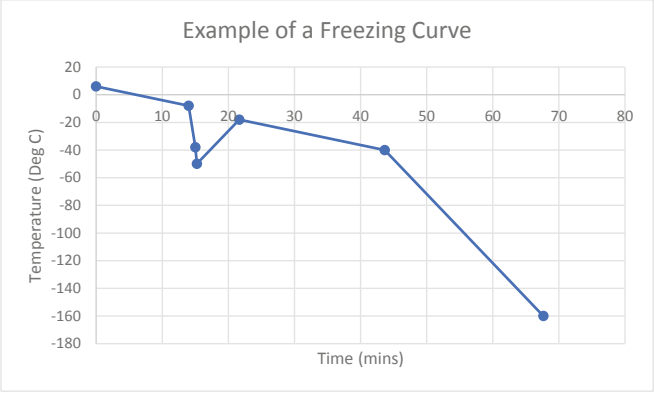
Preparing the HPC Product for Cryopreservation

- Sample of the product is removed for WBC and CD34 count, some laboratories also take a pre-processing bacteriology sample
- Product can be diluted if required according to the laboratory protocol
- Preparation of Cryoprotectant in bag
- Cryoprotectant is cooled on ice packs or in fridge
- Cells and cryoprotectant are mixed together
- Cells and cryoprotectant mixture dispensed into labelled cryogenic storage bags
- Sample is taken post processing for bacteriology testing
- Samples of the product + cryoprotectant are also aliquoted into sample cryovials
- Cryogenic bags are double wrapped and placed into controlled rate freezer or freezer for passive freezing.

6.6 Rate of Freezing

The rate of freezing is very important and influences the post thaw recovery and viability of the product. The optimal cooling rate is different for different types of cells and is dependent on the type and concentration of the cryoprotectant used. The optimal cooling rate for cells and tissues will be defined by the rate that permits some cell shrinkage without the formation of significant amounts of intracellular ice. Tolerances for cell shrinkage and intracellular ice formation vary between cell and tissue types. The effects of cooling rate and concentration of cryoprotectant interact. Cooling rates that are too slow increase the length of time that cells are exposed to the cryoprotectant at the higher temperatures. This can lead to significant toxicity. Also, extracellular ice formation can occur which could lead to dehydration of the cell due to osmosis. In contrast, where cooling rates are too high, the cryoprotectant is unable to enter the cells rapidly enough to prevent the nucleation of ice crystals inside the cells, leading to cell membrane rupture and cell death. The most widely used rate of freezing for HPC products is between 1 and 2 °C/min.

The HPC product can be cryopreserved using a controlled rate freezer or by passive freezing. Controlled rate freezers operate by injecting, into the freezing chamber, an amount of liquid nitrogen which may be determined by a temperature sensor controlling the chamber or the sample itself and comparing this to the temperature required by the set programme. The advantage of using a controlled rate freezer over a non-automated procedure for cooling cell suspensions is that it provides for a reproducible method of freezing each time the procedure is carried out. It is also possible to programme variable rates of cooling at different points during the procedure. If required, it is possible to include into the cooling programme a step which will initiate freezing of the sample and compensate for the latent heat that is released as the cell suspension freezes. This is achieved by including a rapid rate of cooling at the theoretical freezing point of the suspension before returning to the desired cooling rate as demonstrated in the figure below.



The figure above is one example of a freezing curve from a controlled rate freezer. Laboratories may use a different freezing profile however each laboratory should validate their own freezing profile and continuously monitor their results.

Passive rate freezing has the advantage of lower cost, simplicity and speed however the major difficulty in comparing cryopreservation methods is that there is no 'standard' freeze profile.

6.7 Storage of HPC Products

The majority of HPC processing laboratories store cryopreserved bags in the vapour phase of liquid nitrogen or less commonly mechanical freezers. If using liquid nitrogen storage vats, it is now generally recommended that any cells stored for use in transplantation be stored within the vapour phase of liquid nitrogen and not the liquid phase. This is because of the potential for transmission of microbes and viruses across the packaging used for storage. Storage in the liquid phase has caused problems of cross contamination in the past [7], although the vapour phase might also be a potential source of pathogen contamination. Liquid nitrogen should be considered to be contaminated and it is generally considered good practice to double wrap products to further mitigate the risk of cross contamination. Studies have also shown that the accumulation of ice sediment within the storage dewars can be a source of contamination [8].

Although temperatures below −80 °C can be used for relatively short periods of time, up to 6 months, a storage temperature of −150 to −180 °C is recommended for the storage of products for clinical use. Procedures must be in place to avoid significant warming of stored product during the addition/removal of other units of cells. Above the glass transition point (circa −135 °C) there is still free water in the system which is in dynamic equilibrium with the frozen water (ice). Therefore, some new ice crystals are forming as others melt. There is a risk that the new crystals will form inside the cells and hence kill the cells, resulting in a very slow but progressive loss of viability through time [9]. Once below the glass transition temperature, all the water is immobilised and hence new ice crystals do not form. Transient warming events can occur when the lid of the storage dewar is repeatably opened, so it is important that the temperature of the storage dewar is monitored continuously. There should also be backup storage dewars available and a contingency plan should any dewars fail.

6.8 Transport of Frozen HPC Products

The recommended method to transport frozen product is to use dry shippers. Dry shippers are designed to safely transport cryopreserved products at temperatures of around −190 °C. These are mobile, insulated containers that contain a material that

is porous to liquid nitrogen and capable of holding very low temperatures. This means that there is no actual liquid within the container when fully primed. It is essential that the dry shipper is validated to confirm what the holding time is at temperatures of −190 °C and below. Temperature loggers are available for fitting to the lid of dry shippers to enable the user to monitor the temperature within the chamber during transport.

6.9 Further Processing Performed by Stem Cell Laboratories

There are various processing options available to remove, enrich or reduce certain cell types within an HPC product. The procedures used must be GMP compliant and must maintain the sterility of the product.

6.9.1 Bone Marrow (HPC,M) Processing

HPC,M is sometimes collected from a donor for allogeneic transplantation and can require further processing depending on the blood groups of the donor and recipient. There are three types of ABO incompatibility, major, minor and bidirectional.

Major ABO incompatibility—the presence in the recipient's plasma of anti-A, -B or -A,B alloagglutinins reactive with the donors red cells.

Minor ABO incompatibility—the presence of anti-A, -B or A,B alloagglutinins in the donor's plasma reactive with the recipients red cells.

Bidirectional ABO incompatibility—the presence in both the donor and recipient's plasma of anti-A, -B or -A,B alloagglutinins reactive with recipients and donor cells respectively.

Plasma reduction may be required for allogeneic transplants where a minor ABO incompatibility exists between donor and recipient. If required, an additional cell washing step may be included to further reduce the presence of donor antibodies directed against recipient antigens. These procedures are generally performed on an apheresis device using a centrifuge and a single use sterile processing set.

Red cell depletion is required for allogeneic transplants where a major ABO incompatibility exits between the donor and recipient. Bone marrow products can be processed to produce a mononuclear rich preparation which is plasma reduced, volume reduced and red cell reduced. There are automated closed processing systems available all of which use centrifugation.

6.9.2 Selection/Depletion

Post allogeneic transplant donor lymphocytes can attack non-malignant host tissues and organs as they are perceived as 'non-self'. This is termed Graft versus Host

Disease (GvHD). GvHD is mediated by donor cytotoxic CD8+ lymphocytes and lymphokine-secreting CD4+ T helper lymphocytes in the graft. The severity of the GvHD is linked to the degree of HLA mismatch between the donor and the recipient.

In order to reduce the incidence of GvHD following allogeneic transplant a range of positive and negative selection procedures are available. Immunomagnetic cell separation systems can be used to positively select or to negatively select (deplete) cell types for which specific monoclonal antibodies exist. An increasingly wide range of cell-specific monoclonal antibodies coupled to paramagnetic beads are available. These procedures are lengthy and costly and are normally only carried out where there is a high degree of HLA mismatch between the donor and recipient (e.g. haploidentical transplant). They can also be performed when a further infusion of donor cells is required in order to reduce the risk of inducing or exacerbating existing GvHD.

- CD34 Selection
  CD34 +ve stem cells can be positively selected leaving the T-cells responsible for causing GvHD in the residual product. After a washing step the HPC product is incubated with a CD34 antibody conjugated to paramagnetic beads. Following another washing step, the product is passed through a column with a powerful magnet. The CD34+ cells are retained by the magnet allowing the T cell rich fraction to pass into a bag. The CD34 +ve cells are then eluted off the column into the final product bag. The T-cell waste fraction may be used to add a small dose of T cells back to the product if deemed appropriate to aid engraftment or for cryopreservation as aliquots of donor lymphocytes (DLI) for post-transplant therapy.
- TCR α/β and CD19 depletion
  Alpha/beta (α/β) T-cells make up 95% of circulating T cells and are heavily implicated in the development of GvHD. Removal of these cells from an allogeneic HPC product can reduce the incidence of GvHD. Similarly, removal of CD19+ lymphocytes can aid in the prevention of chronic GvHD as this is thought to be primarily B cell mediated. The removal of these cells can be performed using immunomagnetic cell separation system as described above however from a laboratory perspective the TCR α/β and CD19 depletion takes longer to complete then a CD34 selection.

6.9.3 Campath in the Bag

Campath-1H is a humanised IgG1 monoclonal antibody directed against the CD52 antigen on human lymphocytes. It can be added to a bag of HPC prior to infusion in the patient to deplete T cells from the product, however many non-immune cells in the bag also express CD52 so that the antibody may be bound by these cells resulting in an inadequate lymphocyte depletion. Adding an excess of antibody to counteract this may lead to the presence of unbound antibody which once infused can cause immunosuppression in the recipient [10].

6.10 Quality Control of the HPC Product

It is important to have accurate methods for counting the number of cells in the HPC products for transplantation. Samples are taken of each product received in the laboratory for determining the nucleated cell count, the viable CD34+ cell count (measure of the number of stem cells) and the viable CD3 count (measure of the number of T cells for allogeneic products).

It is important to determine the total nucleated cell count prior to cryopreservation and flow cytometry. This is generally determined using a haematology analyser.

CD34+ cell enumeration is the standard method to determine the cell dose required for transplantation. Flow cytometry is used to determine the CD34 count, CD3 count and the viability of the HPC product. There are several flow cytometric analytical protocols for CD34 quantitation but the one most commonly used is the International Society for Haematotherapy and Graft Engineering (ISHAGE) protocol. This was first described by Sutherland et al. [11]. The protocol uses Boolean logic to exclude non-specific background and fluorescent counting beads are included to obtain an absolute cell count. This protocol uses dual staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD45 antibodies and phycoerythrin (PE)-conjugated anti-CD34 antibodies. The addition of a viability dye for example 7-aminoactinomycin D (7-AAD) provides an estimate of the cell viability. 7-AAD has a strong affinity for DNA and is generally excluded from live cells. It is however not a true estimate of viability as it is a measurement of cell membrane integrity which is associated with cell viability.

Flow cytometry analysis can give an accurate result on the cell number and viability of a product however it does not necessarily reflect the ability of the cells to proliferate. In vitro culture assays, colony forming assays (cfu) can be performed to measure the proliferation and differentiation ability of the haematopoietic cells. This procedure utilises viscous or semi solid media with supplements to allow the proliferation and differentiation of the cells. Following incubation discrete visible cell colonies develop which can then be counted. It is important that the culture conditions including the cell preparation, incubation temperature, humidity, CO_2 and O_2 levels are standardised in order to obtain comparable results. The result however from a cell culture assay is not available for 2 weeks whereas the result from a flow cytometry assay is available within 1–2 h.

It is also a requirement to take a sample of the HPC product post processing for assessment of any bacterial contamination. Some laboratories may also take a sample on receipt of the product. Bacterial contamination may arise because of donor infection, contamination during collection, contamination of the collection set or contamination during processing.

6.11 Quality Control Post Cryopreservation

It may be necessary to perform viability tests post cryopreservation and storage for example if products have been stored for a long period or if the storage or cryopreservation procedure have been compromised. A rapid and common cell viability test is still the trypan blue dye exclusion method however the predictive value of this test is low. There have been many studies looking at the cell viability post cryopreservation however as yet there is no standard procedure. Studies measuring viable CD34+ cells and apoptosis by flow cytometry have used 7-AAD, annexin V and SYTO 16 and all can provide additional information on the viability status of the product [12–15]. There have also been several studies using aldehyde dehydrogenase (ALDH) to assess the progenitor cell viability of stem cell products. ALDH is highly expressed in haematopoietic progenitor cells and ALDH bright ($ADLH^{BR}$) cells are highly enriched for CFUs. $ALDH^{DIM}$ cells have been shown to possess little colony-forming ability [16].

The colony forming assay as previously described can also be performed post cryopreservation to determine the proliferative potential of the cryopreserved product. The results of which can provide a good correlation with engraftment potential.

6.12 Conclusion

The procedures within stem cell laboratories are nowadays highly regulated and well controlled. Products are tracked from the collection from the donor, through receipt, processing, storage and subsequent issue to the patient. There are however several areas that are not universally standardised for example the cryopreservation freezing rate profile. Each laboratory should validate their procedures and have documented evidence that the procedures they are following are accurate and reproducible. Regular audits of procedures should be performed to ensure the methods produce the expected results and the products meet specification.

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Storage, Processing and Preservation

7

John N. Kearney

Abstract

The requirement for tissue replacement depends on the type of tissue that has been lost, the size of the defect and the capacity of the tissue to regenerate. If the tissue cannot regenerate then the remaining options are to apply autologous or allogeneic grafts. Tissue allografts may elicit an immune rejection response. If this response is delayed then the graft could still perform a useful temporary function. Alternatively, the tissue could be treated to remove the cells in the graft that elicit rejection. In addition it could be beneficial to preserve the grafts so that they do not need to be used immediately e.g. freeze drying, deep freezing or using high concentration solutes. It is also important to ensure that pathogenic microorganisms are not present on the graft e.g. using disinfection methods for viable grafts or sterilisation techniques for non-viable implants. The latter includes the use of chemical methods e.g. ethylene oxide gas or peracetic acid; or physical methods such as gamma irradiation. In order to confirm effective sterilisation, a sterility assurance level of less than one living microbe per million items would need to be demonstrated. Recently, for viable grafts, it has been shown that combinations of disinfectants can be much more effective in killing microbes, without being cytotoxic to the tissue cells.

J. N. Kearney (✉)
R&D, NHSBT Tissue and Eye Services, Liverpool L24 8RB, UK
e-mail: john.kearney@nhsbt.nhs.uk

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7.1 Introduction

7.1.1 Tissue Replacement

Tissues can malfunction as a result of numerous processes including congenital malformation, disease and damage. Most tissues in the human body have a limited capacity for regeneration, in response to such malfunction.

Skin, being in direct contact with the environment does have regenerative capacity in order to deal with the minor abrasions and lacerations of everyday life. In fact the outer layer, the epidermis, is replaced continuously from below, and the outer skin scales (squames) are shed into the environment. However, the capacity to regenerate skin declines as the depth of the wound increases. Above a certain critical size, full thickness skin wounds fail to regenerate and, in this case, the only way to achieve functional skin is to use a skin graft, either an autograft or allograft.

Bone is another tissue which turns over continuously and hence can repair fractures but removal of a large section of bone resulting from traumatic damage or e.g. tumor excision, will not result in regeneration or replacement. In fact, removal of a piece of bone above a critical size results in a permanent defect. It appears that the healing response is designed to resorb and replace dead bone rather than to regenerate bone that is missing. Therefore, this leads to one of the major strategies in bone tissue transplantation i.e. to replace missing bone with either autologous or donor bone to stimulate the resorption and replacement of the graft with new autologous bone.

Other tissues do not turnover continuously e.g. cartilage. Once formed, there is little turnover of the extracellular matrix and the cells are largely quiescent through life. Therefore, in this case, there is little that can be done to stimulate a natural tissue regeneration response. Here the only hope is to replace defective cartilage with a fully viable allograft, or to stimulate the generation of new autologous cartilage in vitro using autologous chondrocytes [1].

7.1.2 Autologous Versus Allogeneic Grafts

In almost all cases the use of autologous tissue i.e. a graft taken from elsewhere in the body to replace diseased, damaged or missing tissue is the gold standard. Because autografts are “self” they will not elicit an immunological rejection response. If used immediately after harvest they will be fully viable and hence cells within the graft will contribute to the repair process. Indeed for vascularised grafts the vessels within the graft will often enosculate with vessels in the surrounding wound bed thus ensuring rapid re-perfusion of the grafts.

However, there are limitations to the use of autografts. In particular, it is important not to create a non-healing defect at the donor site(s). So, for example with bone, this means taking only small samples, below the critical size, that will allow natural regeneration, whereas for skin it means taking only partial thickness

grafts or very small full thickness punch biopsy grafts. Even then, the patient may suffer significant morbidity at the donor sites including pain, infection, etc. In addition, for certain tissue there are no suitable autografts available e.g. the body only has a single aortic heart valve, therefore a defective aortic valve cannot be replaced by an alternative autologous aortic valve.

For these reasons the practice of tissue allografting developed as an adjunct to autografting, or as the only option where a suitable autograft was not available. Although in principle an allograft transplant could be carried out in one operation, by the retrieval of viable tissue from a living donor (or deceased donor on life support) and transfer directly into the recipient i.e. analogous to an autograft; in practice for various reasons, this is rarely undertaken. The use of a viable rather than a non-viable allograft, may in fact be detrimental and hence this level of organisational urgency unwarranted.

7.1.3 Immunological Reponses to Tissue Allografts

Any tissue or organ that contains living cells is likely to elicit an "acute allograft rejection response" via one pathway or another. The ultimate outcome is that the allogeneic cells will be killed by the recipient's immune response. For organs or tissues that are highly cellularised, and vascularised, the outcome is rapid killing of the vessel endothelial cells leading to thrombosis, vessel occlusion, and death and necrosis of the other cell populations i.e. classical rejection of an organ or skin graft, with easily observable macroscopic changes. In contrast, a piece of cortical bone containing scanty osteocytes may well elicit an acute allograft rejection response owing to stimulation by allogeneic passenger leukocytes, but the consequence is unlikely to be noticeable macroscopically. Indeed, the osteocytes may not be immediately killed but rather opportunistically killed when the bone resorption/replacement process brings the cells in close proximity to the vascular system, which delivers the recipients immunological effectors (leukocytes and antibodies) to the allogeneic cell. Therefore for this tissue, "immunological rejection" is unlikely to be apparent nor is it likely to interfere with the tissue regeneration process whereas for the previous examples it would totally destroy the tissue and any prospect of regeneration.

Many other tissues are between these two extremes. Cancellous bone, for example, contains very few "bone cells" but huge quantities of bone marrow and fat within the trabeculae. These cells would elicit a huge rejection response that would at least delay the regeneration response and may cause some collateral damage or longer lasting inhibition of graft re-colonisation by tissue regeneration cells [2]. It would therefore be good practice to remove at least the trabecular marrow cells prior to grafting. Removing the fat would also accelerate graft incorporation [3].

The only way to prevent the rejection of allogeneic tissue cells is with HLA matching and immunosuppression, as happens with organ grafts. This has been evaluated for skin grafts for use in burns [4, 5]. However, as most tissue grafting is carried out as a life enhancing rather than life saving operation, and there are

significant morbidity and mortality consequences of using intensive immunosuppression, this approach has not been generally adopted.

Therefore, where the viable donor cells will eventually be killed by the immune response, the only circumstances where using a viable tissue allograft will have benefit is if the graft performs a vital function prior to rejection. One example is the use of viable skin allografts. These will gain a blood supply by enosculation with vessels in the wound bed and thus provide true biological closure of the wound and fully functional skin. This buys the surgeon some time to find additional permanent grafts (e.g. by re-cropping donor sites at intervals or growing cultured autografts). In addition, very severely burned patients are to some extent immunosuppressed as a result of the injury so rejection may be significantly delayed, buying even more time. The use of viable skin allografts can therefore be life-saving for severely burned patients.

For most other tissues, there is no benefit to using a viable graft. Tissue banking science has therefore developed methods for banking of tissues in a non-viable state which then allows application of other beneficial processes such as sterilisation to avoid the risk of transmission of disease from donor to recipient or as a result of contamination during processing.

There are however certain "privileged" tissues that do not elicit an "acute allograft rejection response" even though they are fully viable. These are tissues that are not normally vascularised and hence do not become vascularised after grafting. The vascular system is the conduit along which donor cells travel to the lymphoid tissue to elicit an immune response (the afferent arm) and along which recipient effector cells (and antibodies) travel back to the tissue to destroy the donor cells. In the absence of a vascular network, neither can occur. Two examples of avascular tissues are articular cartilage and cornea. Therefore, in most cases, these tissues can be used as viable allografts without being rejected, and indeed do not function adequately or regenerate if non-viable grafts are used. Therefore, development of techniques for banking of viable tissues has also been an important objective for tissue banks. Cryopreservation (or vitrification) is the commonest technique used for long term storage. This is the subject of a separate chapter in this book and will not be covered here. However other short-term banking methods for viable tissues will be briefly reviewed.

7.2 Principles of Tissue Preservation

7.2.1 Preservation of Non Viable Tissue Grafts

The challenge of tissue preservation is one that has faced mankind for millennia. Animal and plant tissues are a major component of the human diet. Both are subject to progressive degradation once harvested therefore methods to prevent degradation of food have been essential to survival. These methods have included drying of the tissue, use of low temperatures and the use of chemical preservatives such as salt.

Although these techniques developed by trial and error, the underlying principles are now better understood. The more recent discipline of "Tissue Banking" has borrowed heavily from this knowledge.

7.2.1.1 Causes of Degradation

One of the major potential causes of tissue degradation is the growth and activity of microorganisms. Whereas in life there are mechanisms to exclude and where necessary, kill microorganisms to prevent their access to tissues; once tissues are removed from the body these mechanisms rapidly disappear. However, as microorganisms that are transferred to recipients can cause serious diseases, methods have been developed to either avoid contamination or to inactivate microorganisms. These methods will be discussed in a separate section later in this chapter. However, it is well known that microorganisms can only grow in the presence of water.

The action of enzymes can also cause damage to both the cells and the matrix of stored tissue grafts. The elaboration of degradative enzymes in life is an important part of tissue turnover, the removal of dead/damaged tissue and in the process of apoptosis. Removal of tissue from the body results in cellular necrosis and the release of degradative enzymes. The presence of water is essential for enzyme activity.

Some of the most powerful degradative enzymes are the hydrolytic enzymes, found for example in lysozomes. Hydrolysis can also be caused by extremes of pH together with raised temperature. As the name suggests, water molecules are essential to the process.

Lipid peroxidation is another potent degradation process. This involves reactions of lipids with reactive oxygen species or "free radicals". Once started, the process continues as a chain reaction. It leads to rancidity of fats in food, but can result in cytotoxic by-products in tissue grafts [6, 7]. In the presence of water, radical formation generates the most reactive species, the hydroxyl radical ($OH\cdot$).

It is clear from the foregoing that all of the degradative reactions that can adversely affect the preservation of tissue grafts, are dependent on the presence of water. This water must be in a free state and able to react i.e. must not be immobilised.

The relationship between degradation rate for each of these processes and water activity is shown in Fig. 7.1.

Water activity ranges from aw = 1 which is 100% free water, to aw = 0. As the water activity decreases, first bacteria then yeasts have reduced growth rates decreasing to zero at aw = 0.9. Moulds are better able to tolerate reduced water activity, however at aw = 0.7 they also cease growth activity.

Enzyme activity decreases rapidly initially but not totally disappearing until aw = 0. Hydrolytic reactions are more tolerant of lower aw initially but approach zero at aw = 0.3. Likewise, lipid peroxidation reaches a minimum at aw = 0.3 but increases either side of this. Therefore, for tissue preservation, an ideal water activity range would be circa aw = 0.2–0.4. This would minimise all the major degradation reactions.

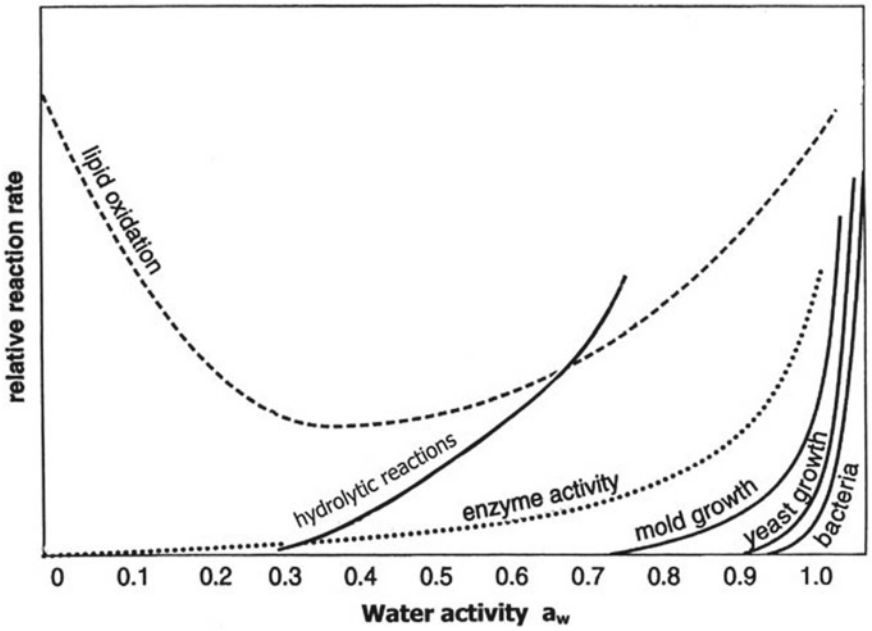

Fig. 7.1 The relationship between water activity and detrimental microbial and chemical reactions. Adapted from *Cell Tissue Bank* [8]

7.2.1.2 Reduction of Water Activity

Water activity can be reduced in a number of different ways. The water can be physically removed by drying the tissue matrix, or by immobilising the water so that it is no longer "active" and hence does not contribute to water activity.

Freeze-Drying (Lyophilisation)

If tissues are dried at ambient temperature, the progressive loss of water results in an increase in the salt concentration in the remaining water in contact with the tissue matrix. This can lead to conformational changes in molecules and to denaturation. In contrast, if the tissue is first deep frozen, and the water removed by sublimation by applying a vacuum, this will prevent molecular unfolding. Once dried, the tissue can be returned to room temperature without risk of molecular unfolding or degradation reactions, as both require the presence of water.

During the freeze drying process, the tissue remains frozen within a refrigerated chamber. A vacuum is then applied to the chamber across a condenser, the condenser being at a lower temperature than the tissue chamber. In this way water molecules sublime and are transferred from the chamber to the condenser [9]. As the process continues, water is lost from the tissue much more slowly, therefore the temperature of the tissue chamber is gradually increased. Using this process a aw of 0.2–0.4 can easily be achieved.

Deep Freezing

One method to "immobilise" the water within a tissue is deep freezing. The water enters the solid phase as ice crystals and hence is no longer available as free water to take part in degradation reactions. The lower the temperature, the lower the water activity. In addition, low temperature itself prevents microbial growth and reduces reaction rates of e.g. enzymatic reactions. In practice, storage temperatures below −40 °C or ideally −80 °C are suitable for the long term storage of tissue matrices [7, 10].

High Concentration Solutes

The major solutes used for preservation of food stuffs have included salt and sugar (in preserving e.g. fish and fruit respectively). In contrast, glycerol has been the major solute used for tissue graft preservation, presumably owing to its long-standing safe use as a cryoprotective agent for tissues and cells. The principle for the immobilisation of water by solutes, is that each solute molecule will sequester water molecules within a hydration shell. The more solute molecules, the more water that becomes immobilised in the hydration shell hence the less free water.

The Euroskin Bank developed a glycerol, solute-preservation method for preserving non-viable skin whereby the skin was incubated successively in 50, 70 and then 85% glycerol [11]. Further, more detailed, characterisation of the glycerol and water fluxes in this system have yielded a more efficient and fully validated protocol [8, 12, 13].

It should be noted that it is important to fully remove these high concentrations of glycerol from the grafts prior to clinical use, to avoid high systemic concentrations of glycerol in the patient. At high doses glycerol does have toxic effects on muscles causing myonecrosis which in turn can result in renal failure and even death [14, 15]. Toxicity studies have shown that the LD 50 for glycerol is 0.00442 mL/g [16].

Fully validated protocols for the removal of glycerol from skin have been developed [8]. This is achieved by repeatedly washing the skin in physiological saline for at least 30–60 min.

7.2.1.3 Cell Removal

For tissues that are stored as non-viable grafts, the dead allogeneic cells serve no useful purpose, and in fact are reservoirs of degradative enzymes and immunogenic molecules. This has led to the development of methods to remove all cells and cell components from the tissue prior to preservation and subsequent clinical use [17–24]. It is important that the decellularisation process does not adversely affect the biomechanical and biological properties of the tissue matrix. At least some decellularised tissues have been shown to incorporate and re-model more rapidly, and to avoid foreign body responses by the recipient [17, 21].

For tissues that do not regenerate rapidly, a further improvement involves the cellular colonisation of the graft in vitro prior to grafting; using either autologous tissue cells or stem cells [25].

7.2.2 Preservation of Viable Tissue Grafts

The preservation of viable tissue grafts is even more challenging than for non-viable tissues. Once removed from the body and hence deprived of perfusion by the vascular system, the metabolising cells within the tissue become dependent on diffusion for the delivery of nutrients and oxygen and the removal of metabolites. For tissues bathed within a nutrient medium at normothermic temperature (circa 37 °C) diffusion from the surface of the tissue into its depths is a very inefficient process.

If cells within the central mass of the tissue are deprived of nutrients and oxygen for prolonged periods they will die and a central necrosis of the tissue will commence. The release of degradative enzymes will then cause the various degradative processes described previously for non-viable tissue, to occur. For tissues where cells are only located on the outer surfaces of the graft e.g. eye cornea, comprising of an outer epithelial layer and an inner endothelial monolayer, diffusion is adequate for maintaining cell viability even when maintained at normothermic temperatures; but for most other tissues reliance on diffusion is inadequate for periods longer than a few hours.

By reducing the storage temperature from normothermic (circa 37 °C) to hypothermic (circa 4 °C), the metabolic rate of the cells will be reduced and hence the demands for nutrients and oxygen. This has been shown to reduce the rate at which tissues lose viability [26]. However, at this lower temperature certain essential biochemical pathways may be inhibited. One example is the Na^+/K^+/ATP pump which maintains higher levels of K^+ and lower levels of Na^+ within cells than in the extracellular medium. To prevent the influx of sodium under hypothermic temperatures, some authors have recommended using a bathing salt solution formulated to reflect intracellular concentrations of Na^+ and K^+ [27].

Another reason why the viability of tissues declines rapidly when isolated from the body and maintained at lower temperatures, may be the effects of free radical damage. In life, the body has many systems to scavenge or inactivate free radicals which would otherwise attack cell membranes and macromolecules. When isolated from the body and maintained at reduced temperature, it is unlikely that the tissue will continue to produce sufficient antioxidants and scavenging enzymes to protect the cells and cell components.

All of these mechanisms combine to render viable non-frozen storage of most tissue grafts as being a very time limited method of storage.

7.3 Principles of Disinfection and Sterilisation

The major strategy for avoiding transmission of viral and other infectious diseases from the donor to the recipient is the rigorous screening of the donor's medical and behavioural history, and testing of blood samples for the presence of viral nucleic acid (NAT) or an immunological response to the virus (antibody detection).

However, there are opportunistic bacterial and fungal pathogens that may contaminate the tissue graft after death, during procurement or during processing within the tissue bank. In addition, certain tissues (e.g. skin and the gut) are associated with a resident microflora in life. Some of these microorganisms may become pathogenic in a surgical wound situation.

Although many tissue banks attempt to avoid contamination of grafts by applying strict asepsis during procurement and subsequent processing, the "sterile" nature of the graft can only be confirmed by final sampling of the tissues for microorganisms. Because destructive sampling of the whole graft defeats the purpose, most sampling regimens are only likely to detect gross contamination. It is reasonable to assume that an immunocompetent patient can eradicate small numbers of bacteria from a well vascularised tissue graft. The problem arises where the graft tissue is not well vascularised and areas of ischemia or necrosis occur. This produces anaerobic conditions ideal for the growth of e.g. Clostridium species, which can be highly pathogenic. Just a few Clostridial spores could lead to a life-threatening disease. This has led many tissue banks to routinely apply disinfection or sterilisation techniques to inactivate bacteria and fungi. Clearly, it is important that these techniques do not significantly damage the tissue graft or adversely affect its function or efficacy.

7.3.1 Definitions

"Disinfection" is a nebulous concept referring to the removal from an environment of microbes that might cause disease. In contrast "sterilisation" is now framed as a statistical concept involving the destruction of all microorganisms with a defined level of probability.

For any given population of microorganisms, the application of a sterilising chemical or physical agent will kill the microbial cells as an exponential function, to a first approximation (Fig. 7.2).

When the number of microorganisms is transformed to log10 this approximates a straight line (Fig. 7.3).

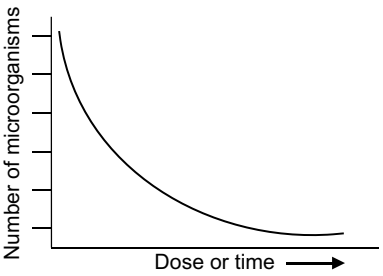

Fig. 7.2 Exponential decrease of microorganisms with increasing exposure to sterilant

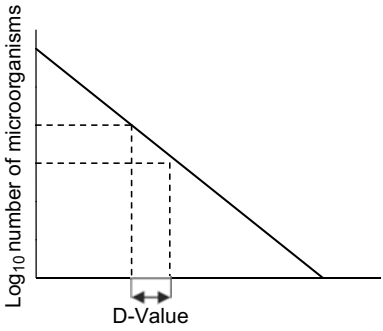

Fig. 7.3 The D-value concept

Therefore the dosage or exposure time required to reduce a given population, tenfold (i.e. 1 log10) is a constant equal to the gradient of the line. This is defined as the D-value (decimal reduction value). Using the D-value concept it is possible to predict the probability of a surviving microorganism after application of a defined amount (or exposure time) to a sterilising procedure; providing the starting level of contamination (the bioburden) is known. In the pharmaceutical industry the accepted level of sterility assurance (SAL) is to have no more than 1 viable microorganism per million units of product, i.e. a SAL of 10^{-6}. Therefore if a product has a starting contamination level of 10^{3} microorganisms per unit, and a SAL of 10^{-6} is required, then the sufficient dose of sterilant required is 9 $\times$ D-value (Fig. 7.4).

If there is more than one species of microorganism present, all at different contamination levels, and with different susceptibilities to the sterilant, then the analysis becomes more complex. Two approaches have been used. One is to assume a "worst case" scenario i.e. take values for the most resistant microorganism and choose a worst case contamination level. If a SAL of 10^{-6} is still achieved, then

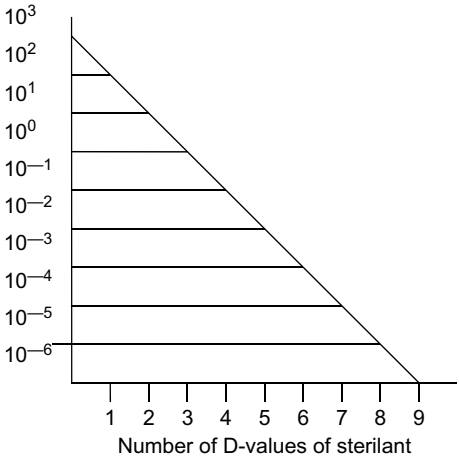

Fig. 7.4 Number of D-values of sterilant required to reduce bioburden from 10^{3} to 10^{-6}

the tissue can be considered "sterile" [13]. An alternative method using actual bioburden levels, and average susceptibility levels of each microorganism has been developed [28]. Once a sterilisation method has been fully validated, it should be possible to use parametric release i.e. if it can be shown that the sterilisation load was subjected to the defined level of the sterilisation process, then sterility can be assumed. In contrast, for disinfection, it is necessary to always test a sample of the tissue for the presence of residual pathogens.

7.3.2 Disinfection Methods

7.3.2.1 Viable Tissues

For viable tissue grafts the most common disinfection method is the use of antibiotics/antimycotics. These agents are used systemically for therapy. They may either kill the microorganism (bacteriocidal) or prevent its growth (bacteriostatic) so that the immune system can locate and kill the microbe. When used in vitro to disinfect tissue grafts such as heart valves, any bacteriostatic effect is not particularly effective as there is no immune system to clear the microbes and once implanted the concentration of the antibiotic will rapidly be diluted thus allowing microbial growth. Therefore it is the bacteriocidal effect which is most beneficial in vitro.

To ensure effectiveness against a range of microorganisms, a cocktail of antibiotics/antimycotics is often used. Care should be taken to fully validate the use of combinations of antibiotics as both synergistic and antagonistic effects may be seen, so that the published Minimum Bacteriocidal Concentration (MBC) for different microbes may no longer be relevant. In addition, the therapeutic use of antibiotics takes place at normothermia (i.e. 37 °C for systemic delivery and slightly lower for topical use). In contrast, many protocols advocate the use of antibiotic cocktails at ambient (circa 20 °C) or refrigerator (circa 4 °C) temperatures. Care should be taken to use antibiotics with a mode of action relevant at these temperatures. For example, penicillin acts by preventing the normal synthesis of cell walls during bacterial growth and therefore requires bacteria to be actively proliferating. At 4 °C few, if any, pathogenic bacteria are able to grow, hence Penicillin is unlikely to be effective at this temperature.

Caution should be taken when using antimycotics. Fungi, like humans, belong to the Eukaryotes whereas bacteria are Prokaryotes. There are many more drug targets against Prokaryotes not shared with humans, than there are for fungi. Therefore whereas antibiotics can be profoundly toxic to bacteria but hardly at all towards human cells, even at high concentrations, this is often not the case for antifungals [29]. Therefore, any novel cocktail should be fully validated for cytotoxicity against critical tissue cells prior to its introduction.

7.3.2.2 Non-viable Tissues

Generally, if a tissue is to be used as a non-viable tissue implant, application of a sterilisation technique would be preferable. However, there are sometimes concerns

surrounding the effects that harsh sterilisation techniques might have on the tissue structure and efficacy. In these cases a less severe disinfection is common. Ethanol is rapidly lethal to non-sporulating bacteria and destroys mycobacteria but is ineffective at all concentrations against bacterial spores.

In addition to ethanol, other alcohols, organic solvents and detergents have been used to disinfect tissues. Specific combinations have been developed to produce proprietary "sterilisation" methods for banked tissues, where a SAL of 10^{-6} is claimed.

Other antiseptics and disinfectants used in the medical setting e.g. hydrogen peroxide and other peroxygens, chlorine compounds and other halogens and even mercurial compounds such as Cialit [30] (used to preserve middle-ear ossicles) have been used to disinfect tissue grafts.

Mild heat treatment has been used to inactivate vegetative bacteria, but care must be taken to avoid destroying advantageous heat labile components (such as growth factors), and denaturation of structural proteins such as collagen. Because the collagen in bone tissue is protected by a mineral coating, this tissue can withstand higher temperatures before denaturation commences. A temperature of 80 °C was generated in a device developed for the disinfection of femoral heads [31], but many bacteria are fairly resistant to these temperatures whereas beneficial biochemicals such as the BMP's are heat labile.

7.3.3 Sterilisation Methods

7.3.3.1 Physical Methods

With respect to physical methods of sterilisation, the temperature required for dry heat sterilisation (over 160 °C) or wet heat sterilisation (in excess of 121 °C) are detrimental to the structure of tissue. Even bone tissue with its protective mineralised coating was shown to function poorly in vivo after boiling [32]. The only physical method currently widely used for tissue sterilisation is the use of irradiation (either gamma or electron beam). This is the subject of another chapter in this book and will not be considered further here.

7.3.3.2 Chemical Methods

For many years, ethylene oxide was the preferred chemical sterilant. When used as a gas it was shown to effectively penetrate tissue matrices including dense cortical bone [33]. More recently however, there has been concern about the genotoxicity of residual gas left in the matrix, for which there is thought to be no safe residual level. Glutaraldehyde has been advocated as a sterilising chemical, and has been used in the manufacture of porcine heart valves [34]. However, in addition to killing microorganisms it also significantly cross-links the collagen within the tissue. This renders porcine tissue much less immunogenic but also causes stiffening of the tissue including the valve leaflets which is disadvantageous and induces rapid

calcification in vivo. Peracetic acid is now frequently used in hospitals for sterilising endoscopes in preference to glutaraldehyde. It has also been used for sterilising bone grafts [35]. Its use for other tissues must be undertaken with care as tendons treated with relatively high concentrations of peracetic acid were shown to have inferior performance in vivo [36]. In contrast, heart valves sterilised using low concentrations of peracetic acid were shown to function satisfactorily in a sheep model (E. Ingham, personal communication). Other chemical sterilising agents used in hospitals and medical manufacturing, such as vapour phase hydrogen peroxide, ozone, etc., have not been extensively evaluated for tissue grafts.

A more recent novel approach has been to use combinations of antimicrobial chemicals which are synergistic in their antimicrobial activity but not with respect to any damaging effects on tissues. As a result, much lower concentrations of the chemicals can be used with negligible adverse effects on the tissue [37, 38].

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Cryopreservation in Tissue Banking

8

Peter Kilbride and Julie Meneghel

Abstract

Successful cryopreservation of cells and tissues ensures genetic stability and preserved function of a banked sample throughout storage. It also provides valuable time for quality and suitability assessments to ensure the best possible patient outcomes. This flexibility lends significant support to the increased adoption of tissue therapies and the development of new protocols and procedures. This chapter considers the design and execution of current cryopreservation protocols, outlining the causes, both physical and biological, of potentially lethal cryo-injuries and how these can be reduced to clinically acceptable levels during freezing and thawing. The appropriate preparation of freshly harvested samples is discussed, including the selection of, and pre-treatment with, effective cryoprotectant compounds and the necessary use of controlled cooling and warming rates. The implications of possible differences between post-thaw survival of low volume samples, typically preserved for quality control functions, and their large volume counterparts is also considered. As well as dealing with cell suspensions the discussion includes current approaches towards the cryopreservation of larger samples, ranging from spheroids, organoids and bioartificial materials to entire, natural organs. The risk to both samples and personnel due to contaminants that inevitably accumulate in liquid nitrogen storage vessels over long banking times is detailed. Finally, alternatives to the use of liquid nitrogen in the management of the entire cryochain due to its safety, sterility and usability issues and difficulties appear necessary and are proposed.

P. Kilbride · J. Meneghel (✉)
Cytiva, Danaher Corporation, Cambridge, UK
e-mail: julie.meneghel@cytiva.com

P. Kilbride
e-mail: peter.kilbride@cytiva.com

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8.1 Introduction

The goal of cryopreservation when dealing with human cells, tissues and organs is to use low temperature to preserve, in a stable state, the structure and function at the level required for therapeutic use at some future time [1]. Tissues and organs are commonly transported hypothermically, at lowered, non-freezing temperatures [2], thereby creating a seriously limiting window for successful storage of between 4 and 24 h for organs before transplantation [3, 4] and, perhaps, a few days for tissues [5–7]. Preservation in this way poses a safety risk for the intended recipient as it does not allow for the 14-day incubation period typically required for sterility testing [8], and the time necessary for biochemical and biomechanical characterisation may not be available. Such time limitations, when added to geographical and tissue-matching constraints can result in ineffective matching of donor organs to recipients. Similarly, unplanned changes in scheduling by patient or practitioner may lead to the discard of invaluable biological material. These timing issues can be avoided if the therapeutic product is cryopreserved at ultra-low temperature [3, 9].

The long-term preservation of tissues by cryopreservation thus allows for more rigorous quality control tests to be performed prior to transplantation for safety and efficacy, but also for full matching to be carried out, thereby reducing the risk of tissue/organ rejection. This is of particular interest when using chimeric techniques that can substantially reduce rejection rates, a cryopreserved organ making sufficient time available to adapt the recipient's immune system to accept the organ without extreme immunosuppression. This would not only help reduce rejection rates but also improve quality of life for patients' post-transplant [3]. Furthermore, patients can receive multiple doses from a single batch of cryopreserved material, in a consistent manner, that would not be possible with unfrozen storage. Cryopreservation can also ease logistical issues related to delivery of cell therapies by accommodating prolonged shipment times and flexible scheduling of delivery of the therapy to the patient.

In this chapter the principles underpinning cryopreservation and the mechanisms leading to cryoinjury are outlined. Procedures that minimise injury and provide successful cryopreservation for a range of tissues and organs are considered, together with issues related to processing or banking of human biological material for therapeutic use. Quality control storage and transport requirements are also discussed.

Successful cryopreservation depends upon reducing the temperature of the biological material to a point where aqueous-based, chemical change is prevented. When ultra-low storage temperatures are employed the samples remain below the glass transition temperature of their cryopreservation solution. Consequently, diffusion-limited reactions are at an absolute minimum and the product should remain unaltered for at least decades and significantly longer periods might be expected. The underlying principles relating to mammalian cell cryopreservation

have been extensively investigated and documented since the landmark discoveries of the cryoprotective abilities of glycerol and dimethyl sulphoxide [10, 11]. These principles have been applied successfully to a diverse range of isolated cells [1], yet extending the knowledge and practice derived from this work to the cryopreservation of complex tissues has proved more challenging.

It should be noted that many of the challenges have indeed been met for the cryopreservation of tissues that provide their therapeutic benefits through specific biomechanical properties e.g. bone samples, heart valves, and blood vessels and do not require live cells to be effective. For materials such as heart valves and decellularised organs, the patient's own cells will use the structure as a base to attach to after transplantation. These systems are typically easier to cryopreserve as damage from cryoprotectants is less of a consideration. Such tissues can be stored for sufficiently long in a mechanical freezer at −80 °C to meet typical, practical needs [12–14]. However, in some cases, physical damage from ice can still be problematic and to avoid this, vitrification techniques may be used e.g. with heart valves [15]. While more concentrated CPAs can be used without fear of cellular damage, very rapid cooling and warming techniques are still required to prevent ice formation, and so new methods such as magnetic nanoparticle and dielectric warming techniques can be used [16, 17].

For individual cells suspended in a fluid matrix e.g. derived from peripheral blood, cord blood or bone marrow, cryopreservation techniques based upon relatively slow cooling rates and the use of cryoprotective agents (CPAs) have proved highly effective [18–20]. However, a great many tissues and composite tissues (including spheroids and organoids) are more highly structured, consisting of groups of cells, either of the same type or a variety of different functional units arranged in an orderly pattern, intimately associated and immersed in an extracellular matrix. Examples include epithelial, connective (cartilage, bone, dense fibrous tissue, adipose tissue), muscle (skeletal, smooth, cardiac) and nerve tissues. For these structured tissues cryopreservation may cause damage at the cellular level (for mechanisms—see below) and may also alter structural elements that affect tissue function. This adds further difficulty to the design and execution of effective cryopreservation protocols.

Any damage suffered by cells and tissues during cryopreservation is due, primarily, to the dramatic changes that occur when ice appears in the aqueous, extracellular matrix. As the amount of ice increases, with time and/or reducing temperature, the injury can progress from impaired function to cell death. Any appreciable ice formation in the intracellular matrix is lethal upon thawing. To minimise such damage, it is necessary to employ chemical cryoprotectant additives, control the rate of cooling and thawing and use ultra-low storage and transport temperatures. Further post-thaw procedures may be required including dilution or rinsing out of CPAs and an incubation period to allow for cellular repair.

8.2 Critical Impact of Ice Formation

Below the freezing point of aqueous solutions (commonly between −5 and −10 °C for the extracellular medium of cryoprotected samples), crystallisation of their supercooled water becomes thermodynamically more probable and ice crystals start to form and grow. This partitions the sample into a solid phase consisting of ice crystals and a liquid phase consisting of the remaining unfrozen water, solutes, ionic salts and the biological material. As temperature decreases the crystal phase increases in volume and the liquid phase decreases correspondingly, becoming increasingly hypertonic (Fig. 8.1). The hypertonic liquid phase creates an osmotic gradient between the extra- and intra-cellular compartments that draws cytoplasmic water out of cells, and cell shrinkage results (Fig. 8.2).

At slow cooling rates there is substantial time for cells to dehydrate and so the volume of free water available to form ice within the cell is minimised, thereby reducing the probability of lethal intra-cellular ice formation. However, if cooling is too slow then the increased time at high solute and ionic salt concentrations may prove damaging, depending upon cell type. Conversely, if cooling is too rapid then intracellular water loss may not be sufficient to prevent significant, intracellular ice formation, with lethal consequences. In addition to the osmotic stresses that accompany extracellular ice formation, cells and tissues can be adversely affected by the mechanical effects of ice crystals, altered solution properties e.g. pH and viscosity and the generation of gas bubbles and electrical fields at the ice/solution interface [22, 23]. Damage during cryopreservation due to exposure to hypertonic solutes during slow cooling (solution effects) and due to intracellular freezing were

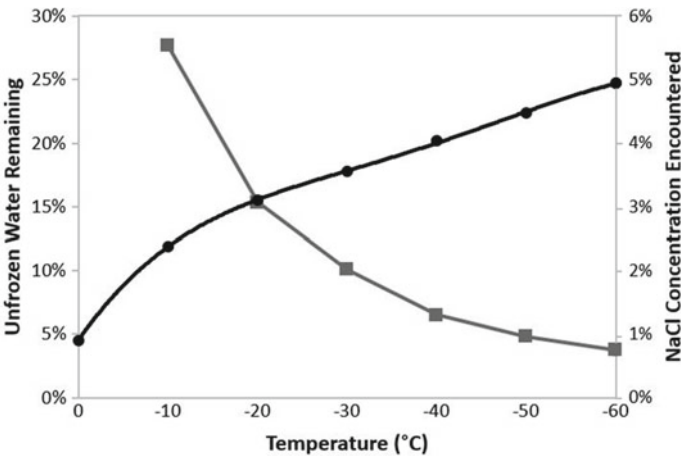

Fig. 8.1 The reduction in unfrozen water (%, squares) and increase in NaCl concentration (% wt, circles) during freezing of a solution of 0.9% wt/v NaCl and 10% v/v dimethyl sulphoxide to −60 °C

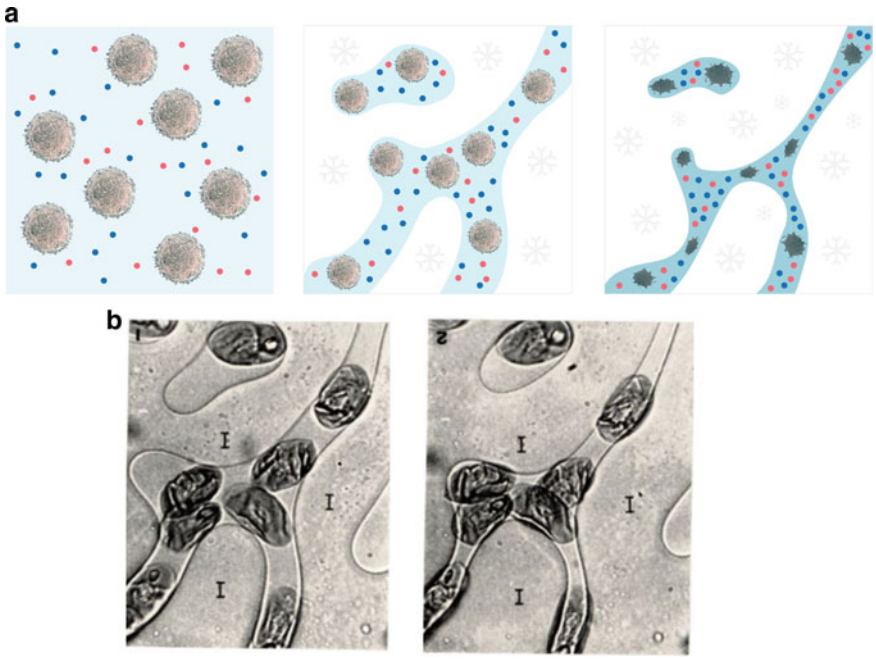

Fig. 8.2 **a** The physical effects of ice formation on suspended cells (left) during cryopreservation. Ice formation creates channels of cryo-concentrated solution with entrapped cells (middle). These become increasingly packed together and shrunken (right). The dots represent various solutes and snowflakes represent the ice fraction. **b** Frozen red blood cells as seen using a cryomicroscope from Rapatz and Luyet [21]. The temperature is reduced between the left and right-hand images, showing increasing dehydration and smaller ice channels

combined into the cornerstone, two-factor hypothesis by Mazur in 1972 [24]. This clearly indicates that successful cryopreservation requires both solution effects be minimised and intra-cellular ice formation avoided (Fig. 8.3).

The goal for successful cryopreservation using relatively slow cooling rates is to reduce, without injury, the amount of intra-cellular water so that when the sample is reduced to ultra-low temperatures that water vitrifies (see below) and lethal intra-cellular ice crystals do not form. This process has to be reversed on thawing. A very wide range of human nucleated cells (e.g. stem cells, lymphocytes, hepatocytes) can be successfully cryopreserved by applying slow cooling rates (c. -1 °C min^{-1}) to temperatures close to -50 °C before transfer to a cryogenic (≤ -140 °C) storage device [19, 25–32].

The optimal cooling rate that enables a maximum recovery of cells after thawing is directly dependant on the rate at which water is able to flow out of cells. This is dictated by the cell membrane permeability to water and the cell surface-to-volume (S/V) ratio. High cell permeability and S/V ratio indicate that the cell will lose water relatively rapidly during cooling in the presence of ice and the cooling rate

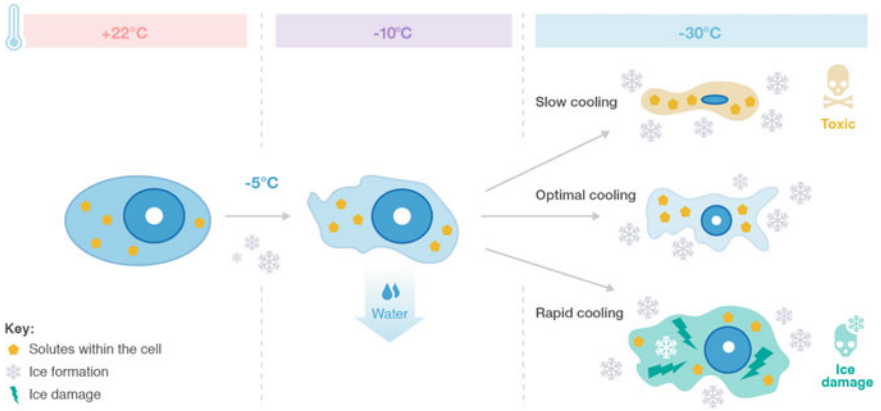

Fig. 8.3 Schematic representation of the two main causes of cell death when using non-optimal cooling rates. As ice locks away pure water in the extra-cellular space, the cell starts to dehydrate, which protects it from intracellular ice formation and consequent damage. Cool too quickly and the cell does not have time to dehydrate enough, resulting in cell death. Cool too slowly however, and the possibility of excessive cryoprotectant and solute toxicity emerges

providing a risk of intra-cellular ice formation will be elevated (Fig. 8.4). Larger or more complex cellular structures such as embryos and tissue pieces, and those with low membrane permeability, will need a longer time for sufficient cell dehydration to avoid intracellular ice formation so an appropriately slower cooling rate has to be employed.

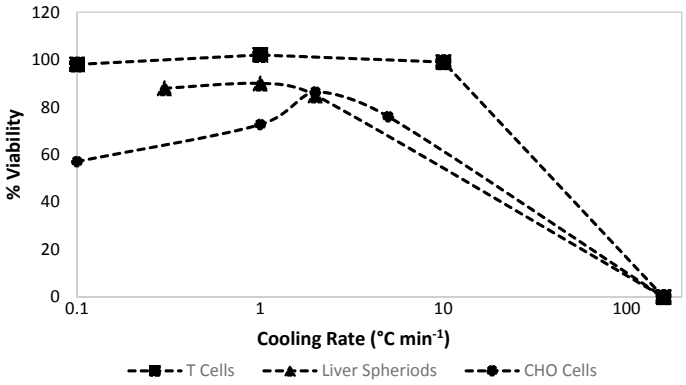

Fig. 8.4 Post-thaw viability of examples of nucleated mammalian cells indicate the optimal cooling rate for successful cryopreservation. For nucleated somatic cells, cooling rates are typically optimal between 1 and 2 °C min^{-1}, although this is cell dependent and many variations exist. Cooling in excess of 100 °C min^{-1} tends to lead to complete cell death, while slower cooling rates can also reduce outcome. Dashed lines added as a visual aid

The primary causes of cryoinjury are thus related to the formation and growth of ice crystals. Consequently, a long sought-after objective has been to find a way to cryopreserve biological material, particularly tissues and organs, in a way that would avoid ice formation. Vitrification, the solidification of water in a glassy rather than a crystalline state provides this opportunity and involves the application of very high cooling rates and/or very high concentrations of solutes. It is theoretically promising but very difficult to achieve in practice, due to the technical difficulties involved in cooling samples fast enough and/or due to the toxicity of the high concentrations of solutes required. Thermal transfer is central to the success of vitrification and the steep thermal gradients that are inevitable when cooling larger samples are often a barrier to success. To achieve sufficiently high thermal transfer, tissue samples may be directly plunged into liquid nitrogen [33], posing a contamination risk as liquid nitrogen is not a sterile fluid (see Sect. 8.8). Furthermore, the cooling rates currently achieved in attempts at vitrification are often not sufficient for anything but the smallest samples (μl volumes) even with a direct plunge into liquid nitrogen [34, 35].

8.3 The Role of Added Cryoprotectants

Controlling cooling at the required rate is not, by itself, sufficient to ensure adequate, post-thaw recovery of viable, functional cells. The addition of a cryoprotective agent (CPA), or a mixture thereof, before cooling begins will be required. The effective CPAs in current use are non-electrolytic solutes of relative low cytotoxicity (at least at low temperature) that can protect biological samples from cryoinjury. Their presence in an incubating medium decreases the amount of ice formed extracellularly, thereby reducing the concentration of electrolytes in the unfrozen fraction of the extracellular matrix. Additional protective mechanisms that are conferred by CPAs depend on whether or not they permeate the limiting cell membrane.

Permeating CPAs increase the intracellular solute concentration, thereby reducing the osmotic imbalance caused by the formation and growth of extracellular ice. This limits the extent of cell dehydration and shrinkage that continues as cooling progresses. These compounds may help to retain intracellular water molecules through hydrogen bonding, and also help stabilise intracellular proteins and organelles during cooling [36]. Since its protectant properties were discovered, using red blood cells and spermatozoa [11], DMSO is, perhaps, the most widely used cryoprotectant that permeates cells by simple diffusion. It is a small, amphiphilic and uncharged molecule (78 Da) with limited cytotoxicity at relatively low concentrations and temperatures (i.e. room temperature and below). Glycerol, whose cryoprotectant properties were discovered in 1949 [10], also with spermatazoa, is also a commonly included component of cryopreservation media. It will penetrate limiting cell membranes but at a slower rate than DMSO [37] so, when used as a cryoprotectant, a longer incubation with the biological sample is required,

perhaps at higher temperature than when using DMSO. Other commonly used intracellular CPAs include ethylene glycol and propylene glycol (1,2-propanediol) [38].

By contrast, non-permeating CPAs e.g. some sugars and polymers of various molar mass, act by increasing the extracellular osmolarity, thus enhancing cellular dehydration. They also increase the viscosity of the extracellular medium, so slowing down the kinetics of ice crystal formation and growth. They may stabilise the cell membrane by interacting with the phospholipid headgroups of its external leaflet [12, 38–41]. Because of their complementary, beneficial effects, cryoprotectant media are often a mixture of membrane permeating and non-permeating CPAs [38].

To ensure successful, biological recovery, the concentration of each CPA used in a particular protocol will require optimisation. Where relatively slow cooling is employed, typical for therapeutic products, the selected level of protectant must be enough to exert the required, positive influence, but not too much to generate an unacceptable level of additional osmotic stress. To avoid cellular shock due to a sudden and significant increase in hypertonicity prior to cooling, the addition of cryoprotectant should be progressive [40, 42]. To achieve the required effect with larger samples it is also important that sufficient time is allowed for the CPAs to diffuse to the innermost tissue regions [43, 44]. However, an overly prolonged exposure to CPAs can also be damaging. Following thawing it may be necessary to remove/dilute CPAs due to their cytotoxicity and/or adverse reactions they might generate when infused into a patient. Careful, serial dilution may be necessary to avoid any rapid, damaging cellular response to the inevitable lessening of hypertonic stress that accompanies thawing.

8.4 Sample Thawing

To ensure maximum recovery and efficacy of a cryopreserved sample, the thawing procedure has to be optimised with as much care as the cooling protocol. Warming rate can have a significant impact on cell damage and recovery. If the cells or tissues have reached water potential equilibrium by dehydration during relatively slow cooling (typical for nucleated mammalian cells and tissues) they will become increasingly rehydrated as extracellular ice melts and, fortuitously, the warming rate achieved by immersion of the sample container in water at 37 °C is likely to be satisfactory. A study using T cells showed that with a slow cooling rate that ensured equilibrium freezing (<10 °C min^{-1} in DMSO-based CPAs), then the warming rate was not critical when reduced to rates as low as 1.6 °C min^{-1} [27]. However, many protocols are still too fast to provide effective equilibrium freezing. In such cases a fraction of the extracellular water will become vitrified, rather than crystallised, during final cooling to ultra-low temperatures. As warming progresses during thawing this vitrified water is likely to recrystallise, initially increasing the hypertonicity of the extracellular solution and the biological materials will experience a

sudden, hypertonic stress once a significant fraction of the ice has melted [45]. This additional level of stress can cause significant, post-thaw injury. For T cells subjected to non-equilibrium freezing, ice recrystallisation during warming was associated with deleterious effects on post-thaw cell recovery, with the problem exacerbated at slower warming rates [27].

Another significant risk associated with thawing is the duration of exposure to CPAs at warm temperatures as ice melts. For example, DMSO cytotoxicity is limited at low temperatures but increases dramatically with rising temperature [42]. In current practice, thawing is commonly achieved by direct immersion of the frozen sample in a warm (37–40 °C) water bath [12, 18, 25, 29–31], with precise conditions difficult to standardize. A widely acknowledged difficulty is in determining when thawing is complete, which is commonly done by largely subjective visual assessment or adoption of a timed incubation previously found to be effective. If there is a delay in removing an already-thawed biological specimen from the CPA-containing, freezing medium serious post-thaw injury due to cytotoxic effects can result. As a wide range of controlled-rate freezing devices are available to ensure that precise temperature reduction can be achieved during cryopreservation, it is to be hoped that there will be a similar growth in the availability of automated, standardized thawing devices to improve control in this critical area [46].

8.5 Spheroid and Organoid Cryopreservation

Most cryopreservation, with perhaps the notable exceptions of cord blood, peripheral blood and bone marrow derived products, has historically been carried out on biological materials not intended for direct therapeutic use. Rather, they have been used for the storage of cell lines, reproductive medicine, storage of supernatants for future testing, and plant and conservation work. However, novel therapeutic treatments are also becoming prevalent, from bioartificial livers to tissue scaffolds to retinal spheroids. This opens up new opportunities and challenges for cryopreservation. These new treatments invariably require cryopreservation on economic and logistical grounds for widespread uptake, however successful cooling and warming can be more difficult than for individual cells. This is related to the tissue architecture which, if damaged, can impact the performance of individual cells. In tissues with multiple cell types, different cellular populations can have different optimal cooling rates, which can impact overall performance, and the large volume of samples can limit heat and mass transfers [43, 47, 48].

Cryopreservation of organoids and spheroids is becoming increasingly important as their therapeutic use and value in research and disease modelling is growing [49]. Organoids are small, self-organised clusters (typically a few mm in diameter) that develop in culture within an extracellular matrix and are initiated from a specific patient-derived organ. They proliferate and organise into a 3D structure containing a range of cell types from the organ of origin and have a realistic, but limited, microanatomy [50]. Spheroids are 3D multicellular monocultures that also retain

their initial cellular structure and function. They are commonly established from isolated tumour cells and have also been established from other cell types including stem cells, hepatocytes, and neuronal cells [49, 50]. A particular benefit of organoids is their diverse range of functional cell types and a major challenge for successful cryopreservation is to find a cooling rate that retains both the function and population balance between them. For the essentially monocultural spheroids, cryopreservation must also preserve the required level of cell functions.

The 3D structure will affect cellular diffusion, impacting on CPA uptake and removal and cellular dehydration. Thermal transfer across the 3D structure will also be influenced, affecting the cooling and warming rates experienced by cells at different internal locations. Each of these dynamic processes will be slowed as distance from the organoid or spheroid surface increases. Consequently, a slower cooling rate, for example may be required to ensure adequate cell dehydration across the 3D structure following ice nucleation. This has been demonstrated for HepG2 cells where a required cooling rate of c. −2 °C min^{-1} for isolated cells falls to −0.3 °C min^{-1} for spheroids [47, 51]. Conventional slow cooling has thus been successful for spheroids derived from pancreatic islets [52, 53], bioartificial liver [54], and prostate tissues [55]. Cryopreservation protocols may, with further development, be routinely applicable for functional organoids established, for example, from thymus, pancreas, mammary, kidney, lung and liver [50, 56].

In some instances, the satisfactory cryopreservation of these 3D structures using slow cooling has been unattainable and vitrification has been used. Employing the necessary rapid cooling techniques and high solute concentrations has provided good post-thaw outcome for retinal organoids [57] and embryos, these being viewed as a special type of organoid [58]. However, this technique is limited by the small sample volumes that can be processed at any one time with, typically no more than a few organoids of <1 mm each in each droplet to be vitrified.

8.6 Toward the Cryopreservation of Organs and Biopsies

Attempting to apply methods based on current cryopreservation protocols to whole organs introduces its own set of challenges. These relate primarily to the thermal and diffusion gradients that inevitably occur in larger, 3D structures and to the diversity of structure and function that has to be preserved. Currently, effective full or partial organ cryopreservation is not achieved due to damage caused by ice formation and prolonged exposure to CPAs. Notable exceptions occur in a limited number of organs which can be dissected, and tissues transplanted without losing the essential function of the organ—this includes thymus, ovaries, cartilage and skin. These organs seem less sensitive to ice damage on cryopreservation, presumably as their macrostructure is less important to their function than in other organs such as heart or muscles. Following cryopreservation by slow cooling, paediatric thymus slices have been shown to function comparably (in terms of induction and support of T-cell development) to fresh human thymus slices

transplanted into mice [59]. Thymuses removed from infants undergoing cardiac surgery are normally sliced, treated, and then transplanted immediately, or discarded if no suitable recipient is presented. Cryopreservation of thin slices offers the potential of transplantation into several human babies born without a thymus, to reconstitute their T-cell immunity.

Transplanted human ovaries from cryopreserved ovarian tissue segments have been shown to result in live births when using a standard slow cooling protocol [60], complementing work on ovarian strips [61, 62]. In the past few years whole sheep ovaries have been cryopreserved and, following thaw and re-transplantation, have resulted in fertile offspring [63]. However, the ability to reproducibly cryopreserve entire human internal organs, to be stored frozen, has continued to be elusive [64].

Cartilage cryopreservation presents a unique challenge as the sample is, typically, a thin slice of cartilage with bone attached to one side, so the diffusion of cryoprotectants can only occur through one side of the tissue. Due to the lack of a blood network, CPAs can take many hours to diffuse through the tissue and therefore low-toxicity agents must be chosen [44]. To resolve this difficulty, high concentrations of CPAs are added initially to increase diffusion rates, and then steadily reduced until the final concentration is achieved throughout the tissue [65].

Alternatively, vitrification techniques are being developed for large-organ cryopreservation [66–68]. A study with rabbit kidneys showed strong functional recovery from almost half of the preserved whole kidneys [69] and this technique is being pursued with a view to scale-up to accommodate human kidneys [70]. Vitrified ovarian tissue slices have been transplanted successfully to provide follicles for subsequent IVF procedures producing two live births so far [60, 71, 72]. Still, the reports to date suggest that the outcomes for conventional slow cooling and vitrification of ovarian slices are comparable [72].

Finally, biopsy samples are frequently cryopreserved and stored in biobanks for future use e.g. being thawed several years later and compared with a patient's subsequent medical history since excision. In such cases, living cells are often not required to extract data, so suboptimal cryopreservation techniques that result in cell death are commonly employed. There would be great advantage in the development of improved techniques that preserved both viability and biopsy structure, allowing much more data to be extracted when required. Methods such as freeze-substitution [73] and high-pressure freezing [74, 75] have been proposed to achieve this.

8.7 Quality Issues

Therapeutic treatments, where cells are often directly transfused into a patient immediately post-thaw, require the highest level of rigour during cryopreservation, storage, and thawing conditions to ensure patient safety and traceability. The necessary resources and procedures should be precisely documented as standard operating procedures and a monitoring system established to ensure that they are

followed. The responsibility for updating and circulating procedures should be clearly allocated. For the biological material being processed, acceptable quality may be defined as meeting the required criteria for structure and performance at the necessary time after thawing. For those therapeutic products destined for direct clinical administration this will be immediately upon thawing, whereas other products may benefit from recovery time under laboratory culture conditions, for example.

To avoid unacceptable wastage, it is prudent to have a number of sub-samples of the bulk sample available for assessment, these having been cryopreserved in the same way and at the same time as the bulk material. Using cryobags, for example, discrete segments of the bag or cryovials are cryopreserved together with the much larger volume cryobag. These are intended to be used for quality control to determine the characteristics and efficiency of the bulk sample prior to end use. Such practice assumes that cells recovered from these quality control segments/cryovials are identical to, or at least satisfactorily represent, those within the bulk sample [76]. This can be problematic, however, as the smaller containers necessarily have thermal characteristics that differ from those of the larger bag. These differences affect heat transfer during cooling and thawing and so, despite being processed together, the bulk sample and the smaller sub-samples may have differing post-thaw outcomes [77, 78]. Discrete segments of umbilical cord blood bags intended for quality control thus provided poorer post-thaw assessments to their bulk sample counterparts when cryopreserved under identical conditions [78]. In this instance it is clear that the quality control results can be viewed as a 'worst-case' outcome, a perspective that may be more widely relevant and certainly an important area for further investigation.

8.8 Sterility, Handling and Contamination Issues

Liquid nitrogen (−196 °C) is the standard coolant used in the majority of controlled-rate freezers and is the medium of choice for the long-term storage of the cryopreserved product. Its advantages are that it is easily liquefied, widely available and relatively inexpensive. However, liquid nitrogen also comes with a number of drawbacks. There are risks when handling related to accidental contact with the ultra-low temperature liquid and to asphyxiation if evaporating nitrogen reaches toxic levels in an enclosed workspace, for example. Safe working practices need to be rigorously applied where liquid nitrogen is being used [79, 80].

Liquid nitrogen can also be a source of microbial contamination of stored biological samples and of the protected environment in clean rooms, for many microorganisms survive immersion in liquid nitrogen and can proliferate when returned to physiological conditions. Liquid nitrogen is produced through fractional distillation of air at high pressures and initial production, typically, has a very low microbial count. However, further contamination can occur during storage and distribution. Additionally, on arrival in a hospital, manufacturing site, or laboratory, the nitrogen is likely to be decanted into a coolant storage vessel, such as a

pressurised Dewar, before final transfer to an open liquid nitrogen storage container or the supply container of a controlled-rate freezer. There is an obvious risk of contamination of the storage environment when using contaminated liquid nitrogen to top up a storage vessel [81–84]. Leaking vials, cryobags or other containers can also act as source of biological contamination, as can pathogens in the air when the Dewars are opened to add or remove samples. Further, using contaminated liquid nitrogen as the coolant in a controlled-rate freezer can contaminate the laboratory environment as the machine vents nitrogen, particularly damaging to clean room environments. There is also a potential risk of contamination of sample container surfaces during processing in a freezer reliant upon vaporising nitrogen for direct cooling of the samples. Microbial contamination is not only a potential hazard for cryopreserved samples but may also pose a risk for operators. Transfer Dewars and dry shippers that are warmed once the sample has been delivered and are then to be returned to the sender for re-use, are also a potential source of contamination. These vessels can accumulate pools of condensate which can become contaminated with microorganisms. When the container is subsequently refilled with liquid nitrogen any such contamination is effectively cryopreserved and risks being deposited onto stored samples during a subsequent transport cycle (Table 8.1).

Table 8.1 Microorganisms cultured from swabbed, inner surfaces of liquid nitrogen storage tanks and from sampled, sedimented detritus

	Nitrogen phase	Swab culture	Sediment culture
1 (20 years)	Liquid	*Acinetobacter lwoffi* *Corynebacterium* sp.	*Acinetobacter lwoffi* *Pseudomonas oryzihabitans* *Sphingomonas paucimobilis*
2 (20 years)	Liquid	*Pseudomonas fluorescens/putida* *Corynebacterium* sp. *Staphylococcus hominis*	*Acinetobacter baumannii* *Acinetobacter lwoffi* *Bacillus* sp. Gram-negative bacilli *Pseudomonas fluorescens/putida* *Aspergillus fumigatus* *Phaecilomices* sp. *Aspergillus glauctus*
3 (15 years)	Liquid (detritus) Gas (swab)	Negative	*Enterobacter amnigenus* *Staphylococcus auricularis* *Delftia acidovorans*
4 (15 years)	Liquid	*Aspergillus fumigatus* *Bacillus* sp. *Staphylococcus capitis* *Staphylococcus haemolyticus*	*Acinetobacter lwoffi* *Shewanella putrefaciens* Gram-positive bacilli *Gemella* sp. *Staphylococcus auricularis* *Rhodococcus equi* *Corynebacterium* sp.

The left column shows the tank identification number and the length of time the tank had been in service, without cleaning (from [85])

Critically important is managing any risk of contamination that could lead to infection in the recipient of a therapeutic product, an example being a limited outbreak of Hepatitis B [83]. Samples are ideally stored in hermetically-sealed vials or cryobags (standard cryovials are not medical devices) and in the vapour phase above liquid nitrogen (c. −170 °C), not the liquid phase. Gasses within the sample will reduce in volume during cooling creating negative pressure that can assist the entry of contaminants if they are not perfectly sealed. The external surfaces of thawed samples should be sterilised in some way e.g. with wipes before opening. Storage and transport tanks are difficult to decontaminate [86] and, where stored samples are involved, the process assumes availability of a second, clean storage vessel. Safe removal of stored material into a second tank is critical and, notwithstanding such efforts, sterility will again begin to be compromised once liquid nitrogen is re-introduced to the newly-sterilised vessel.

Consequently, care must be taken when storing samples for therapeutic, or other, uses. Liquid nitrogen should also not be used in clean rooms as it may contaminate the air, and so controlled-rate freezing should be carried out using a nitrogen-free device, or at a separate location.

8.9 Conclusion

Successful cryopreservation of isolated, mammalian cells at ultra-low temperatures can be achieved using 'conventional' slow cooling protocols, applicable to both small (cryovial) and large (e.g. blood bag) volumes. A clear understanding of the chemical and physical issues affecting cell freezing led to the development of these protocols and will also support new applications and improvements. Slow cooling procedures are being adapted for the successful cryopreservation of complex tissues and 3D structures with increasing success as knowledge and experience increase. Vitrification by rapid cooling also has application for some of these more complex materials but increasing size (e.g. whole organs) poses a significant practical challenge to achieving the required rates of cooling. Reliance on liquid nitrogen as a coolant and storage medium can raise difficulties for transport and the various risks of microbial contamination. These difficulties have to be managed, but can be a barrier to successful adoption of cryopreservation in some clinical and clean room situations. The increasing availability of equipment and procedures that do not rely on a liquefied coolant may resolve many of these difficulties.

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Sterilisation by Irradiation

9

Artur Kaminski, Izabela Uhrynowska-Tyszkiewicz, and Waclaw Stachowicz

Abstract

Sterilisation refers to any process that eliminates/inactivates transmissible infectious agents. There are two main types of sterilisation physical and chemical one. Radiation sterilisation is a physical process based on the action of radiation energy with the matter. The term ionising radiation covers all types of radiations carrying the energies capable to produce cascades of ionisations in the matter. Sterilisation efficacy of ionising radiation lies in its good penetrability in the matter followed by killing effect on pathogens. For that reason irradiation became a very frequently used sterilisation method in tissue banks that decided to use terminal sterilisation of non-viable tissue grafts. Authors describe in the chapter types of irradiation used for sterilisation of human tissue grafts: electromagnetic rays e.g. gamma and X-rays and corpuscular radiation e.g. fast moving mono-energetic electrons. Following subchapters are focused on the specificity of radiation sterilisation with gamma rays and electron beams, then dosimetry methods applied for radiation sterilisation are described. Detailed mechanisms of action involved in the inactivation of micro-organisms is the next raised very important issue. As an important topic in tissue banking practice radiation resistance of micro-organisms is also described. Finally, factors affecting the effectiveness of radiation sterilisation and the sensitivity of micro-organisms to irradiation are discussed.

A. Kaminski (✉) · I. Uhrynowska-Tyszkiewicz
Department of Transplantology and Central Tissue Bank, Medical University of Warsaw, Ul. Chalubinskiego 5, 02-004 Warsaw, Poland
e-mail: artur.kaminski@wum.edu.pl

W. Stachowicz
Institute of Nuclear Chemistry and Technology, Ul. Dorodna 16, 03-195 Warsaw, Poland

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Keywords

Radiation sterilisation · Ionising radiation · Gamma rays · Electron beams · Dosimetry

9.1 Introduction

Sterilisation refers to any process that eliminates/inactivates transmissible infectious agents (pathogens) containing nucleic acids e.g. vegetative and spore forms of bacteria and fungi, parasites, viruses etc. There are two main types of sterilisation distinguished by the characteristics of sterilisation agent used, namely physical and chemical sterilisation.

Radiation sterilisation is a physical process based on the action of radiation energy with the matter. For sterilisation purposes ionising radiation is applied. The term **ionising radiation** covers all types of radiations carrying the energies capable to produce cascades of ionisations (ions formation) in the matter. Two types of ionising radiation are used for sterilisation purposes: electromagnetic rays with wave length below 100 nm (0.5 ÷ 5.0 meV) e.g. gamma and X-rays and corpuscular radiation i.e. fast moving mono-energetic electrons (3.0 ÷ 10.0 meV). Sterilisation efficacy of ionising radiation lies in its good penetrability in the matter followed by killing effect on pathogens.

Both types of high energy radiations penetrate into the matter resulting in induction of ionisation of constituent molecules. However, both represent two different types of ionisation: direct or indirect [1, 2].

Electrons possess both mass and charge. Therefore, they interact directly (direct ionisation) by electrostatic interaction with atoms which are close to their tracks which results in ionisation and formation of secondary electrons. These secondary electrons, in turn, are capable of initiating further processes of a similar character. Each interaction decreases the energy of fast electron and after several interactions its kinetic energy will be lowered and velocity reduced. At a defined depth electrons became thermalised and their activity is spent. It means that there will be no ionisation events in deeper layer of the material. Therefore, in contrast to photons, the range of penetration of high energy electrons is limited and depends on the initial energy of incident electrons, atomic number of absorbing material and its density. Moreover when high energy electrons pass close to atomic nuclei, they lose a considerable portion of their energy liberated in the form of photon radiation called *bremstrahlung* (German originated term for decelerating radiation). The higher atomic number of the source material, the higher is the decelerating radiation. In soft tissues the stopping of electrons is negligible, a little bit higher in bone tissue. In both cases it does not influence the course of radiation sterilisation [2].

The ionising radiation can induce not only ionisation events in the matter but also other physical and chemical processes. It happens if the energy absorbed raises an electron in an atom/molecule to a higher energy state without ejection of electron, breaking of chemical bounds or inducing nuclear reaction. It is possible to distinguish three consecutive stages of the interaction of ionising radiation with the matter: (1) physical stage—excitation and/or ionisation of its atoms/molecules. (2) Physicochemical stage—free radicals formation and (3) chemical stage—reactions between free radicals and free radicals with atoms/molecules of the matter. In biological systems as a result of radiation induced physical, physicochemical and chemical processes some changes of biological properties are observed (biological stage). The appearance of high chemically reactive particles such as free radicals, ion-radicals etc. may result in changes on the molecular level which in turn lead to changes on the cellular level. For example, destruction of proteins by the mechanism of peptide links breaking, amino group NH_2 removal (deamination), disulfide bridges formation etc. leads to changes in proteins conformation and to decline the function either of enzymes and of structural proteins. Changes in lipids such as lipid peroxidation may result in changes in the permeability of cellular membranes and may lead to release of lysosomes contents, changes in processes of protein synthesis in rough endoplasmic reticulum (RER), formation of toxic substances, disturbance of mitochondrial oxidative phosphorylation. Apart from biological membranes the DNA is a primary radiobiological target. Irradiation-induced DNA lesions may consist in breaks in DNA strands, alteration to bases (their oxidation, alkylation, hydrolysis, adduct formation), destruction of sugars as well as crosslinks and dimmers formation. All these varied and interrelated changes occurring at the biological stage are formed within seconds, hours, days, and throughout the life of the cell and can lead either to the premature cell death by the mechanism of apoptosis or oncosis, or, in the case of nonlethal lesions, be passed on from parent cells to their progeny.

It was estimated that a secondary 1 meV electron passing through the liquid medium produces about 40.000 ionisations and about the same number of excitations. Ionised molecules are neutralised in a very short time (10^{-11} s) in reaction with low energy electrons appearing in excess during radiation treatment and become highly excited parent molecules. Most of the excited molecules lose the excess energy in contact with surrounding molecules while a small fraction of them decompose into free radicals which may undergo further transformation in reaction with radicals, atoms and molecules collocated in their neighbourhood. This is a typical way of the formation of radiolytic products in the condensed phase. In crystalline matrices radiation induced ions and radicals may survive in deep traps for a longer period.

The different mechanisms of the absorption of gamma rays and fast electrons has important practical implications.

9.2 Specificity of Radiation Sterilisation with Gamma Rays and Electron Beams

Gamma and X-rays reveal much better penetrability inside the irradiated material as compared with fast electrons. In consequence, more uniform distribution of dose inside the graft is expected under gamma exposure. Instead, electron beams are more effective in producing ionisation per mass unit when compared with gamma photons. It means that to reach the same level of sterility gamma radiation has to be applied much longer (several hours) than electron beams (several minutes) [2].

On the other hand, penetrability of gamma rays is higher than electrons. Consideration on the problem of the penetration and homogeneity of ionizing radiation in the bulk of grafts relies on the assumption that the average density of the grafts is 2 g/cm^3 the number better fitted to the real value then 1 g/cm^3 (density of water) typically taken earlier in this type of consideration.

The reduction of the dose of gamma rays by 50% occurs in 2 g/cm^3 model system at the depth of 6 cm while the same effect is obtained at the depth of 1.8 cm only if the beam of electrons is applied. Consequently, at the depth of 2.6 cm electron beam (EB) irradiation is practically reduced to zero level. Such situation never appears with gamma rays which are absorbed exponentially (Fig. 9.1). The specific feature of ionising radiation is a dose build-up inside irradiated objects. It is effected by a violent ionisation in the upper inside layer of irradiated product not followed by neutralization process fast enough. In deeper layers of the product effected by proceeding thermalisation, the excess electrons are neutralised fast and the dose decreases as a function of absorbed radiation energy. The effect is pronounced with electron beam irradiation. As a consequence of this effect at the depth of 1.3 cm the dose of 10 meV electron beam is by 27% higher than in the surface layer (Fig. 9.1).

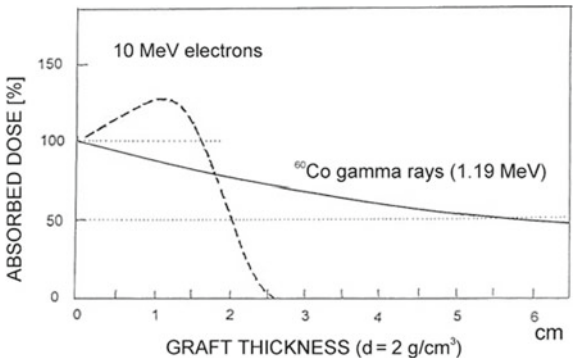

Fig. 9.1 Dose depth distribution of ^{60}Co gamma rays and beams of 10 meV electrons in a model matrix of the density 2 g/cm^3. One-side irradiation. Dotted line—10 meV electrons. Continuous line—^{60}Co gamma rays. From Stachowicz [3]

In order to improve the homogeneity of irradiation dose inside the graft it is advisable to apply two-side radiation treatment. With a two-side gamma irradiation the acceptable depth-dose distribution is obtained inside the graft 15 cm thick (Fig. 9.2), while with two-side EB irradiation, the thickness of ca 4.7 cm will meet the requirement (Fig. 9.3c). Four graphs presented in Fig. 9.3 show evidently how much the distribution of dose inside the graft depends on its thickness. With the matrix only slightly thicker (5.2 cm) the dose in the middle of a graft will be lower than accepted for safe surgery (Fig. 9.3d). On the other side, with a graft of lower thickness (3.7 cm) the dose inside the matrix will be enormously higher then exposure (surface) dose. This would not be acceptable from the point of view of mechanical resistance of the implant (Fig. 9.3a). In this case an acceptable decrease of the exposure dose by ca 30% could be acceptable solution. By sterilisation of "light grafts" (skin, cartilage etc.) or thin bone bars, the EB irradiation from one side could be satisfactorily enough. The decision whether to adapt one-side or two-side EB treatment or whether it is necessary to modify sterilisation process in some detail depends on the operator of irradiation facility.

Radiation sterilisation is classified as a "cold" process what means that in the course of irradiation the temperature is not changed significantly and does not influence the process. This is generally true but one needs to remember that, the last step of the absorption of ionizing radiation described earlier as electron thermalisation process is followed by local emission of heat inside the graft. The temperature rise of tissue allografts irradiated with the dose of 35 kGy of gamma rays does not exceed a few degrees. Since EB irradiation is more effective then gamma irradiation, the thermalisation process inside the EB treated graft becomes more effective too and more heat is locally emitted in a unit of time. Therefore, it is advisable to conduct EB sterilisation (25 ÷ 35 meV) of grafts in two steps to deliver the energy in two smaller portions [2].

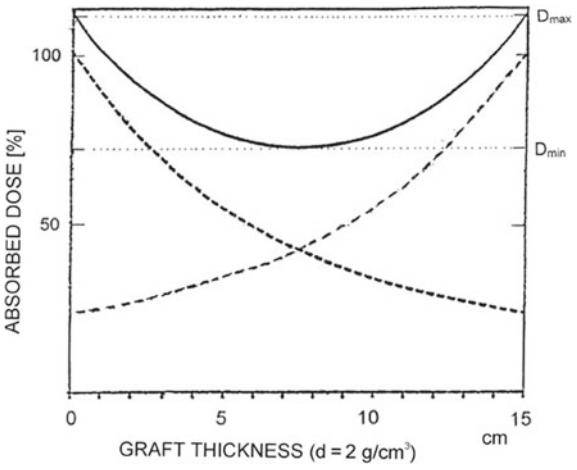

Fig. 9.2 Dose depth distribution of ^{60}Co gamma rays in a model matrix of the density 2 g/cm^3. Two-side irradiation. Thickness of irradiated model matrix 15 cm. From Stachowicz [3]

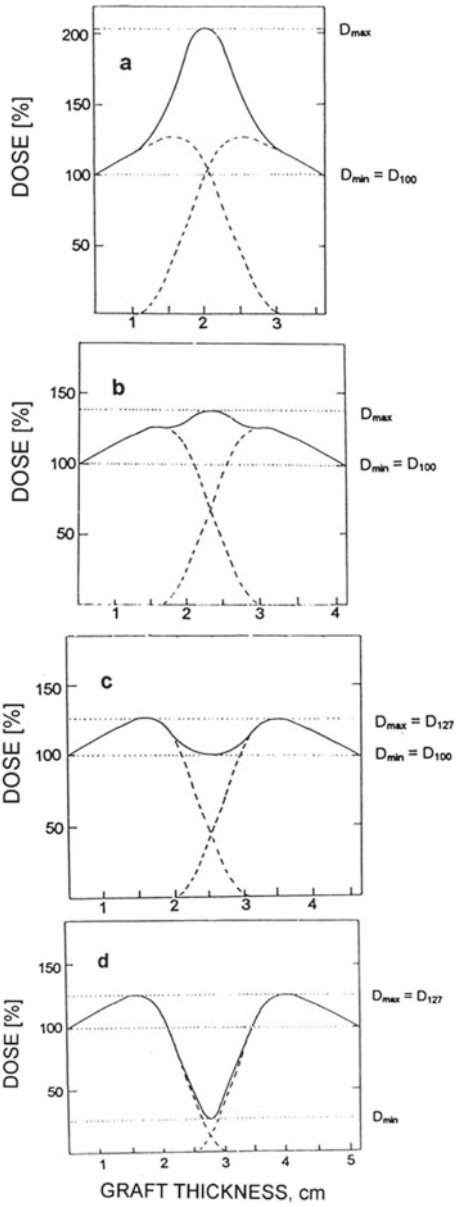

Fig. 9.3 Dose depth distribution of a beam of 10 meV electrons in a model matrix of the density 2 g/cm^3. Two-side irradiation. **a** thickness 3.6 cm: the dose in the centre is two times higher than the surface dose; **b** thickness 4.2 cm: the dose in the centre is by 50% higher then the surface dose; **c** thickness 4.7 cm: the dose inside the matrix is by 27% higher then the surface dose; **d** thickness 5.2 cm: the dose in the centre is by 75% lower then the surface dose. Graph **a** illustrates the over-dosage in the centre of the graft, while graph **d** the case with a dose in the centre which is to low to be accepted. From Stachowicz [3]

Tissue grafts prepared for radiation sterilisation are usually sealed in double or triple bags made of polymeric or laminated foils 0.02–0.05 mm thick. A foil must be resistant against higher doses of radiation and non-reactive against organic substances, like fatty acids, for example, that may be present in the tissue graft. Polycarbonates, polystyrene, polyesters, polyethylene and their laminates are

commonly in use. It is recommended to apply commercial materials specifically produced for this purpose. For radiation sterilisation with gamma rays hard, thick-wall, plastic or glass containers are also acceptable. It has to be remembered, however, that some not significant part of the energy of ionizing radiation is absorbed in such package.

9.3 Dosimetry for Radiation Sterilisation

Absorption of ionizing radiation is non-homogenous in its nature and special attention has to be paid to a proper modelling of irradiation procedure. It is obvious that any piece of bone prepared for grafting is not of the same density and that each graft differs from the other in its shape and local densities. This implies variations of the absorbed irradiation energy i.e. variations of dose absorbed inside each of graft. It is why dosimetry problems are more complex with allografts then with medical materials sterilized by radiation but manufactured with the use of plastics. It has to be remembered that control of dose is an essential requirement for radiation sterilisation of tissue grafts [4].

The absorbed dose D (simply called dose) is the amount of the energy absorbed per unit mass of irradiated product at the unit point in the region of interest. The SI unit of dose is 1 gray (Gy) corresponding to 1 J of energy absorbed in 1 kg of a product. In a practical situation, D is given as an average value, however, the upper and lower limits of dose delivered to the product should be formulated too. Any way, the absorbed dose or "sterilisation dose" the term applied commonly to tissue grafts is in fact the average dose only. It is practically the exposure (surface) dose optimised from the point of view of microbial and structural (mechanical and biological properties) demands [5]. However, in order to assure desired sterility, more detailed information on the distribution of dose inside the graft are needed.

The adapted exposure dose must be calibrated with reference dosimeters. In addition, the individual dose measurement on each tissue graft must be done. For this purpose dose-meters attached to every package or bag with a graft are used. The following are commonly used:

(i) A sensitive water or graphite calorimeter adapted for the estimation of absorbed dose and dose rate of electron beam (EB) irradiation. It is based on electrometric measurements of the increase of the temperature during exposure [6];
(ii) Ferrous sulfate solution (Fricke dose-meter) for the estimation of absorbed dose and dose rate during gamma-ray irradiation. The concentration of ferric ions produced by radiation oxidation of Fe^{2+} ions are measured spectrophotometrically [7].

Both dosimeters should be related to international standards. It is achieved by the use of transfer dosimeters controlled by an authorised international dosimetry center [8].

The important function of routine dosimeters (plastic films, solutions, powders, glasses) is to monitor package-to-package or bag-to-bag dose variations and to facilitate the evaluation of dose distribution in tissue grafts.

The detail information on dose distribution through the product is in practice not as important as the determination of two extremes:

(i) a maximum dose D_{max} absorbed in the graft which must be lower than or equal to the highest acceptable dose, and
(ii) a minimum dose D_{min} absorbed in the graft which, in turn, must be greater than or equal to the lowest acceptable dose.

By sterilisation of allografts the most important is the knowledge of D_{min} since this number corresponds to the lowest dose in some part of a graft in the which the sterility must be assured to guarantee the sterility of the latter in total (see also Fig. 9.3).

D_{max} must be always lower than critical level of dose accepted for a given type of a graft. The methods for the determination of both minimal and maximal doses with the use of routine dosimeters will be discussed.

From a fairly large number of routine dosimeters presently applied in irradiation facilities, the most suitable for dose measurements on tissue grafts seem transparent plastic dosimeters distributed in the form of blocks or tapes adapted for the use in spectrophotometry. The dose estimation is based on spectrophotometric measurement of absorbency (optical density) which is proportional to the dose applied. The measurement of absorbency at a suitable wavelength, usually in near ultraviolet allows to determine the dose with a reasonable accuracy.

Plastic dosimeter most frequently used is based on poly-methylmethacrylate (PMMA) and permits to measure the dose from 0.1 to 50 kGy. It is usually available in small blocks 2–3 mm thick. There are several types of PMMA dosimeters in use which absorb at different wavelengths. A family of plastic film dosimeters from 0.01 to 0.20 mm thick includes radiochromic film dosimeters containing dyes sensitive to radiation or pure polymer dosimeters changing their colouring during the irradiation. Poly-chlorostyrene films with the content of triphenylmethane dye, polyethylene tetraphtalate or pure polyvinyl films are most frequently used.

Polyvinyl chloride (PVC) dosimeter is relatively cheap and enables to measure the dose at 398 nm within the range $5 \div 50$ kGy. It can be also used in the form of long tapes to be co-located on the packages with graft positioned lengthwise the irradiation container to control the homogeneity of electron beam and conveyer speed during EB irradiation. The PVC dosimetric films in cartoons are used for the determination of D_{min} if placed below a single bag with tissue graft in the button of a carrier. The measurement can be done with one-side irradiation to decide whether this method could be adapted for a given kind of graft. Thick grafts can be

controlled by a "sandwich method". In order to adapt this technique it is necessary to perform a model experiment. A representative graft is cut in two equivalent parts and between these parts a sheet of dosimetric foil is collocated. Spectrophotometric examination gives the answer whether the graft of this type can be sterilized properly by EB irradiation. The procedure is called *dose mapping* (Fig. 9.4). When applied to commercial gamma irradiator with a multi-side exposure system, the distribution of tens of smaller dosimeters throughout one representative package with tissue grafts is needed. The examination of all dosimeters allows for the proper estimation of the extreme values of dose in the package. There is no need to adapt this procedure if single bags with tissue grafts are irradiated by gamma rays. Recently l-alanine dosimeter and bone dosimeter have also been used [2, 9]. Both polycrystalline l-alanine and bone powder give rise in electron paramagnetic resonance spectroscopy (epr) to stable and specific signals shown in Fig. 9.5. The amplitude of the central line (h_c) or the integrated area of the epr signal is proportional to absorbed dose of gamma or EB radiation. The dose-dependence curves of the response of both dosimeters are shown in Fig. 9.6. The advantage of bone powder dosimeter is that its composition is adequate with the composition of bone, the tissue most frequently used for grafting [10, 11]. Bone dosimeter is produced from bovine bone (*femur*) powder with average grain size of 0.02 mm. The preparation comprises special cleaning, sieving and lyophilization of the powder. Bone dosimeter can be used within the range of doses from 0.05 to about 40 kGy of gamma rays or beams of accelerated electrons. The curvature in dose versus signal

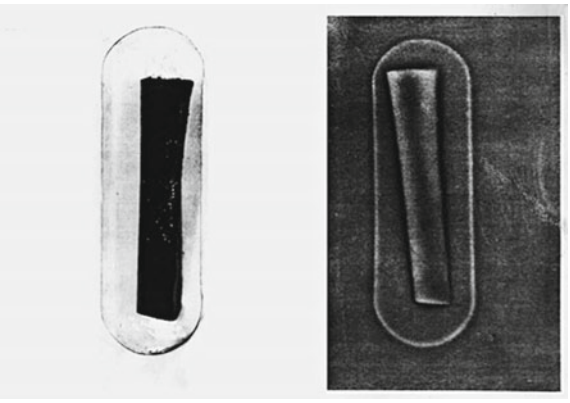

Fig. 9.4 PCV mapping of a small bone bar in a plastic container. One-side irradiation. On the left a photo, on the right mapping with PCV foil. The areas of different degree of darkening indicate different levels of absorption of ionizing radiation inside the graft. Deeper darkening (gray) corresponds to a suitable, relatively uniform absorption of ionising energy in the bulk of graft. Light colour indicates almost full absorption of ionising energy in the graft but followed by markedly lower absorption in this part of graft which is opposite to the surface exposed to radiation. From Dziedzic-Goclawska and Stachowicz [13]. Advances in Tissue Banking, pp. 261–321

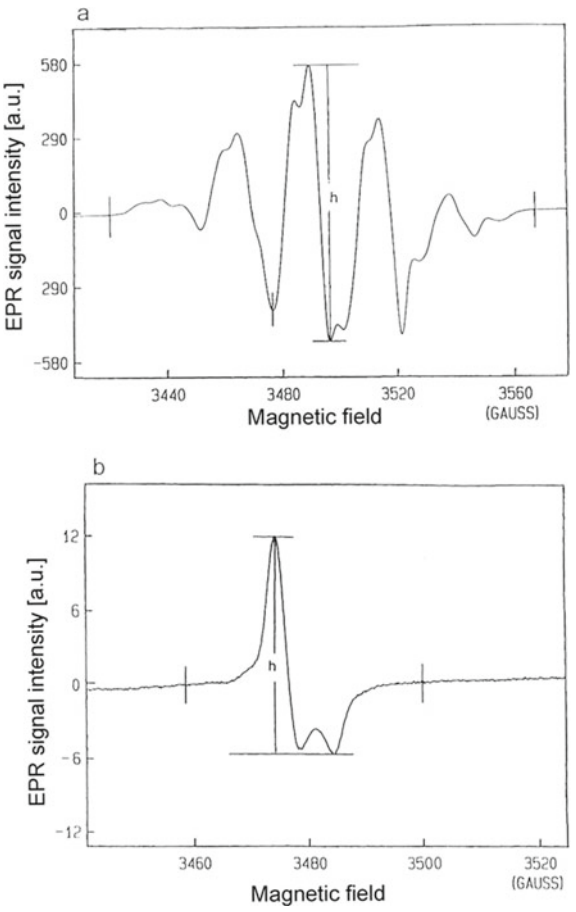

Fig. 9.5 Epr signals (first derivative) recorded with l-alanine (**a**) and bone (**b**) dosimeters. The estimated dose is calculated from the proportion of the heights (h) of the peaks. From Dziedzic-Goclawska and Stachowicz [13]

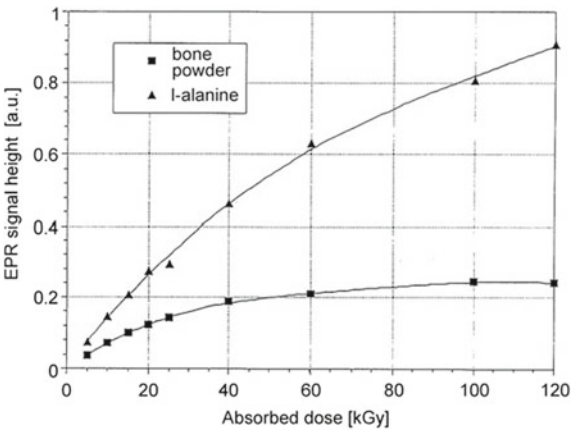

Fig. 9.6 Relationship between the heights of the epr signals recorded with l-alanine and bone dosimeter as a function of dose of ^{60}Co radiation. From Ziaie et al. [14]

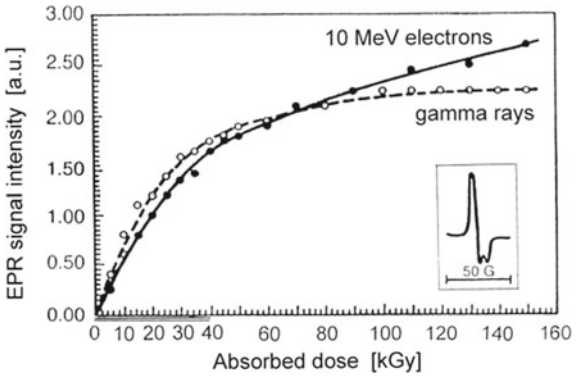

Fig. 9.7 Relation between the dose of ionizing radiation and the intensities of the epr signals recorded with bone dosimeter. Dotted line—^{60}Co gamma rays, continuous line—beams of 10 meV electrons. From Stachowicz [3]

intensity relationship around 25 kGy makes the dose estimation at higher doses less accurate [10, 12]. It has been proved that the error of dose estimation below 25 kGy is from 3 to 4%, while above this level is increasing gradually to 8% around the dose of 40 kGy.

Routine dosimeters are calibrated with reference dosimeters described earlier and all of them are based on the readings from dose-dependence response curves or alignment charts. In the case of significant difference in the dosimeter and product densities the corrections are sometimes needed (Fig. 9.7).

The response of reference and routine dosimeters to gamma rays and EB irradiations is not much different. Slight difference not exceeding a few percent has been reported. In addition to the described dosimeters to each bag with allograft so called "go-not-go" radiation-sensitive indicators are routinely attached. The role of indicators is to ascertain visually whether given graft was or was not irradiated. During radiation treatment indicators change their coloration from yellow to red, for example. It has to be stressed that dye indicators cannot be used for the measure of dose and for that reason cannot be never considered substitute for routine dosimeters.

9.4 Mechanisms Involved in the Inactivation of Micro-organisms by Ionising Radiation

The major target sites in micro-organisms that are susceptible to ionising radiation are nucleic acids (DNA—deoxyribonucleic acid, RNA—ribonucleic acid). The damaging process may be caused directly by ionising radiation or indirectly through radiolysis of water and the production of highly reactive, short-lived hydroxyl radicals (*OH). In the presence of water, the indirect mechanism predominates. The

presence of oxygen enhances the damaging effect. Oxygen reacting with hydroxyl radicals produces peroxide radicals and peroxides cause various kinds of damage to DNA. Both direct and indirect effects of ionising radiation may cause single or double strand breaks of DNA. DNA intra-strand cross-links and damage to the DNA bases or DNA sugar may also occur. Ionising radiation induces structural damage in DNA which inhibits DNA synthesis, causes errors in protein synthesis, and this leads to cell death. It has to be kept in mind that at low doses of radiation, several bacteria possess the ability to repair damage to DNA due to the action of repair enzymes (e.g. DNA polymerase I) and recombination. Repair of single-strand brakes and double-strand brakes (which are of course more difficult to repair) produce radiation-resistant mutants such as *Deinococcus radiodurans (Micrococcus radiodurans)*.

9.5 Radiation Resistance of Micro-organisms

The radiation resistance of micro-organisms is genetically determined. Gram-negative bacteria are more sensitive than Gram-positive bacteria. Usually, spores are more radiation resistant than the vegetative forms of bacteria. The most resistant fungi may be as resistant as bacterial spores, while viruses are, in general, more resistant than bacteria. Prions are extremely resistant to most chemical and physical sterilising agents, including ionising radiation.

Enzymes, pyrogens, toxins and antigens of microbial origin are, in general, very radiation-resistant, compared to living cells. Therefore, the number of micro-organisms present prior to radiation sterilisation is of importance when dealing with medical materials, regardless of the radiation resistance of the contaminating population.

There are many factors that can modify the radiation resistance of micro-organisms. Protectors such as alcohol, glycerol, reducing agents, dimethyl sulphoxide (DMSO), proteins and carbohydrates increase resistance. On the other hand, the presence of water and oxygen will enhance radiation damage. Irradiation at low temperatures increases, while at higher temperatures decreases, the resistance of micro-organisms. These factors should be taken into consideration when setting the sterilisation dose for any product.

An acceptable sterilisation procedure for any type of product, including tissue allografts, depends on defining the most resistant micro-organisms which could be present and the density of each of these.

The results of the radiation sterilisation procedure depend on the amount of energy transferred, the number of contaminating micro-organisms and their resistance to ionising radiation (initial contamination) characterised by the D_{10} values.

The commonly used term “bioburden” describes the population of viable (active) micro-organisms that are present on or inside a material or product before sterilisation. This is one of the factors influencing the efficiency of radiation. Clearly, the lower the bioburden is, the more effective the process occurs.

The D10 value, usually expressed in kGy, is the dose of irradiation necessary to reduce the initial microbial population by 1 log 10, i.e. by 90%. This value can be read directly from the dose-inactivation curve or calculated using the following equation:

$$\text{D-value} = \frac{\text{radiation dose}}{\log N_0 - \log N}$$

where N_0 and N represent 1-log difference in viable numbers. D-value differs greatly among different types of micro-organisms and, occasionally, considerable variation among different strains of the same organism is observed. The response of micro-organisms to radiation also depends on external conditions.

The concept of "sterility assurance level"—SAL is derived from kinetic studies on microbial inactivation, i.e. the probability of viable micro-organisms being present on or inside a product unit after sterilisation. For example, SAL 10^{-6} would assure that less than one out of a million contaminants would survive on or inside the product following sterilisation. Depending on the risk posed by the use of various specimens, different values of SAL (10^{-3}, 10^{-6}) may be recommended. For medical devices which are in contact with blood, for parenteral solutions as well as for tissue allografts, a value of SAL 10^{-6} or lower is required.

Radiation sterilisation of health care products, which was introduced in the middle of the 1950s, has recently been more frequently used [15]. The sterilisation dose recommended for these products is 25 kGy, and this is the dose that is commonly used in the UK, in the USA and in many other countries [16, 17]. In some Scandinavian countries, the recommended dose is up to 45 kGy. The recommendation of a dose of 25 kGy is based upon the bioburden and radiation resistance of micro-organisms that are found on health care products. International standards specifying procedures for the validation and routine control of the process used for sterilisation of health care products have been prepared by the International Organization for Standardization.

The same dose of 25 kGy has been recommended for sterilisation of tissue allografts and is routinely applied in many tissue banks. Although this dose may not be entirely satisfactory, as explained below.

In cases of health care products that are manufactured under defined and clean conditions, it is easy to establish the average bioburden, which is usually low and has a standard distribution, thus the recommended dose of 25 kGy has been accepted for most of these products.

With respect to human tissues that are collected from cadaveric (or even from living) donors, it is very difficult or simply impossible to determine the bioburden each time, since initial contamination may vary greatly from tissue to tissue and from one donor to another.

The problem is additionally complicated by the possible presence, in human tissues, of pathogenic viruses such as the human immunodeficiency virus (HIV), hepatitis viruses (HBV, HCV), cytomegalovirus (CMV) or others. Data concerning the sensitivity of these viruses to ionising radiation are scarce. The effectiveness of ionising radiation to inactivate them in tissues that have been collected from

cadaveric donors has not been well documented and the mechanisms of viral responses are unclear. This is mainly due to the fact that there are no suitable tests to study their inactivation, no appropriate animal model exists and no suitable method of in vitro culture of the highly differentiated target cells for these viruses has been developed [18–23].

In retrospective studies, transmission of hepatitis C virus (HCV) through non-sterilised cadaveric tissue allografts has been reported, while allografts irradiated with a dose of 17 kGy did not evoke infections in graft recipients [19]. There are no data on the sensitivity of hepatitis B virus (HBV) to ionising radiation.

The majority of studies have been carried out on the inactivation of HIV. It has been postulated that the dose of irradiation needed to reduce the viral load by 1 $\log_{10}$—the D_{10} value is 4 kGy or even 5.6 kGy. Taking into consideration the required SAL 10^{-6}, assuming the average HIV bioburden to be about 10^3 virions/ml for the state of acute infection and a D10 value of 4 kGy, a reduction of 9 (6 + 3) units or a dose of 36 kGy would be required [20]. On the other hand, if the D_{10} value is 5.6 kGy, then a dose of >50 kGy would be needed to inactivate HIV. These results are in agreement with data that has been published by Fideler [7] who, using a polymerase chain reaction (PCR), found that in order to inactivate HIV in fresh frozen bone–patellar-ligament–bone grafts, a dose in the range of 30–40 kGy is required. The sensitivity of HIV to ionising radiation also depends on the temperature of irradiation. The reduction of the virus titre of 5–6 $\log_{10}$ was achieved at doses 50–100 kGy in frozen plasma (−80 °C) and 25 kGy at 15 °C.

Considering the high D_{10} value for HIV, even a dose of 35 kGy cannot be treated as the sterilisation dose, but it is impossible to increase the irradiation dose with impunity, as high doses of ionising radiation (over 50 kGy) evoke many physico-chemical changes in tissue allografts which may affect their biological properties [21].

The selection of a radiation dose is a compromise between a dose that is high enough to inactivate as many micro-organisms as possible and low enough to preserve important biological properties of tissue allografts. It is recommended to implement a dose of 35 kGy, which certainly provides a more adequate assurance of sterility for tissue allografts than does the commonly used dose of 25 kGy.

The dose of irradiation is generally applied as one step process for both gamma and EB. Newly proposed fractionated method of irradiation for soft musculoskeletal tissues sterilisation with EB allows for better preservation of its biomechanical properties, that are similar as compared to fresh-frozen allografts. Fractionation of dosages used to decrease tissue damage, similar to irradiation of tumours in oncology allows to achieve sterility of tissue grafts [24].

The validation of radiation sterilisation processing of tissue allografts should be performed by adequate measurements of the absorbed dose, set a priori and required to achieve the specified sterility assurance level (SAL). In addition to proper dosimetry systems, it is advisable to use radiation-sensitive indicators. The purpose of these “go-not-go” indicators is the check visually whether or not a graft has been irradiated. An indicator should be attached to every graft package and it should entirely change colour after irradiation. It should be stressed that indicators are not dosimeters and should never be used as a substitute for routine dosimeters.

9.6 Summary and Conclusions

Several factors can affect the effectiveness of radiation sterilisation and can modify the microbial sensitivity to ionising radiation.

One of the factors influencing the effect of irradiation is bioburden, i.e. the number of micro-organisms present on or inside tissues before sterilisation.

Since toxins, pyrogens and antigens of bacterial origin are very resistant to irradiation, in comparison to living cells, regardless of the radiation resistance of the contaminating population, it is strongly recommended to avoid recontamination of tissues during retrieval and processing and to prevent micro-organism proliferation by temporary storage of tissues at low or freezing temperatures before sterilisation.

The presence or absence of water and oxygen plays an important role. In the absence of water (e.g. in air dry or lyophilised samples), the resistance of micro-organisms is increased. On the other hand, in the presence of water, an indirect effect of ionising radiation predominates [25]. Oxygen enhances the damaging effect to micro-organisms and increases their sensitivity to irradiation. During radiolysis of water, highly reactive, short-lived hydroxyl radicals (*OH) which react with oxygen are produced, causing the formation of peroxide radicals and peroxides which intensify damage to the nucleic acids of micro-organisms.

Therefore, if lyophilisation is used as a preservation procedure, it would be better to leave some water in the tissue than attempt to remove as much water as possible. It has also been recommended that the vials containing tissues be refilled with inert gaseous nitrogen to remove as much atmospheric oxygen as possible. However, since oxygen increases the sensitivity of micro-organisms to ionising radiation, this procedure should be discontinued.

It should be noted that irradiation at low temperatures increases, while at higher temperatures decreases, the resistance of bacteria and viruses. It has been found that the resistance further increases when lyophilised viruses are irradiated at a low (−79 °C) temperature as compared to fresh-frozen irradiation at −79 °C.

All these factors influence the effectiveness of radiation sterilisation and should be taken into account when setting the radiation dose [26].

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Screening the Tissue Donor for Infectious Agents

10

Alan Kitchen

Abstract

Like all substances of human origin, tissues are capable of transmitting infectious agents from donor to recipient. The screening of tissue donors for blood-borne infectious agents helps minimise this risk. To ensure the reliability and accuracy of screening a number of critical elements need to be considered. The quality of the samples provided needs to be assured, especially when tissues are collected from deceased donors. Although the core range of transmissible infectious agents is the same for blood and tissue donors, additional markers of some infectious agents may be included for tissue donors. The screening strategy and algorithms used for tissue donors may differ from those applied to blood donations. The number of tissue donors and the retrieved tissues is often less than the numbers required, and unnecessary loss of tissue donations because of non-specific screen reactivity must be minimised. This can be achieved through careful and scientifically based selection of the screening algorithm used. In addition, effective confirmatory testing of all screen reactive samples should be performed, with good confirmatory testing it is quite appropriate to consider releasing screen reactive tissues based on confirmatory results. To ensure the algorithms do provide the required outcomes, the most suitable assays must be selected, ensuring the highest possible sensitivity without loosing too much specificity. However, as with all substances of human origin, zero risk is not achievable, and even after reliable and high quality screening, some residual risk, albeit very low, remains.

A. Kitchen (✉)
Independent Consultant, Transfusion and Transplantation Microbiology, London, UK
e-mail: adkitchen@btinternet.com

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10.1 Introduction

The transplantation of tissues from donor to recipient is now a fundamental part of modern medicine. A range of tissues including bone, skin, tendons, heart valves, corneas etc. are collected from suitable donors and transplanted into those recipients for whom tissue transplant would have clinical benefit.

In the same manner as blood transfusion, transplantation is not without risks, one important risk being that of the transmission of infectious disease via the transplant; transplantation transmitted infections (TTI). Although it is clear that not all tissues carry the same risk, such distinctions cannot be easily quantified and as far as possible all tissue donors should be treated in the same way in terms of the pre-donation selection process and consequent laboratory screening applied to tissue donors.

Therefore, in a parallel manner to the screening of blood donors and donations, all tissue donors must be screened for any evidence of exposure, or risk of exposure, to a range of transmissible infectious agents prior to release for clinical use. This is achieved through a) the donor selection process followed by b) the laboratory screening of blood samples collected from those donors deemed to be suitable by the donor selection process, for evidence of current or previous infection with specific transmissible infectious agents. Although the basic principles applied to the selection and screening of tissue donors are the same as for blood donors and donations, and indeed donors of any other substances of human origin (SOHO), there are some important differences between blood and tissue donors which may result in differences in the screening strategies adopted. The critical differences are centred around:

- The source of tissue donations includes both living and deceased individuals
- Tissue donors may have received blood components and/or blood products and/or crystalloids/colloids prior to the collection of the samples used for laboratory screening
- Whilst the specific infectious agents screened for are broadly the same between blood and tissue donors, the specific screening targets used for the individual infectious agents may differ because of regulatory requirements.

This text considers some of the basic principles of the screening of tissue donors for the range of infectious agents that may be present in the donor at the time of retrieval of the tissue(s) and which may therefore be present in the tissues retrieved and consequently likely to be transmitted through transplantation of the donated tissues. These agents include viruses, bacteria, parasites and prions. The subject of the bacterial/fungal contamination of tissue products as a result of the collection, processing and storage processes is not covered in this particular section. However when considering screening programmes and the differences between the screening of blood donors/donations and tissue donors (and indeed donors of other substances of human origin) the key issues are not so much the infectious agents themselves—

the core relevant infectious agents are common to all donated products, but the overall appropriateness and quality of the screening programmes in place—the appropriateness and effectiveness of the donor selection process, and the reliability and accuracy of the screening results generated by the laboratory.

Another important difference between the screening of blood donors/donations and tissue donors is that in the case of blood donors/donations the donation itself is screened; the laboratory samples are collected during the blood donation process via the venepuncture used to collect the donation. However the tissue donations themselves are not screened, rather it is the donor who is screened using a venous blood sample collected at or around the time of tissue retrieval; this sample is deemed to be representative of any infectious agent that may be present in the tissue. Consequently when describing blood donation both the donor (donor selection) and the donation (laboratory screening) are screened, whilst when describing tissue donation it is only the donor who is screened.

Although countries may have specific Tissue Establishments, increasingly Blood Services are becoming involved in many aspects of tissue banking. Certainly in relation to infectious disease risk and the laboratory screening of tissue donors, Blood Services are ideal organisations to take on the responsibility of screening as the core screening principles and requirements between blood donors/donations and tissue donors are broadly the same. Blood Services have the laboratory screening systems in place to be able to easily and efficiently deal with tissue donor screening requirements, integrating the required tissue donor screening into the existing laboratory screening of blood donations.

10.2 Sample Quality

Probably the most critical factor in the overall reliability of the tissue donor screening programme is sample quality. Poor quality samples produce poor quality results, with the potential resultant loss of sensitivity and/or specificity. Sample quality is determined by a number of factors, although different assays and assay formats may be more or less tolerant of sample quality: whether the sample was collected from a heart-beating or non-heart-beating individual, the age of the sample, the overall quality of the sample (in respect of haemolysis, lipaemia etc.), the volume of sample collected/available, any haemodilution through the administration of iv fluids such as blood components/products and/or crystalloids/colloids.

10.2.1 Living v. Deceased Donors

10.2.1.1 Donor Selection

Similarly, the screening of the donors themselves, the donor selection process, is also determined by the nature of the donor. The selection process applied to living

tissue donors is similar to that applied to blood donors, except that such donors do not generally volunteer in the same way as an "altruistic" blood donor. Living tissue donors may often have been approached directly and a direct history may therefore be taken from them. However in the case of deceased donors, although the process is the same, the history is generally second hand and may not be totally correct or accurate [1]. Thus the inherent risk associated with deceased tissue donations may be higher than that of living donations and the screening strategy developed accordingly.

10.2.1.2 Laboratory Screening

A fundamental factor when considering the screening of tissue donors for transmissible infectious agents is the status of the donor, living or deceased, and consequently whether the sample(s) collected were collected pre- or post-mortem (heart or non-heart beating). The screening of living tissue donors for infectious agents is effectively no different to the screening of blood donors/donations, in that the blood samples used for screening are collected from living individuals. The characteristics of the samples should be identical, see below in respect of the issue of haemodilution, and the screening is performed using assays specifically designed, developed and supplied to test venous blood samples collected from living individuals.

Importantly it should be noted that although tissues may be retrieved post-mortem, blood samples for screening may have been collected ante-mortem whilst the donor is still heart beating; samples collected up to 7 days ante-mortem, in the absence of any further intervention which could introduce an infectious agent and any possible haemodilution, are usually deemed to be suitable to screen tissues retrieved post-mortem.

It is the screening of tissues from deceased donors, where the blood samples are also collected post-mortem (non-heart beating), that may present some specific problems which would need to be overcome to ensure accurate and reliable screening results. These problems are associated with the overall quality of the samples obtained and their validity as suitable substrates for the assays used; the time post-mortem of collection, the integrity of the sample, volume, level of haemolysis, biochemical changes post-mortem etc. However, as long as these issue are understood and the assays used specifically validated for use with such samples [2], the overall quality of the screening programme, the accuracy and reliability of screening results, should be the same whether the samples are collected ante- or post-mortem.

Blood samples collected post-mortem may be different from those collected ante-mortem for a number of reasons. The sample is collected from a now static fluid that is settling into its cellular and liquid phases, in which coagulation may have occurred with the resultant biochemical changes including red cell damage, and where cell death is taking place with the release of a wide range of cell chemicals into the surrounding tissue fluids. The overall results of this may be gross haemolysis and/or other changes to the "blood" collected, leading to the possibility of degradation of the screening targets that would be present in blood of an infected individual. In some cases there may be the release of substances that may be

inhibitory to the screening assays used. However the changes that occur post-mortem obviously vary between cadavers, depending upon factors such as: age, cause of death, time between death and the cold-storage of the cadaver, time pre-mortem under direct clinical care and the interventions used, other underlying conditions (that are not contra-indications to tissue donation), and time post-mortem of sample collection. The occurrence of post-mortem changes per se do not necessarily make any sample collected a less suitable substrate for screening. The screening of post-mortem samples from deceased tissue donors has been performed for many years in many countries with few, if any, known adverse outcomes. In addition assays can be validated for use with deceased samples, and indeed some commercially available infectious disease screening assays for blood-borne viruses have undergone some limited validation work by the manufacturer with the resultant inclusion of claims that the assays can be used to screen samples collected post-mortem and under certain conditions.

An important factor in the screening of deceased samples is the time, post-mortem, that the sample was collected. The sooner after death the sample is taken the less likely it is to have been effected by any post-mortem changes. Interestingly there are currently few published papers that look critically at the time post-mortem for sample collection. Regulatory requirements within the EU require sample collection within 24 h post-mortem, and in the US there is an expectation that tissues would be collected as soon as possible post-mortem, and within 24 h, but this is currently not subject to any specific regulation. There are published studies on aspects of deceased testing that include samples collected up to 48 h post-mortem and without any problems being encountered [3, 4], but specific studies on sample suitability related to time of collection are lacking.

10.2.2 Haemodilution

In almost all circumstances tissue donors would be expected to be patients in some way, and consequently are likely to have had clinical intervention prior to any tissue donation. Although there may be clinical interventions which would automatically render some patients unsuitable as tissue donors there are many which would not in themselves prevent donation. However there are some interventions which involve transfusion of blood components or the infusion of blood products or crystalloids/colloids and which, whilst in themselves not necessarily a bar on donation, may stop donation if the volume infused is such that the dilution effect on any circulating screening target present would take it, below the detectable level.

This situation is not uncommon and most Tissue Establishments/Blood Services have guidelines in place to identify situations in which it is known that (potential) tissue donors have received fluids which may result in haemodilution, and then calculate the degree of haemodilution likely to have occurred. A very broad accepted level is that of up to 50% haemodilution, above this may result in a dilution of any target to the extent of not being reliably detected by the screening

assays in use. However there are limited data to support this and some assays may tolerate a higher level of dilution; serological assays (targeting Ag and/or Ab) in general are more sensitive to dilution than molecular assays (targeting the infectious agent's nucleic acid).

It is important that Tissue Establishments determine the means by which any degree haemodilution can be calculated and the accuracy of that calculation. They should then ensure that the laboratory undertaking the screening of the donors has validated their assays for use with samples diluted up to the extent which the Tissue Establishment has set as the maximum safe level. Being able to identify and deal with haemodilution is important as the unnecessary loss of tissue donations because of possible haemodilution, the extent of which cannot then be accurately and reliably calculated, is something to be avoided.

The immediate pre-mortem history of the donor must therefore be known if there is any likelihood of adverse effects due to haemodilution and at the very least the sample collection site chosen to try to minimise the risk of collection of a sample containing a significant amount of any infusion. There is a reported case of the failure of screening to identify an HIV infected tissue donor specifically because of massive fluid infusion shortly before death with the resultant dilution of HIV antibodies to below that detectable. Interestingly it is possible for the antibody levels to return to that normally seen, and detectable, within 48 h as the tissue fluids naturally re-distribute themselves [5]. However, most of the studies performed [6] have indicated that the modern screening assays used will tolerate a significant dilution of plasma before approaching the point at which a false negative result may occur. This situation is most likely to occur following significant acute blood loss and this fact will be apparent when assessing any potential deceased donor. There are FDA guidelines which seek to help clarify the situation, and these require tissue banks to develop algorithms to determine the degree of haemodilution and the action to be taken in terms of the validity of screening results [7]. Within the UK Blood Services there is a clear algorithm that can be followed in cases where haemodilution has occurred, that enables the degree of haemodilution, and thus sample suitability, to be assessed.

10.2.3 Screening Targets

Whilst the group of blood-borne infectious agents for which blood donations are screened does provide the basis for the screening of tissue donors, there may be some differences in the specific screening targets used for the individual infectious agents. There are minimal biological or scientific reasons for any differences, the reasons tend to be regulatory, and especially when blanket regulation is applied internationally, and reflect variability in the overall quality and reliability of the screening performed in different countries. To ensure overall 'safety' in respect of infectious risks associated with tissue products, especially when those products may be supplied to other countries, a more comprehensive laboratory screening programme may be required to ensure that any possible risk of transmission of

infection is identified. In a country which has a high quality and reliable screening programme for blood donations it is highly unlikely that including additional screening target requirements for the screening of tissue donors would identify anything additional of any clinical significance.

10.3 Range of Infectious Agents

The range of infectious agents for which tissue donors need to be screened, both mandatory and additional, is generally the same as for blood donations. Essentially the specific markers used as screening targets for the individual infectious agents are also generally the same, although regulatory requirements may include the use of some additional screening targets for some of the infectious agents. In some cases regulations may require screening for additional infectious agents; this may vary from country to country depending on the level of regulation required or in place.

Table 10.1 lists a range of infectious agents that have been reported to be transmitted via blood and blood components. Although only a few of these agents have been reported to be transmitted via tissue transplantation, it is reasonable to consider that all of these agents could be transmitted if present in a tissue donor and

Table 10.1 Infectious agents reported to have been transmitted via blood transfusion or tissue transplantation

Viruses	Bacteria
Hepatitis viruses Hepatitis A virus Hepatitis B virus Hepatitis C virus Hepatitis D virus (requires co-infection with HBV) Hepatitis E virus	*Treponema pallidum* (syphilis) *Borrelia burgdorferi* (Lyme disease) *Brucella melitensis* (Brucellosis) *Yersinia enterocolitica/Salmonella* spp. Staphyloccocal spp./pseudomonads/*Serratia* spp. Rickettsiae: *Rickettsia rickettsii* (Q fever)
Retroviruses Human immunodeficiency virus 1 and 2 Human T-cell lymphotropic virus *Herpes viruses* Human cytomegalovirus Epstein–Barr virus Human herpesvirus 8	**Protozoa** *Plasmodium* spp. (malaria) *Trypanosoma cruzi* (Chagas' disease) *Toxoplasma gondii* (toxoplasmosis) *Babesia microti/divergens* (babesiosis) *Leishmania* spp. (leishmaniasis)
Parvoviruses Parvovirus B19	**Prions** Variant Creutzfeld Jakob Disease (vCJD) [classical CJD has been transmitted by corneas and dura]
Miscellaneous viruses West Nile virus Dengue virus Zika virus Japanese Encephalitis virus	

consequently in the donated tissues. Thus the same approaches in terms of identifying specific donor risk need to be followed, albeit with the obvious issues in the pre-selection of deceased donors.

The transmissible infectious agents that are of specific interest can be considered in three screening categories:

- those for which all tissue donors must be screened—mandatory screening
- those which are recognised and commonly encountered threats, but for which only specific tissue donors need to be screened because of specific identifiable risk—additional or selective screening
- those which are rare or unknown threats and which are dealt with on a case by case basis—other screening.

In addition to defining the infectious agents themselves, the specific screening target(s) for each infectious agent must also be defined. The available targets vary according to the infectious agent and theoretically there are 3 potential targets for any infectious agent—nucleic acid, antigen, antibody. However it is the suitability of each of these targets, in the context of the screening of asymptomatic donors for evidence of infection that has to be considered. Target suitability depends on the interactions between the biology of the infectious agent, the host response to infection, and the sensitivity and predictiveness of the available tests. Figures 10.1, 10.2, 10.3 and 10.4 provide a generalised overview of the different circulating markers that can be detected following infection with HBV, HIV and HCV respectively. The figures depict the relative timeframes for the appearance of the different markers for each of these agents, and from these the most useful markers in terms of reliable detection of infection can be determined. A pre-requisite for any

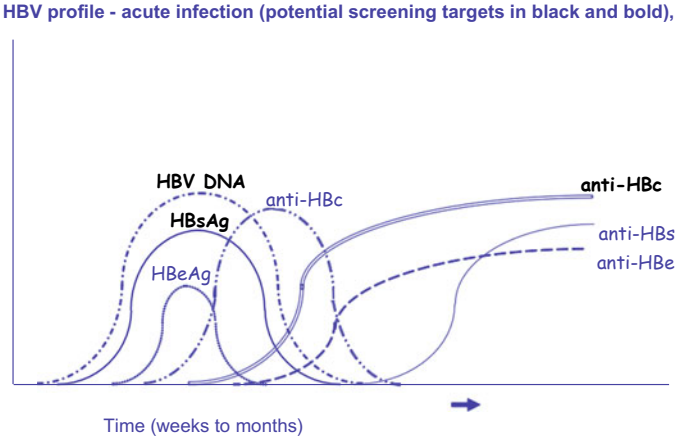

Fig. 10.1 HBV plasma markers—acute infection

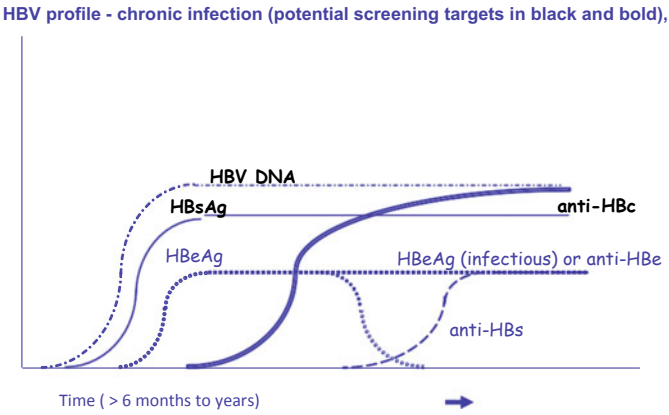

Fig. 10.2 HBV plasma markers—chronic infection

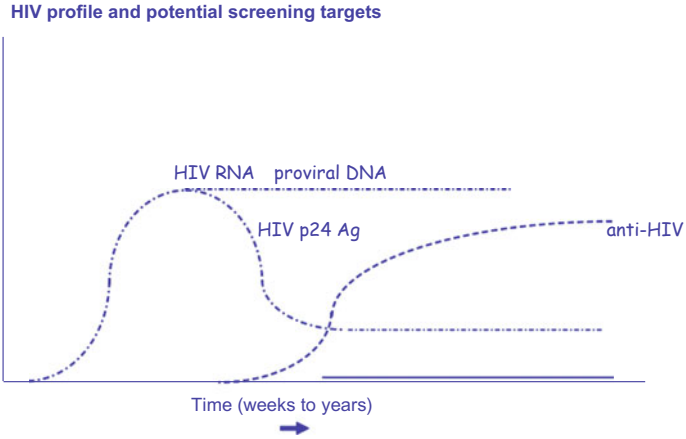

Fig. 10.3 HIV plasma markers—early infection

effective screening programme is always a thorough understanding of the biology of the agent and course of the infection in the host.

Even though it could be argued that some tissue products are processed in ways that remove or inactivate any infectious agent that could theoretically be present, the use of tissue products collected from a donor who may be infected is not considered to be appropriate practice, irrespective of the processing methods used. No pathogen inactivation or removal methods can be considered to be totally effective and therefore must be used in conjunction, not instead of, screening. If a donor is known to be infected with any of the transmissible infectious agents there

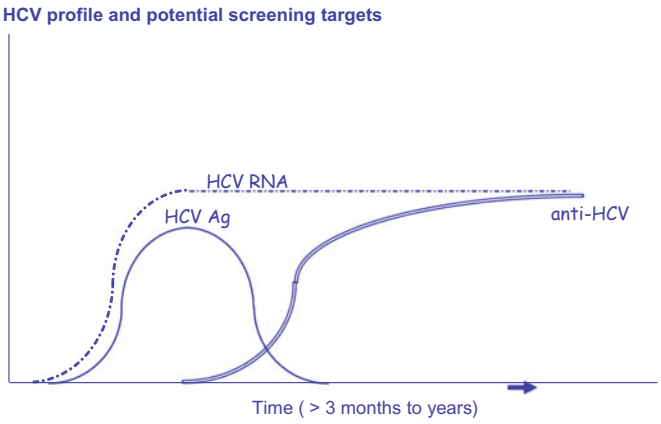

Fig. 10.4 HCV plasma markers—late infection

is a clear risk of transmission and therefore the products should not be released for clinical use.

10.3.1 Mandatory Screening

The infectious agents that are currently considered to be mandatory for the screening of tissue donors in most countries with developed healthcare systems and formal regulatory authorities are hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency viruses 1 + 2 (HIV), and *Treponema pallidum* (causative agent of syphilis). These 4 infectious agents are those for which there is published, irrefutable evidence of transmission through blood and other body fluids, and therefore the potential for transmission through a range of tissue products. The agents are generally present in populations worldwide, although the prevalence and incidence does vary significantly from country to country, and present such a general risk that, in theory, any donor could be infected, irrespective of absence of (known/identified) risk or symptoms.

It is important to understand that exposure risk is something that the pre-donation questioning and selection process seeks to identify, but donors may have been exposed to an infectious agent through a route that they were not aware of. In addition all of these infectious agents may give rise to asymptomatic sub-clinical and chronic infections, and donors may appear to be fit and well at the time of donation whilst actually being infected and thus infectious. This is especially important in the screening of deceased donors as they cannot be questioned directly, and the selection process is dependent upon what family and close contacts believe, which may not necessarily be correct.

Table 10.2 Infectious agents and suitable screening targets

Infectious agent	Screening target		
	Nucleic acid	Antigen	Antibody
HBV—hepatitis B virus	X	X	X[1]
HCV—hepatitis C virus	X		X
HIV—human immunodeficiency virus	X	X	X
HTLV—human T-cell lymphotropic virus			X
Syphilis—syphilis			X
HEV—hepatitis E virus	X		
CMV—cytomegalovirus	X[2]		X
Plasmodium spp.—malaria	X[3]		X
T.cruzi—Chagas disease			X
WNV—West Nile virus	X		

[1]Screening for anti-HBc may be included
[2]CMV DNA may be required for some non-blood donations depending on CMV Ab status
[3]Malaria DNA may be utilised, if available, in addition to Ab for organ donors with a recent exposure history or with serological evidence of malaria infection at some time

All donors are therefore theoretically at risk of being infected with one or more of these agents and so universal donor screening is necessary. However, as mentioned above, as well as identifying the infectious agents, the specific screening target(s) for each infectious agent must be identified. Table 10.2 lists these specific screening targets and the mandatory screening requirements currently in place for all donors/donations screened by the UK Blood Services.

10.3.2 Additional/Selective Screening

There are infectious agents for which there is published, irrefutable evidence of transmission through blood and other body fluids and which can cause significant disease, but where risk is, and can be more easily, mitigated because the agent is either not endemic or naturally present in the general population in that country, or where there are particular exposure risk factors that can be specifically identified in a donor. Specific examples of infectious agents for which additional screening may be required include West Nile Virus (WNV), *Plasmodium* spp. (causative agents of malaria), *Trypanosoma cruzi* (causative agent of Chagas' disease).

To be considered as a potential risk there is an absolute requirement for exposure of the donor to the infectious agent either through living in or by visiting an endemic area. Donors who are at risk can therefore be easily and specifically identified at the point of donation and only those donors require any additional screening.

10.3.3 Screening for Other Infectious Agents

In addition to the well known, characterised and commonly screened for infectious agents, there is always the potential for the sporadic transmission of other infectious agents. These may be existing agents which have a generally low prevalence and incidence, donors are rarely exposed to, often tightly geographically restricted, are rarely transmitted, but which nonetheless may be transmitted if present in the donor at the time of donation. If any screening is required routine screening, universal or selective, is not usually put in place, rather screening of individual donors for the specific infectious agent is performed on an ad hoc basis as the need arises.

10.4 Emerging Infectious Threats

Emerging infectious threats are an important issue for donor/donation screening for any substances of human origin. The status of an infectious agent which is not usually a significant concern/risk changes when the incidence and prevalence increase, either through increasing incidence in endemic countries, through spread into previously non-endemic countries or through increased travel into endemic countries.

There may also be yet unidentified infectious agents that would be transmissible, but which at this time have not appeared as the causes of any identifiable transfusion or transplantation associated infections.

It is not possible to provide a definitive list of all possible current and future infectious threats, Tissue Establishments must put in place, or link into, monitoring for emerging infectious agents which may impact on their ability to collect and issue tissue products. As well as infection risk there is also the issue of sufficiency —the emergence of an infectious agent which may not be transmitted through transplantation, but which may limit the ability to collect tissues if sufficient of the population become infected to limit donor availability and even the availability of staff to retrieve tissues. The majority of recent global threats have been arboviruses, all giving rise to acute infections, but which may be asymptomatic for part or all of the duration of the active infection. However not every infectious agent that affects humans is a potential transfusion/transplantation threat, indeed the number of potential threats is only a small proportion of those infectious agents currently known to man. The 2020/21 global coronavirus pandemic is an example of the appearance of an infectious agent which has not been demonstrated to be transmitted via blood transfusion or tissue transplantation, but has the potential to impact tissue transplantation by limiting the availability of tissue donors.

One major problem that may affect the ability of any screening programme to include a particular infectious agent is the potential limitation either in the appropriate screening target(s) being accessible and/or the appropriate screening assay(s) being available, and on the screening platforms in use. This is may significantly hinder the development of effective and appropriate screening strategies for some

infectious agents, resulting in a greater reliance on the donor selection process and donor deferral rather than laboratory screening. Such reliance on the donor selection process is not always possible or effective, especially in the screening of deceased donors.

10.5 Screening Strategy

To be effective laboratory screening requires the development and implementation of an effective screening strategy to ensure that the laboratory screening performed is targeted correctly, that the outcomes are accurate and reliable and then used effectively and appropriately. When considering the screening strategy and programme to be applied to the screening of tissue donors, the need to be able to apply the same system to both living and deceased donors must be recognized.

Although laboratory screening is still the final decider in the release of the donation for clinical use, the experiences of Blood Services in the assessment of blood donors has shown the value of the donor selection process in reducing the risk of collecting a donation from a "high risk" donor, namely a donor who may have been recently exposed to a transmissible infectious agent, has been infected and is infectious, but where the infection may not be detectable at that time with the tests in use. The obvious restrictions in the depth of the donor selection process that can be applied to deceased tissue donors means that there is a much greater reliance on laboratory screening to determine the safety of the tissues retrieved. However, apart from these differences, there are no other differences between the screening of living or deceased tissue donors.

There are two basic elements to a screening strategy, firstly defining the infectious agents for which screening is needed together with the specific screening target(s) for each infectious agent, and secondly the testing algorithm adopted.

As discussed above, the range of infectious agents that need to be considered to ensure tissue safety is similar to those for blood donors/donations and generally well defined. Likewise the specific screening targets for each of these infectious agents are also well defined. However, these targets may differ depending upon the relative inherent risk associated with different donation types. Additional screening is added where the inherent risk is higher.

The second key element of the screening strategy is the specific screening algorithm adopted. The algorithm essentially defines how the screening assays are used in terms of the initial and (any) repeat testing, and how the final screening result is determined. In most cases the screening algorithm is the same for both blood and tissues.

10.5.1 Screening Algorithms

There are three algorithms, see below, that may potentially be suitable for the screening of tissue donors to determine the fate of any tissues retrieved, i.e. suitable for use or to be discarded. Although every donation is valuable, it is appropriate to point out that blood donations are more numerous than tissue donations, blood donors can donate regularly and it is far easier to replenish stocks. Thus the specificity of the screening and the algorithms, whilst important, are not so critical for blood donations. This difference in supply of blood and tissue donations has led some Tissue Establishments to consider carefully the action to be taken when the screening assay is reactive. In the case of blood donations, screen reactive donations (usually repeat reactive—Algorithm 2) would usually be discarded and the status of the donor then investigated by a Reference Laboratory. In the case of tissue donors, given the much lower numbers of such donors together with the potential for one donor to provide a range of tissues, some Tissue Establishments consider that screen reactivity itself should not automatically result in discard of the tissues retrieved, rather the tissues retrieved may be suitable for use depending on the results of the confirmatory testing performed by the Reference Laboratory to investigate the screen reactivity. Tissues retrieved from those tissue donors whose screen reactivity is conclusively demonstrated to be non-specific may be considered to be suitable for release for clinical use.

1. Perform the initial screening test (Assay A) and use the results obtained to determine the status of the donor. Negative—tissues suitable for clinical use, Reactive—tissues may be unsuitable for clinical use (see text—tissues may be released if reactivity is confirmed to be non-specific)
2. Perform the initial screening test (Assay A) and repeat any initial reactives, in duplicate, using the same assay. Use the 2 out of 3 rule (the 2 identical results out of the 3 results obtained are considered to be the true screen result) to determine the donation fate. Negative—tissues suitable for clinical use, Reactive–tissues may be unsuitable for clinical use (see text—tissues may be released if reactivity is confirmed to be non-specific)
3. Perform the initial screening test (Assay A) and repeat any initial reactives, in duplicate, using a different screening assay (Assay B). Use the 2 out of 3 rule to determine the donation fate. Negative—tissues suitable for clinical use, Reactive —tissues may be unsuitable for clinical use (see text—tissues may be released if reactivity is confirmed to be non-specific).

The 3 algorithms are clearly quite different and have different uses, depending upon the type of donors/donations being screened, the levels of infection in the donor population, the screening assays available and the complexity and effectiveness of the quality management system. Although all 3 algorithms are effective and will ensure similar levels of product safety, it is the associated specificity that is perhaps of more interest and relevance when dealing with tissue donations, and

hence the strategy described above which would not automatically discard tissues because of screen reactivity.

Algorithm 2 is used to screen blood donations by the majority of Blood Services in countries with developed healthcare systems. It offers an effective approach, with an acceptable level of specificity. Indeed in those countries that use Algorithm 1, the unnecessary wastage of blood donations can be very high. Algorithm 3 is based upon the use of a second assay, Assay B that has at least equal sensitivity to Assay A, that is used to test the initial screen reactives. The algorithm is based on the fact that most non-specific reactivity is assay specific, and therefore the majority of cases of non-specific reactivity seen with Assay A, are unlikely to be seen with Assay B, whilst any true reactivity will always be seen in both assays. The algorithm increases the specificity of screening significantly, reducing the unnecessary loss of donations just to those that are reactive with both assays, generally those where infection is more likely to be confirmed. However although very effective, Algorithm 3 is more challenging, both technically and from a quality management perspective, and is generally not an algorithm that is felt to be practical in a routine mass screening environment.

As more and more Blood Services become involved in the screening of tissue donors the overall strategy and algorithm used becomes more critical as in most cases these have been developed specifically for blood donation screening. Although these strategies and algorithms may actually be quite suitable for the screening of tissue donors, this does need to be determined prior to tissue donor screening being implemented. This is particularly critical for deceased tissue donations where the samples may have been collected post-mortem and may therefore not be optimum substrates for the screening assays used and where overall the specificity may consequently be lower than that of blood donation screening. Although a number of authors have reported problems with the specificity of deceased screening programmes [8–11], the deceased tissue screening programme currently in place within the English Blood Service (NHS Blood and Transplant), at this time a combination of Algorithms 2 and 3, is not encountering these levels of screen reactivity and the overall specificity of the screening programme is relatively high [12].

10.6 Screening Assays

The in vitro laboratory screening performed can be divided into serological (antigen and/or antibody) and molecular (nucleic acids). To be able to understand the specific role and value of the two different approaches, the basic biology of infection must be understood.

Following exposure to an infectious agent there is a period during which the infectious agent either enters the body's cells and starts to replicate or is captured and eliminated by the body's passive defence mechanisms. Once the agent has entered the body's cells and started to replicate it is highly likely that a productive

infection will follow. There is a period during which the infectious agent continues to replicate locally until there is sufficient to start to spread to other suitable target sites within the body. During this time levels are low and generally not detectable. However as the agent starts to spread (generally via the circulation), when levels are high enough it can then be detected.

At this point nucleic acid can start to be detected, and depending upon the infectious agent, antigen may also be detectable. Subsequently as the agent spreads the immune response is initiated, symptoms appear and antibody levels start to rise and become detectable. However, again depending upon the agent, at this point nucleic acid levels in the circulation may start to fall as the immune system starts to combat the infection and a balance ensues. In the case of a number of the blood borne infectious agents the infectious agent then sequesters itself in its preferred cells in the body and a chronic or long-term infection develops, the antibody produced simply indicating infection at some time and possibly mediating the infection, but not necessarily conferring any immunity to the agent. In the case of other infectious agents, those that give rise to acute infections only, the appearance of antibody in the circulation marks the start of the resolution of infection and the clearance of the agent from the body, and subsequent immunity, for at least a short period, to that particular infectious agent.

10.6.1 Serological Screening Assays

Screening for the presence of specific serological makers of infection has been in use for many years and is performed on dedicated equipment, which allows high work rates with high reliability and inbuilt quality through high levels of process control. The assays are sensitive, reliable, and cost-effective screening and will identify the vast majority of donors who are genuinely infected (early, recent, ongoing and old infections). Table 10.2 outlines the serological markers for which tissue donations collected within the UK Blood Services are screened.

There are a number of different types of immunoassay available, but all have broadly the same principles. In general immunoassays comprise a solid phase and a liquid phase, and the assay is performed in a number of stages, each of which has an incubation period and a wash to remove the excess materials at the end of the incubation. The solid phase carries the immobilised components of the assay, those designed to capture the specific target that may or may not be present in the sample and is coated with either antigen (Ag) or antibody (Ab) or a combination of both depending on the assay. Any captured target is then detected using a compound which detects any Ag/Ab complexes formed and usually has an enzyme attached to it. This enzyme triggers a detection reagent, either colour or luminescence, that then signals the presence of the compound and thus the target, through the development of colour/luminescence which can then be measured.

The screening assays available from the major international manufacturers are highly sensitive and specific immunoassays that are designed to detect specific target as early as possible in the infection process. They are usually run on

dedicated automated systems with full process control. This ensures highly accurate and reproducible results and thus the overall reliability of the screening process.

10.6.2 Molecular Screening Assays

Large scale molecular screening is now commonplace in many Blood Services and in almost all situations has been used to augment rather than replace serology. Large-scale molecular screening requires the use of dedicated automated systems which perform both nucleic extraction and amplification. Prior to amplification any target nucleic acid in the blood sample needs to be extracted, this extract is then used in the amplification phase. At this time there are only a limited number of mass screening platforms available, and the assays available are limited to those produced by the manufacturers of these platforms. However the platforms available offer highly sensitive and specific molecular screening assays which detect very low levels of nucleic acid. The platforms themselves are fully automated with full process control.

Currently there are two key technologies used by molecular screening assays and both involve amplification of any target to levels which are relatively easily detectable. Although this chapter is not the appropriate place to provide an in-depth review of these methodologies, an overview follows. Polymerase Chain Reaction (**PCR)** is a temperature dependent method that allows exponential amplification of short DNA sequences present within a longer double-stranded DNA molecule, in this case the genome of the infectious agents being screened for. It entails the use of a pair of primers, short sequences of nucleotides that are complementary to a defined sequence on each of the two strands of the DNA. These primers are extended by a DNA polymerase so that a copy is made of the target sequence. After making this copy, the same primers can be used again, not only to make another copy of the input sequence but also of the short copy made in the first round of synthesis. This leads to logarithmic amplification. For RNA viruses the RNA must be copied to DNA first using a reverse transcriptase (RT) step.

Transcription Mediated Amplification **(TMA)** is an isothermal method that allows exponential amplification of RNA or DNA targets in, as for PCR, the genome of the infectious agents being screened for. TMA technology uses two primers, one of which contains a promoter sequence for RNA polymerase, and two enzymes: RNA polymerase and reverse transcriptase. In the first step of amplification, the primer with the promoter sequence hybridizes to the target rRNA at a defined site. Reverse transcriptase creates a DNA copy of the target rRNA by extension from the end of the promoter primer. The RNA in the resulting RNA: DNA duplex is degraded by the RNase activity of the reverse transcriptase. Then a second primer binds to the DNA copy and a new strand of DNA is synthesised from the end of this primer by reverse transcriptase, creating a double- stranded DNA molecule. The RNA polymerase recognises the promoter sequence in the DNA template and initiates transcription. Each of the newly synthesised RNA, amplicons

re-enters the TMA process and serves as a template for a new round of replication. Again, as in PCR, this leads to logarithmic amplification.

Molecular screening offers a (theoretically) higher sensitivity than serological screening for identification of early infections, but at the same time is less effective in detecting existing/old infections. However the more recently emerging infectious agents which have had impact on the supply of a range of donated products have been predominately acute infections for which molecular screening is the only way in which infectious donors/donations may be identified.

Molecular techniques do have a distinct advantage over serology in that each sample tested is internally controlled. Each sample has a specific internal control, an engineered length of nucleic acid, added prior to the nucleic acid extraction process and this length of nucleic acid is extracted and amplified concurrently with any specific target that may be present, and which is then detected using a separate detection system. Thus every sample should score as positive with the internal control, irrespective of the presence of the specific target of interest, uniquely, validating each negative result. Failure of the internal control indicates a failure of the process at some point, whether it be due to a failure of the molecular reagents themselves or due to inhibition of the reagents by something within the sample itself, and invalidates the results for the sample. This has a specific advantage in the screening of samples of poor quality which could contain inhibitors of the assays used.

10.7 Residual Risk

The overall effectiveness of the screening of tissue donors is without question. For many years serological screening was the only approach available for the screening of tissue donations and there is no published evidence, excepting in cases of haemodilution [5], that this approach has led to the transmission of infection as a result of serological testing failing to detect the presence of serological markers in an infected individual. The inclusion of molecular testing, at least theoretically, increases the level of safety even more, although the incremental benefit over that gained from serological screening alone is difficult, if not impossible, to quantify accurately. This is due to the relatively small numbers of tissue donations screened, the even smaller numbers of confirmed positive donors and the virtual impossibility of accurately determining the incidence of infections in tissue donors. Overall the microbiological safety of tissue donations that have been properly and effectively screened for infectious diseases is at least equal to that of blood donations.

However, laboratory testing is not perfect and there are occasions (for a number of reasons) when an assay may not detect its target. Consequently no screening programme has absolute sensitivity and it is possible that infected donors could be undetected with the resultant entry of an infected donation into the supply chain. This is referred to as **residual risk**, i.e. the risk of infection from an infectious donation not identified by the screening programme. However, this is generally due

to the lack of detectable target, rather than the assay failing to detect target. In brief, assays fail to detect target either because the assay has missed something that is present or because there is no or insufficient target present for the assay to detect, although the donor is infected and the donation may be infectious. The first scenario arises either due to poor assay performance (analytical sensitivity) or due to poor performance of the assay (operator/system error). However, if well evaluated and validated highly sensitive assays are being used, and the screening is being performed within a well designed and managed quality management system with well trained and competent staff, then this scenario should rarely, if ever, occur. The second scenario, lack of detectable target yet with infectivity present, is more likely to occur, no matter how good the screening programme and quality management system, as it is related to the normal natural history of infection in an individual.

10.7.1 Window Period

The major threat, in terms of residual risk is therefore from donors who are infected yet negative on screening as the normal screening target(s) are not present, or are present but below detectable levels. This situation occurs naturally very early during the course of infection, at a time when a productive infection has arisen, but before the specific screening target(s) have either been produced or their levels have risen to those detectable by the assays in use. It also occurs later in some infections when the infection is starting to resolve, the level of the screening target has declined to below that detectable, yet infectivity remains. These very specific situations are referred to as the "Window period" of an infection, infectious yet undetectable with the screening assays used. In relative terms, specific antibody appears later during the course of infection than antigen, and antigen later than nucleic acid. Therefore if the screening target is antibody alone there will be a period of time when donations from infected donors would give negative screening results despite the presence of nucleic acid and/or antigen. The same applies when comparing antigen (with or with- out antibody) screening with molecular screening.

The overall risk of collecting a donation from a donor who may be in the window period of infection is dependent on the overall level of infection in the population, and specifically the incidence of infection in the donor population. The higher the incidence of infection, the greater the risk that a donor could have been recently infected. At the same time the ability to detect an infected donation is dependent upon having an effective and appropriate screening programme in place. A screening programme with a relatively poor sensitivity is more likely to fail to detect recently infected donors. However the overall risk is also dependent upon the actual number of donations collected, and although the incidence of infection may be the same in the blood and tissue donor populations, as the populations are so very similar, the overall numbers of tissue donations collected is many orders of magnitude lower than the number of blood donations. Thus it can be argued that the overall probability of actually encountering a donation from a tissue donor who is in

the window period, assuming a screening programme with equal if not greater sensitivity, is much lower than that for a blood donation.

The window period is thus the main reason today that donations, be they tissues or blood, have an associated residual (albeit very low) risk, and screening programmes and strategies are developed to ensure that the window period is reduced to as short a period as possible.

10.8 Conclusions

Overall the current screening of tissue donors prior to release of retrieved tissues is highly effective at preventing transmitted infections. The basic principles applied to the screening of blood donations do apply as there is considerable overlap in the need to identify low risk donors, the range of transmissible infectious diseases, the screening assays used and the screening strategies developed. Certainly there is little, if any, difference between the screening of surgical tissue donations and blood donations, as the samples are all collected by standard venepuncture from living individuals. The screening of deceased tissue donations is different inasmuch as the nature of any samples collected post-mortem may be different to those collected pre-mortem, although the screening assay themselves are the same. It is the reliability of the screening process in relation to any samples collected post-mortem that needs to be assured, although not all samples from deceased tissue donors are collected post-mortem, some samples were collected immediately prior to death, but whilst the donor was still heart-besting.

In addition consideration should be given to the specific screening algorithms used, as those commonly used for blood donation screening may not always offer the most appropriate approach for the screening of tissue donors. The unnecessary loss of tissue donations due to poor specificity is unacceptable because tissue products are generally in shorter supply, often with many potential recipients on waiting lists. Furthermore a deceased donor may provide a large number of different tissues for clinical use, and an unresolved non-specific screening result could result in the loss of a significant number of clinically valuable products.

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Contamination in the Tissue Way

11

Pieter Petit

Abstract

Tissue Establishments (TE) guarantee maximal sterility in handling donated tissue. The complete proces that tissues go through from donor to recipient, called "the tissue way" carries many risks for contamination. The tissue way lacks safety checks and use insensitive methods that are not standardized allowing high failure rates in discovering and identify contaminants. The tissue way includes 5 steps: the donor, explantation, transport, the tissue bank and the microbiology laboratory that collects and tests all samples for sterility. All steps will be discussed in relation to the different kinds of contaminating microorganisms, its origins and modes of transmission. Weak points are discussed and optionds are given to improve and prevent it tissue contamination. Specific subjects are discussed, including durability of cardiovascular tissue, mycobacteria, spore forming bacteria, and the use of antibiotics and how to proces tissue in a closed fully automated system with maximal safety.

11.1 Introduction

Tissue Banking has the aim of delivering good quality and safe tissue for use in recipients. It is the responsibility of the Tissue Establishment(TE) to control the whole process from retrieval of tissue from the donor right through to implantation of tissue in the recipient.

There is still a great need for tissues. Worldwide more than 70,000 organs (and >100,000 corneas) are transplanted. Human tissue allografts are used >2 million times a year to save lives or significantly improve the quality of life for many recipients. Clinical results from donated tissue are very good, with very few

P. Petit (✉)
Franciscus Vlietland Hospital, Rotterdam, The Netherlands

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complications and is a comparatively cheap and easy to use treatment. The biggest risks associated with tissue transplantations are the infections because of the presence of contamination with micro-organisms. The rate of a transmitted infection is unknown but likely to be minimal (1%) in solid organ recipients and even much lower for cornea and tissue recipients [1–4]. For most tissue products it is not possible to carry out terminal sterilisation of the product, making it essential that tissue processing is done in a sterile environment with continuous monitoring of sterility throughout the process. There are many points during tissue processing where introduction of contamination is possible, including the donor themselves, the retrieval process, the processing of the tissue product and the microbiology sampling. The risk of contamination is higher when tissue is retrieved from post-mortem mortuary (non-heart beating) donors.

It is important that TEs monitor and check for the possibility of contamination throughout the whole process of tissue retrieval, processing and storage. Not all TEs however carry out testing in a similar manner and the methods used to check for the potential presence of contamination vary and can be of varying sensitivity. Irrespective, the TEs remain responsible for ensuring tissue products issued for clinical use are of good quality, and as sterile as current methods allow.

In order to identify the presence of any potential contamination it is important that in-process testing is carried out throughout the whole process. In-process testing includes the requirement to test the impact of staff during tissue processing (air movement and dispersing of microorganisms) and the environment where the tissue is handled. It requires a professional multidisciplinary assessment of the potential presence of contamination from multiple sources that include the donor, the retrieval process, the environment where retrieval takes place, the transport of the tissue product, the environment where tissue product processing is carried out, and last but not least the medical Microbiology Laboratory responsible for testing the samples taken to look for the potential presence of contamination.

The in-process testing starts by taking representative samples at every step and location and condition involved, together with full identification of any microorganisms present up to species level. Biological safeguarding of the tissue product requires a whole chain approach. These microbial controls are not only aiming to exclude the potential presence of contamination but are also serve for controlling the process i.e. being able to answer the following: is the product safe; if not what is the contamination; what caused it; , what was its source; what can be done about it. Especially this last point is too often not considered! [5–11].

How and where to look for the source of contamination, and above all what to do about it, depends upon which micro-organisms are expected and which are isolated.

11.1.1 Bacteria and Fungi

Contamination refers to bacteria and fungi already present within and persisting in tissue products that are meant for clinical use in patients. Tissue products used clinically must be sterile otherwise great harm is potentially caused to their

recipients. All micro-organisms can be transmitted, but in this chapter we will only deal with the risk of transmitting bacteria and fungi (and not viruses and parasites).

Bacteria are living cells too small to be seen. They are ubiquitous and present on a scale of millions. Contamination in Tissue Banking happens mainly with commensal bacteria i.e. bacteria that normally colonise(live) on human beings, on the skin, in the airways and in the gastrointestinal tract. These bacteria are normally harmless to human beings and may even be beneficial to us. Even in wounds commensal bacteria will not cause infection unless present in heavy numbers (more than 100.000 per gram tissue).

Every bacteria and fungus has its own natural habitat that provides it with optimal living conditions. All living matter have their own commensal bacteria (also known as their bacterial flora). Such commensal micro-organisms are non-pathogenic, i.e. they do not cause disease except under very specific circumstances [12]. This suggests that it may not be clinically significant if some commensal micro-organisms are still present on transplanted tissue products. There are several publications showing that there are no resultant infections after implantation of non-vital tissue products that are positive for commensal flora [13–17] and there are no reports of endocarditis after tissue implantation [18]. Commensal micro-organisms are always present and are a benefit to us, defending us against unusual and pathogenic bacteria that are not normally present in human beings. Human beings are exposed to numerous transient bacteria present in the air around us or by contact with animals, water, food or soil; such bacteria stick to the skin or airway or enter through the intestines. There is a continuous bombardment of bacteria and fungi on humans, but these transient bacteria normally cannot survive long and will disappear in minutes to hours without causing any harm. For an infection to result there needs to be a break in the normal human defences (for example a wound); even a real pathogen needs a break in the normal human defences to cause an infection.

Micro-organisms are universally present so that all retrieved tissue products have evidence of contamination [19, 20]. The question is with what micro-organisms, what level of contamination and what was the source of the contamination.

It is important to better understand these contaminating bacteria. Bacteria are very small and multiply very quickly: in optimal growth conditions they duplicate in number every 20 min and keep on multiplying as long as they are able to, dependant on available nutrition. One bacterium, under optimal growth conditions becomes more than a million bacteria within 8 h. In such a situation, eliminating 99% of the bacteria will still leave 10,000, which will become a million again within 2.5 h. In Tissue Banking 99% elimination of bacteria is not enough.

Bacteria can be made visible by staining them, using a Gram stain, which stains bacteria red or blue. Red-stained bacteria are called Gram negative bacteria, while blue-stained bacteria are called Gram positive bacteria. The Gram stain allows the shape of the bacteria to be identified as either a rod or a coccus (round); it also identifies how the cell wall is built: blue (Gram positive bacteria) is a thick permeable wall that stands dryness, and red (Gram negative bacteria) is a thin fatty wall with pores (called porins) that needs humidity. Due to these properties bacteria

normally found on the skin are mainly Gram positive (blue), while the gastro-intestinal tract and airways (mucosal surfaces) normally have mainly Gram negative bacteria (red) as they need humidity to survive. Being aware of the properties of the bacterial wall through Gram stain allows an assessment of what antibiotics can be used to treat effectively.

In Tissue Banking most of the contaminants present should be bacteria normally present on human skin because human beings continuously shed skin cells with bacteria while the airway/gastro-intestinal tract are internal so that contamination from these can only be transmitted by coughing, speaking or direct contact. Happily most contaminants are commensal micro-organisms or the transient bacteria that originate from the environment. A third group of micro-organisms, pathogenic bacteria are however problematic; humans can be carriers of such micro-organisms i.e. these are bacteria that have to live on humans and can be passed on from one person to another. In healthy people even these pathogenic bacteria won't cause any harm, but they can lead to severe infections if there is just a small break in defences: a small wound (*S.aureus, Streptoccus haemolyticus*) or they are swallowed with food (*Salmonella spp*) or breathed in with aerosolised water (*Legionella spp*). Most TEs reject tissue products if these highly pathogenic micro-organisms are found in any of the samples and will not use the tissue product even if post-decontamination the tissue samples tested are found to be negative for their presence [21].

The full identification of any contaminants present can allow the origin of the contamination to be surmised [22]. By assessing the circumstances of retrieval, environment and the processing carried out at the different stages of tissue banking it may be possible to identify where contamination is likely to have happened. Such information would help evaluate how to prevent and manage the contamination [23, 24] (Tables 11.1 and 11.2).

Table 11.1 Origin of contaminating bacteria in percentages from the total of contaminated tissue in 4 Tissue Establishments

	Percentage (%)	
Skin	47–77	Gram positive cocci: Staphylococci, Micrococci Gram positive rods: Corynebacteria, Propionibacteria
Airway/mouth	13–23	Gram positive/negative cocci: Streptococci, Neisseria Gram negative rods: Haemophili, Prevotellae, Porphyromonas
Gastro-intestinal	5–25	Gram negative/positive rods: *E.coli*, Enterobacter, Klebsiella Anaerobes: Bacteroïdes + Clostridia (spore former)
Environment	2–10	Gram negative rods: Non-fermenting Pseudomonas-like bacteria Gram positive: Arthrobacter
Air	1–4	Gram positive cocci: Micrococci Spores from Bacillus, Spores from Fungi

Table 11.2 Highly pathogenic micro-organisms and their source as part of the total contamination rate in one big Tissue Establishment (>10,000 donations)

Tissues[a]	Donors[b]	Respiratory tract[c] (%)	Gastro intestinal tract (%)	Environment (%)	Skin	TOTAL of high pathogens (%)
MST	HB	3.2–4.8	7.8	0.2	–	11.2–12.8
	NHB	1.5–6.8	6	0.1	–	7.5–12.8
CVT	HB	2	6	–	–	8
	NHB	4–24	21	1.2	–	26.2–46.2

[a]*MST* musculoskeletal tissue, *CVT* cardiovascular tissue [b]*HB* Heart beating, *NHB* non-Heart beating

[c] Range quoted is due to the fact that most streptococci are commensals and no species names were given. The % of the total quoted are with and without streptococci

No highly pathogenic micro-organisms were isolated from skin, with most of the pathogenic micro-organisms being isolated mainly from the respiratory and gastro-intestinal tracts. Such contamination is usually due to perforation of the gastro-intestinal and/or respiratory tract during retrieval, although other sources include the retrieval staff themselves (respiratory pathogens) and the microbiology laboratory carrying out the testing of the contaminating samples (spore formers and respiratory pathogens).

Not checking for the potential presence of contamination pre-decontamination, instead relying only on post-decontamination sampling, may lead to a false reassurance. Moreover, the presence of dead bacteria in the tissue products may lead to endotoxic shock, and may provoke a reaction or rejection of the tissue. Bacterial spores (as well as latent bacteria) may start growing in the transplanted tissue after a delay leading to infections in the recipient that may be difficult to fully identify and resolve. Relying on a decontaminating process that cannot guarantee a 100% sterility without assessing for the potential presence of contamination pre-decontamination is therefore considered poor practice. Knowing which contaminating bacteria are present in the tissue product allows the potential source to be identified and the contamination to be managed appropriately.

There is wide variation in practice as regards the type of samples taken for sterility testing, culturing techniques used, and decontaminating protocols [21, 25, 26]; often, due to cost and lack of standardisation of the microbiological tests used. This may lead to no- identification all potential contaminants. Carrying out most of the testing required in house by the TE would help reduce the costs of such testing. In doing this though, it is important to carefully develop protocols that will ensure all possible contaminants can be identified. To standardised such protocols there is current work ongoing with the aim of setting up an external quality assessment (EQA) system for the microbiology aspects of tissue banking [21, 27]; the results of such EQAs would help clarify which techniques would be most reliable.

From available information, the main source of contamination in Tissue Banking is the skin of staff handling the tissue at any stage of the process, and the next main

source of contamination are areas with uncontrolled environments such are the mortuary and the microbiology laboratory. The contamination rates vary widely between TEs. One of the most important factors for such variation is the technique used to take samples for cultures and the media used for culturing. In the quality round carried out by the author and other colleagues in 2016 it was clear that the media used to culture tissue samples were in many Tissue Banks insufficient to identify all relevant micro-organisms that may be present. Further most Tissue Banks do not check for the presence of residual antibiotics leading to the risk of false negative culture results [28]. The aim of tissue processing is to prevent and eliminate any risk of bacterial/fungal contamination that may be acquired during the procurement, processing and storage of tissue products 5 [8].

11.2 The Tissue Way, P.L.C. Petit [22]

11.2.1 Donor

Screening: The Tissue Way starts with an accepted donor (after a medical, travel and social history screening and risk assessment) without evidence of infection. Initial screening is often done according to national guidelines and can differ from country to country and even from region to region [29]. The reason for this can be the regional endemic occurrence of specific diseases such as fungi (e.g. Histoplasmosis) and parasites (*Tryponazoma cruzi*, *Leishmania spp* and *Toxoplasma gondii*) for which additional serology or NAT testing is needed. Information about the cause of death (was it traumatic; was resuscitation required?) and whether invasive procedures, operations or treatments (such as antibiotics) were carried out need to be known to allow a risk assessment to be carried out by the TE as to the risks of bacteraemia [30] and endogenous contamination from the donor due to colonisation with pathogenic and resistant bacteria due to a stay in hospital (particularly on the intensive care unit). A traumatic cause of death (a road accident) or invasive procedures increase the risk of contamination by gastro-intestinal and airway bacteria 3–4 times [31–34].

a. Environment: Most often death happens in a hospital and the donor is moved to an operating theatre or a dedicated retrieval suite for tissue retrieval. The donor should be cooled as soon as possible. The more a donor is transported and manipulated the more bacteria inside the body are transpositioned and can migrate from the gastro-intestinal and respiratory tracts to other places. When

the donor is cooled within 6 h of death there is no evidence of any transmigration (if not manipulated) of micro-organisms for up to 72 h [35, 36]. In such circumstances the donor adds little to contamination: the level of donor commensal flora is reducing from the moment of death [30, 37]. During tissue retrieval, depending on the technique used and the experience of the staff carrying out the retrieval, the airway and gastrointestinal tracts should remain intact so that no contamination will occur from these tracts. If perforation does occur of one or both of these tracts, this should be documented to allow a full assessment to take place. Further consideration should be given to cleaning the room after such a retrieval with a sporicidal disinfectant (due to the spore forming bacteria that are normally present in the intestines), or even more aggressive chemical disinfection to make sure that the environment will be as clean as possible for the next retrieval (recommended disinfectant to be used is for example hypochlorite).

b. Risks: In 25% of donors an unidentified bacteraemia (12.5% skin commensals) is present at the time of death [30, 31, 38, 39] and needs to be identified. A bacteraemia will cause contamination of all organs or tissues but it will be difficult to identify using current samples (Swab, fluid sample, tissue biopsy) to look for the potential presence of contamination. Following death, translocation of bacteria is increased by transport and movement of the donor's body - the more contact with the environment and the more translocation of bacteria occurs. If tissue retrieval is carried out within 6 h of death or the donor is cooled within 6 h, the donor's body as a source of contamination is almost nil [30, 37].

The rate of tissue contaminations quoted for living donors, multi-organ donors and tissue-only donors vary tremendously, but are lowest in living donors and highest in tissue only donors; in the latter group of donors tissue contamination may be as high as 88.3% of products [40] in cardiovascular tissue and 54.8% in musculoskeletal tissue. Further contamination of tissue products retrieved in the mortuary tends to be much higher than when tissue retrieval takes place in an operating theatre [32, 41–45].

In summary: in non-heart beating donors (NHBD) contamination is higher and more often with multiple strains when compared to donation from heart-beating donors(HBD), and contamination of cardiovascular tissue (CVT) is higher than that of musculoskeletal tissue (MST). The rate of contamination is highest from tissue-only donors, followed by multi-organ donors and least in live donors [31, 36, 40, 46–50].

c. Principles of Donor Testing:

1. Tissue from donors need to be tested for the presence of residual antibiotics in their tissue especially after hospital admission where they are likely to have been treated with antibiotics just prior to their death. Tissue containing traces of antibiotics may lead to false negative tissue culture results. Antibiotic residues are found particularly in heart tissue donors (proof of

principle Saegeman, Jashari, Petit 2018) and in culture samples taken after tissue decontamination [51–53].

2. To identify the potential presence of endogenous contamination a blood culture should ideally be taken prior to proceeding with tissue retrieval. Endogenous contamination with haematogenous spread of micro-organisms through the whole body and to every organ is caused by traumatic bacteraemia or post-mortem bacteraemia due to transmigration or translocation [54–56]. Clostridium bacteraemia is often only detected by carrying out a blood culture (spores that can germinate in blood culture media). Previously unidentified bacteraemia is reported in as many as 25% of donors [31, 32, 38, 39]. The advantage of carrying out blood cultures post-mortem is their additional and sometimes only (such as in Clostridium contaminations) detection of pathogens; more tissues are positive if the donor is found to have a positive blood culture, allowing a better evaluation of the donor if there is a blood culture result available.
3. Hospitalization of donors: if the donor was a hospitalised, particularly if admitted to intensive care or there was trauma requiring operation prior to death, non-commensal bacteria with high resistance patterns have to be expected. The best way to identify such contamination in the donor with the least false positive contamination results is to take tissue and fluid samples and inoculate them straight on to bacterial media or inject them into blood culture bottles and incubate them at both 35 °C (to identify bacteria) and 20-25 °C (to identify fungi). Fungi have to be expected when tissue retrieval is carried out in mortuaries with an uncontrolled air supply (therefore likely to be carrying many spores). It is important that the media used are enriched or have additives to ensure that fastidious bacteria can be identified, and also to neutralise any toxins and antibiotic residues [57–59]. A significant increase in positive cultures for all types of bacteria was accomplished by the Tissue Bank in Treviso once they started using BACTEC instead of BHI [40]. BACTEC is a blood culture medium, validated for the culture of liquids other than blood [60] but not validated for tissue samples although proof of principle for tissue is already proven (personal information Jashari, Saegemann, Petit 2018). Blood culture media or enriched media (BHI and Thioglycollate but with additives/supplements like Fastidious Organism Supplement) have the advantage of being a closed system, can be injected and kept in a normal incubator for culture in the TE until it shows growth. Only if growth is identified would the sample then need to be referred to the microbiology laboratory for formal identification of the contamination present. Such sampling can be done both for testing of tissue biopsies and also for liquid samples.

Sources and risks of contamination from the donor

Source of contamination	Skin micro-organisms	Respiratory or gastro-intestinal tract	Environmental air	Endogenous from donor	Outside body	Place of death (hosp.?)	Process controls	Extra risks
Donor	±	±	+	±	+	++	Blood culture	Cause of death Perf[a]. or trauma HB/NonHB[b] Place of expl.[c] Delayed cooling

+ = risk ++ = frequent risk +++ = risk at all times ± = incidental risk – = no risk
[a]*perf* perforation [b]*HB* heart-beating [b]*NHB* non-heart-beating [c]*expl* explantation

11.2.2 Tissue Retrieval in mortuary (1) or operating theatre (2)

1. **Mortuary**

a. Environment: in a mortuary or a dedicated tissue retrieval suite there is usually no control of the air environment and the surfaces are often only given a minimal (household style) cleaning. Usually these areas have a water source through a tap, cupboards, a refrigerator and other equipment that is not sterile and often filled with different kinds of storage material which will provide a good environment for bacteria to thrive on. Further dust particles in such environments can contain and spread bacteria via uncontrolled airstreams.
b. Risk in Mortuary: in an environment that is not sterile and where there is no control of the air environment there is a significant risk of contamination through the air at all times often with difficulty to treat environmental bacteria (non-fermenters with antibiotic resistance). In the mortuary contact with environment is inevitable: in the mortuary refrigerator the outside of the donor's body will be in contact with dirty surfaces where many different types of contaminants may be present. The donor's body is however cooled and the numbers of microorganisms present on the body decrease with every hour of cooling. Further prior to proceeding with tissue retrieval the donor's body is meticulously prepared, disinfected and covered to maintain a sterile operation type field; this will therefore not be a cause for tissue contamination unless during retrieval the respiratory or gastrointestinal tracts are perforated in error. In the absence of these the main source of contamination are the staff carrying out tissue retrieval, with the risk increasing with the number of individuals present and influenced by the techniques used [61, 62]. The lack of air control in the mortuary means that high rates of bacteria and spores in the environmental air will contaminate (for days and weeks) anything present in the room. Due to all these factors the rate of contamination can be as high as 83% for

cardiovascular tissue and 52% for musculoskeletal tissue (with multiple strains in 42% and 10% respectively) [17, 18, 20, 30, 31, 40, 63–67].

c. Principles of Testing: usually following retrieval the retrieved products are placed in sterile containers that are sent to the TE; there they take relevant samples for microbiology testing. This is a reliable way of testing for the potential presence of contamination with minimal manipulations of the donated products. Otherwise tissue samples, swabs and biopsies, would need to be taken during the retrieval process for all products retrieved. This is complicated and is likely to lead to contamination of the samples taken. The potential presence of contamination due to a bacteraemia can be tested for by taking a blood culture BEFORE retrieval starts (see earlier). The risk of contamination from the environment on the other hand is mainly caused by donor manipulation, micro-organisms present on the retrieval team and from the environment. The latter has to be tested by taking samples during retrieval and processing and can be identified by taking samples such as surface swabs and tissue biopsies:

1. Contamination from the environment, caused during retrieval mainly by skin and airway bacteria from the retrieval team, can be identified by taking swabs (especially for musculoskeletal products) from every retrieved product However swabs have a low sensitivity for detecting the presence of contamination compared to entire bone cultures [68, 69]. Sponge swabs or swabs immediately placed in a broth (during retrieval) have better sensitivity. Even better would be to collect and filter the transport fluid in which the tissue/organ was transported and culture that sample. Using filtered transport fluid makes quantification of the level of contamination impossible (unless cooled on CO_2 ice) but quantification has a very low predictive value (see under Microbiology Laboratory). It is much more important to identify which bacteria are present than to quantify the bacteria that are present [66, 70].
2. Contamination of tissues by bacteria from the respiratory or the intestinal tract is possible if there has been a perforation by trauma; similar damage can also be caused during the retrieval procedure. Air currents in the room during retrieval can also lead to contamination during the retrieval procedure. Experience from infections caused in operating theatres shows that the staff and their behaviour while in the operating theatre are the cause of most transmissions of bacteria through the environmental air to the operation site. Due to the fact, that, the intestinal tract contains spore forming bacteria (e.g. Clostridia), these spores can remain in the environment for weeks and will be difficult to clear. Specific cleaning with sporicidal disinfectants would be necessary. As previously mentioned, if the donor is cooled within 6 h of death contamination from the donor will be minimal; on the other hand if warm ischaemic time is increased (i.e. donor is not cooled until after 6 h from the time of death), particularly if there is a lot of transport or manipulation of the donor's body this will favour the growth and migration of commensal flora from the gastrointestinal and airway tract, leading to tissue contamination, especially so in the warm temperatures of

summer [48]. In such cases contamination may be secondary to any kind of micro-organism so that it is important that the medium used to look for the potential presence of contamination is a richly supplemented medium that supports growth of both aerobic as well as anaerobic micro-organisms, including fastidious bacteria and fungi.

In summary the recommended way to identify the potential presence of contamination on tissue products is to take blood cultures from the donor, and once retrieved all tissue products should be safely packaged and sent to the Tissue Bank who will then be responsible for taking all the samples required to identify the potential presence of contamination [32, 36, 41–43, 49, 71].

Risk from donor	History of trauma, operation or other invasive manipulation, known diseases Viral testing is required to assess the risk of blood borne infections	Donor manipulation Techniques used at retrieval Potential perforation of the airway or gastro-intestinal tract Contact with environment
Risk from operating theatre	Theatre is NOT a clean room, with dust particles and doors opening	Number of individuals and their behaviour during the retrieval, the techniques used to carry out the retrieval, potential perforation of respiratory/gastro-intestinal tracks and manipulation of the donor body
Risk from mortuary	Non-sterile room and non-sterile air streams	Number of individuals and their behaviour during the retrieval, the techniques used to carry out the retrieval, potential perforation of respiratory/gastro-intestinal tracks and manipulation of the donor body

d. Environmental controls are important: settle and contact plates, especially from the retrieval table and near the doors should be the minimum environmental monitoring carried out. A glove print from the surgeon onto plates at the end of the procedure should be taken as well. Air quality should also be monitored using a volumetric air sampler with particle counting on regular intervals to guarantee or at least be aware of the air quality and the occurrence of spores from fungi and bacteria and fine particles. Such plate cultures can be carried out by the TE. If no growth is seen there are no concerns. If, on the other hand there is growth from these plates then quantification and robust knowledge of the type of micro-organisms identified is important to judge how clean the environment is and whether there is a significant risk of contamination. If the environmental monitoring is positive it is possible to compare the results with the culture results of samples taken from the donor/ tissue; if the same micro-organisms are identified, then the source of contamination can be identified allowing it to be dealt with effectively.

2. **Theatre**

a. Area: in operating theatres a controlled sterile environment is established, but mainly around the operating table. The room is cleaned after every operation, in-coming air is sterile, and all instruments and equipment used are sterile. Theatres have a system that blows air over the donor as to make an air block around the donor that is difficult to penetrate (but door-openings and activity of people do so). However, there is no airlock at the entrance of the operating theatre, the room is not dust free so that the area around the operating table is probably equivalent to a class C or even D according to clean room classification.
b. Risk in operating theatres: the main source for contamination are the staff working inside the theatre, mainly the retrieval team. People in operating theatres are dressed with gloves, face masks and surgical gowns; however the risk of contamination persists due to the continual shedding of human skin with bacteria. Men shed as much as 10 × more skin as women. Clothing does not stop this. It has been shown that while working in operating theatres every person sheds approximately 2000 skin scales with bacteria on them every minute, and two thirds (2/3) of these skin scales are able to penetrate and pass through the surgical gowns. The more active an individual is, the more skin scales are shed, which can increase up to 1000 × more if people are running [72] or working steadily [73–75]. A further source of possible contamination is talking and shouting, which make it possible to transmit airway bacteria, even through a face mask, and especially if it gets wet with saliva. Outbreaks are documented where the source of contamination was shown to be staff members colonised with *S.aureus* (carriers) transmitting high numbers of the micro-organisms through the air, on skin scales and through contact with surfaces [76–78]. Micro-organisms passing through face masks and clothing [79–82] can and will cause transmission and contamination of the environment; this can be very substantial with increasing number of individuals and airstreams (e.g. opening doors) and movements going on. The main source of contamination is by skin commensals through the environmental air [73–75, 83–87]. If during retrieval perforation of the intestinal and/or airway tracts occurs, the environment becomes contaminated with intestinal and airway commensals. Identification of micro-organisms that are normally resident in the gastro-intestinal tract, such as Clostridium, *E.coli*, Enterococci and Bacteroïdes indicate that the source of contamination is likely to be secondary to a perforation of the intestines, while contamination with micro-organisms that are normally present in the respiratory tract, such as Neisseria, Streptococci, Haemophili or *S.aureus* [76] indicate that the likely source of contamination is either through perforation of the airway tract break or from staff carriers. Contact with such commensals can easily transmit them through face masks, surgical gowns and gloves as well as the organs/tissues that are being retrieved.

Hand washing prior to carrying out a retrieval is critical, as that removes 95% of superficial micro-organisms. To prevent contamination by the remaining 5%, gloves are worn. However it is important to realise that there is evidence that orthopaedic surgeons perforate their surgical gloves in 53% of operations and 14% had *S.aureus* on their hands while on their gloves only skin-microbes from the patient were identified. Contaminated and punctured gloves and contaminated surgical gowns will very often lead to tissue contamination during retrieval through contact [88, 89].

Therefore the risk of contamination is significant during retrieval. The risk is higher if retrieval is carried out in a mortuary or other tissue retrieval suite because the environment is unclean with non-sterile airstreams that make it possible to transmit bacteria and spores to anything present in the room. However it is worth remembering that more than 63% of the environment in a surgical theatre is also contaminated (blades, gloves, needles) [75] with skin and air micro-organisms that originate mainly from the operating team, so that the risk of tissue contamination remains substantial even in operating theatres.

Risks and source of contamination at tissue retrieval in operating theatres and mortuary or dedicated tissue-retrieval suite

	Skin	Respiratory/gastro-intestinal tract[a]	Environmental air	Inside environm	Outside environm	Place	Process controls	Extra risks
Theatre	++	±	±	+	±	Clean	Not taken	Human activity
Mortuary	++	±	++	+	++	Dirty	Not taken	Techn.exper. Manipulation Nr. of people present

+ = risk ++ = frequent risk +++ = risk at all times ± = incidental risk − = no risk

[a]If no perforation at explantation!

c. Principles of Testing: this will be the same as in the mortuary i.e. it is recommended that once the organs/tissue are retrieved, they are packed and sent to the TE who will then take samples for cultures. It is again important to also take 2 or more negative controls (e.g. culture sterile injection fluid) without adding antibiotics as evidence of the sterility of the transport method used and the laboratory carrying out culture testing.
d. Environment: It is equally important to carry out environmental monitoring in operating theatres. This should be done according to a maintenance protocol for air and cleaning monitoring, as well as contact (fingerprints) and settle plates during retrievals to monitor what is happening during the retrieval process. As far as the author is aware this is not normally done but is an important consideration to gather information about the risk of contamination.

Recommendations to reduce the risk of contamination: keep ischaemic time as short as possible [30, 90], retrieve with as few staff as possible, ensure retrieval staff are well trained, with disciplined behaviour, use proper disinfection, and carry out environmental monitoring, including glove prints from the retrieval staff.

11.2.3 Transport

Transport of cardiovascular, musculoskeletal, cornea, skin or any other tissue products is done using a closed system usually in a Perspex box on CO_2 ice. Introduction of contamination during transport is therefore unlikely so no additional testing is required unless there is an accident, delay in transport, controlled temperature fluctuations or there is damage to the package.

11.2.4 Tissue Establishment

a. Area: the Tissue Bank is the most sterile aspect of tissue processing. The staff involved in tissue banking is normally well trained in using an aseptic technique and is used to work in a controlled environment that is dust free (grade B-C environment). In the TE, tissue processing is carried out in a Laminar Air Flow cabinet to avoid tissue product contamination through the environmental air. In clean rooms the air pressure in the room is controlled and higher than outside, and the air comes in through a HEPA filter. Further there are no water sinks or taps, no cabinets, no refrigerator etc. In such an environment contamination is only possible from the operator carrying out the tissue processing; to minimise this risk their skin, airway and hair are covered (sterile gowns plus full face mask plus hat and a plastic screen behind which the work takes place). There is minimal handling of the tissue product using gloved hands within the cabinet. This is the best place for tissue and fluid samples to be taken for sterillity testing both before AND after the tissue decontamination. Antibiotic-residues can be rinsed (thought to be not very effective with the high concentrations of antibiotics used) or neutralized (with resins and anti-toxin factors in blood culture media), but these processes are not yet validated. The European and the US Pharmacopoeia recommend that any factor that interferes with microbial growth during sterility testing must be eliminated, so tissue samples should be without substances that are toxic to micro-organisms, such as antibiotics. There is current work ongoing to validate the antibiotic neutralizing effect of the BACTEC/BacTalert media (2018, Jashari, Sagemaen, Petit). Once the detoxifying or antibiotic neutralizing step is carried out, more tissue biopsies should be taken to

confirm sterility: 2 for culturing, one to keep in reserve in the eventuality of needing to repeat test, as well as sending a negative control (e.g. sterile water sample) to check the sterility in the Microbiology Laboratory where the samples are sent for testing. At the present time (2018) many TEs culture samples that are likely to contain high concentrations of antibiotics, leading to the possibility of false negative culture results [21, 51].

b. Risk: Clean rooms are necessary for the processing of tissue products [67] to minimise the risk of introducing contamination during processing. By using a Laminar Air Flow cabinet in a clean room and trained and very disciplined staff, the risk of introducing contamination during processing within a TE is minimal. If contamination does indeed occur during the tissue processing this can be identified through the settle and contact plates and glove prints that are done routinely during clean room processing i.e. though routine environmental monitoring of the clean room environment.

c. Principles of Testing

1. To identify the presence of contaminations one must be aware which bacteria are likely to be present. In Tissue processing this will be the microbial contaminants that may already be present in the retrieved tissue, and much less frequently flora that is normally resident on skin from the staff that handle the tissue during retrieval and processing. If the Tissue Bank uses the proper conditions during processing, decontamination and storage of the tissue in the Laminar Air Flow cabinet, then contamination through the environmental air, or from water or through contact with the staff which is very unlikely. In general, all samples for culture pre- and post-retrieval (after decontamination) can be processed by the Tissue Bank under controlled sterile conditions in the manner described under retrieval. A pre-processing culture sample as well as a final post-decontamination, pre-final packaging sample are the minimal samples that need to be tested in order to prove the safety of the processing carried out for tissue products. Even better would be to take additional samples during processing, in double for culture results, and at least one additional reserve sample that can be re-sent for testing if there is any doubt as to the results due to the potential for false negative results (see below in Table 11.3). Such samples taken for culture can be processed, at the TE until confirmed as either negative or positive. This would minimize the risk of false positive results due to sampling. The protocols to follow should be developed in collaboration with the retrieval team and the microbiology laboratory. It is important to ensure that the final samples taken post-decontamination prior to final packaging of the tissue products do not have residual antibiotics present that may lead to false negative culture results [51]. Removing antibiotics can be done either by rinsing the antibiotics or by neutralising them; validation of either method would be important. Antibiotic neutralization can be achieved by using blood culture media for which there is proof of principle for both liquid samples as well as for solid tissue [51, 59, 91].

Table 11.3 Sensitivity(Se) of sampling methods and sampling error(SE)

	Sensitivity (%)	Sampling error	
Blood culture taken from donor Bone marrow aspirates	25	++++	[17, 20, 32, 55, 92]
Swab	39	++++	[54, 68, 69, 93–96]
Sponge swab	<44	+++	[68, 69, 92, 97]
Swab in broth	54.9	+++	[68]
Biopsy (small, risk of sampling error, poor sensitivity, introduces manipulation)	50–55	+++	[18, 68, 94, 95, 98]
Destructive biopsy	77.9	++	[93, 96]
Transport/representative fluid	> = 90	+	[94, 99]
Filtered transport fluid	>90	±	[60]
Fluid in blood culture medium	>90	±	[60]
Culture in toto	92	±	[20]

Sampling error [42, 54, 68, 100]

The residual possible sources of contamination during processing in a TE are from the accidental use of contaminated or perforated gloves; the risk of cross contamination if tissues from more than one donor are handled at the same time inside the cabinet, (considered to be poor practice), environmental contamination, if unsterile equipment is in the clean rooms.

Risk and source of contamination in tissue banks

	Skin	Respiratory/gastrointestinal tract	Environmental air	Inside environm	Outside environm	Place	Process controls	Extra risks
Tissue Bank	±	–	–	–	–	Sterile	Taken	Mistakes

± = incidental risk – = no risk

2. Quantitative Cultures are in most situations not possible and unreliable during tissue processing: there is usually a time lag between culture samples being taken, either by the retrieval team or by TE staff, and the sample being processed by the laboratory, usually of several hours and sometimes days during which time any contaminants present will multiply or die. This would lead to totally different quantitative results compared to the original composition taken from the donor (and present in the tissue product).

Culture samples should ideally be processed within 6 h of sampling. Results are likely to become unreliable if sampling and/or processing of the samples is delayed.

3. Contamination from Retrieval or the Microbiology Laboratory

The only way to identify the presence and source of contamination is by taking samples for culture throughout the entire process of tissue banking: during retrieval, environmental monitoring and glove prints of the retrieval team, and including negative controls during sampling by the TE (together with samples taken for culture from the final product after decontamination and neutralisation of the antibiotics or other disinfectants are used). Such assessment of results can allow the source of contamination to be identified which in turn would allow corrective action to be done to avoid further contamination in the future and potentially save a lot of tissue. It is recommended that the importance of avoidance of both false negative and false positive results is clearly discussed with all staff i.e. the retrieval team, TE staff and the microbiology laboratory staff to ensure the safety of tissue products while avoiding the potentially unnecessary discard of such products.

d. Environmental monitoring: it is important that regular environmental monitoring is carried out of the operating theatre, clean room (with Laminar Air Flow cabinet), and the microbiology laboratory, looking at the quality of the environmental air, sterility of the surface and the cleaning protocols used according to documented maintenance protocol. Settle plates and contact plates taken both during processing and at rest, together with glove prints from the operators taken at the end of the process are required. For the purposes of environmental monitoring quantification and global recognition of colonies would be sufficient, without the need for full species identification of contaminants present.

Samples taken should be cultured on rich media. Robust general media such as BHI, TSB and Thioglycollate are not sufficient. The aim of taking negative controls and tissue sample cultures is to identify whether any contamination has taken place. It is important for the TE to be aware of all forms of contamination present during tissue processing. The identification of some contaminants does not necessarily mean the tissue product needs to be discarded; such decisions will depend on the type of micro-organism identified and at what point in the process i.e. pre- or post-decontamination. Many skin and airway bacteria are fastidious or need specific enriched media like blood culture media (BACTEC and BacTalert and others) or supplemented media with additives, such as Fastidious organisms supplement and longer incubation time (1–2 weeks).

It is essential that tissue processing is carried out using an aseptic technique and ensuring that environment used is also sterile and is continually monitored.

e. Results and Responsibility

When releasing a tissue product for clinical use it is important that the results of all samples taken throughout the entire process of tissue retrieval and processing are reviewed to allow a final decision on the clinical suitability (or otherwise) of the

product. It is also good practice to follow up tissue recipients to ensure there were no post-operative complications that may have been due to contamination of the tissue product.

11.2.5 Microbiology Laboratory

The microbiology laboratory remains one of the biggest but unquantified risks for the introduction of contamination of samples taken during tissue processing. However contamination introduced by the microbiology laboratory is only of the culture samples sent for processing and not of the tissue product itself. However, such sample contamination will lead to the unnecessary discard of tissue products which may well have been safe for clinical use.

a. Area: The microbiology laboratory is an open and dynamic area for potential contamination -taps and sink; air streams can all produce aerosols thereby spreading bacteria (e.g. streptococci) while working on open benches with numerous culture samples and material from multiple patients. In a microbiology laboratory there is usually no air control and the work stream is not controlled (unlike in a clean room). Such laboratories usually contain several different types of equipment: refrigerator, culture machines, stoves, centrifuges, water baths, cupboards for storage, all of which are a source of dust and environmental contamination of the surroundings (surfaces and air). Staff working in a microbiology laboratory do not use protective clothing that covers up all skin areas i.e. they do not wear whole body suits, gloves, whole face masks and hats. Entry to the laboratory is not normally controlled so that air-streams are generated as doors are opened and closed. Biosafety requirements are such, that all work with open cultures of micro-organisms, must be performed in a containment cabinet Class I-II [101]. There is the risk of cross-contamination in such cabinets. To identify the source of such contaminants is very difficult. The rate of false positive results can be very high in a microbiology laboratory (personal experience of 35 years, many times proven contaminants but never systematically recorded). The many incidents of transmission of pathogens infecting laboratory staff [102] proves this, because these infections are recognisable. The results from the Quality Rounds carried out by the author and other colleagues also support these findings [21].
b. Risk: Of all the potential sources of contamination of tissue products mainly by bacteria that are normally present on skin (gram positives), the microbiology laboratory is a particularly high risk area. Therefore the laboratory should carry out culturing of the samples from tissue products in a “clean room” [67] environment.

What are the sources for contamination in microbiology laboratories:

1. The biggest source of contamination is the skin of the staff working in the laboratory.
2. The second source of contamination is the respiratory tract from the laboratory staff sneezing, coughing and talking while working in the laboratory. Bacteria can be transmitted even if face masks are used (which are often not used in microbiology laboratories). The respiratory tract is a frequent source of Streptococci which can be carried easily in the air-streams in the laboratory.
3. A third source of contamination is the gastro-intestinal tract: microbiology laboratories often process faecal material. Micro-organisms such as Clostridium, Bacteroïdes, E.coli or Enterococci only originate from the intestines.
4. And finally the environment is a potential source for incidental contaminations.

Risk and source of contamination in the microbiology laboratory

sources for contamination	Skin	Respiratory/ gastro-intestinal tracts	Air	Envir. inside	Envir Outs	Working sterile	Process controls	Risks
Microbiology laboratory	++	++	++ +	–	+++	Dirty	Not tested	Open area many people minor sterility Not in LAF

+ = risk ++ = frequent risk +++ = risk at all times ± = incidental cause − = no risk

Working with potential sterile material should be done at least in a Laminar Air Flow cabinet and if possible in a clean room with controlled sterile air supply If not: false positive results are possible	Much incidental contamination of "samples" occurs in the microbiology laboratory because of the non-sterile conditions and the difficulty in identifying the potential source of contamination unless environmental monitoring is carried out; most microbiological laboratories do not carry out environmental monitoring during open bench work

c. Principles of testing:

1. For the identification of commensal flora and environmental flora it is necessary to use enriched or supplemented (with fastidious organisms' supplement or blood) media and media that support fungal growth for a prolonged period of incubation at different temperatures. Thioglycollate and Brain Heart Infusion or Trypticase Soy Broth are not sufficient on their own, but with enrichment (with blood and other supplements) they would be able to identify most types of contaminants, including anaerobes. It is important to ensure that all contamination that may be present is identified. Such enriched media would also allow the identification of spore formers (all present in commensal flora of the body aerobe and anaerobe) and fungi that are able to grow slowly in tissue products, but may not be easy to identify

when using general media. Prolonged incubation (up to 2 weeks) is necessary to ensure that slow growing bacteria (for example Proprionibacterium and Corynebacterium) and fungi are identified.

2. Further to fully assess the clinical significance as well as the potential source of a contaminant it is necessary to identify the micro-organism up to species level. Furthermore, individuals may also carry transient bacteria from the environment that survive only short periods (minutes till days) on their skin because people are in touch with their environment all the time. These bacteria originate from plants or animals or water or other human beings, especially on their hands and skin. Further sources of contaminants are the mouth, nose and saliva. Plants, animals, water and soil all have their own bacteria which are recognisable. If individuals have regular contact with animals or gardens, bacteria derived from these sources can be identified from their skin and airways (and gastro-intestinal tract). Further, in the microbiology laboratory such bacteria are identified everyday with laboratory technicians having contact with these bacteria and transmitting them to the media and agar plates they work with. Only by optimal culture conditions would it be possible to identify the source of contamination and how to reduce the risk of further similar contamination (refer to Table 11.4).

Conclusions for the microbiology laboratory:

a. Tissue is a high risk material the samples from which need to be processed and cultured in strict sterile conditions. Such work should ideally be carried out in a Laminar Air Flow cabinet as a minimum.
b. Identification of micro-organisms to species level is important.
c. Testing the right samples is important (see Table 11.3).

3. The Tissue sample taken for culture must be representative of the donor and donor condition at the pre- decontamination stage and of the final product at the post-decontamination stage. In this way the safety of the product can be guaranteed. Undiagnosed bacteraemia occurs frequently in donors and can only be identified by taking blood cultures prior to starting tissue retrieval. When taking such blood cultures, the blood sample should be injected straight into a blood culture bottle. Bacteraemia leads to donor contamination that cannot be identified by swabbing or by taking a tissue biopsy from the donor's organs/tissues. Other samples should give enough material, fluid or tissue for culture on agar plates and broth and if possible these tissue samples also should be cultured straight away and in a closed system (at the retrieval stage or TE) or sent to the microbiology laboratory for handling it in a LAF-cabinet within clean conditions without delay. Delaying processing of the samples beyond 6 h is not recommended due to changes in the contents of the samples leading to the risk of both false positive and false negative results. Blood culture medium is an enriched medium and therefore can neutralize the toxic effects of the tissue as

Table 11.4 Reference list of low and high pathogenic micro-organisms and their origin

	Genus	Species	Virulence	Comments/origin
O2	Enterobacteriaceae Fermenting Gram Neg Rods	Escherichia coli	High	Intestinal commensal
		Klebsiella pneumoniae, oxytoca, sp	High	Carrier gi/airway environment
		Enterobacter aerogenus, cloacae, sp	High	gi.commensal
		Serratia	High	Transient gi
		Citrobacter freundii, sp	High	Gi commensal
		Pantoea agglomerans	High	Plants
		Aeromonas sobria/hydrophila/sp	High	Moist env./potable water
		Plesiomonas shigelloides	High	Seefood/tropic
		Proteus vulgaris/mirabilis/sp	High	Gi commensal
		Haffnia albei	High	Gi Carrier, aquatic environment
		Morganella morganii	High	Gi commensal
		Salmonella sp	High	Animal
		S.typhi/paratyphi	High	Human carrier gi
O2	Non-fermenting Gram Negative Rods	Pseudomonas aeruginosa, fluorescens, sp	High	Moist env. Soil plants fruits
			high, dep	
		Stenotrophomonas sp (= Xanthomonas sp)	High, dep	Water env. soil plants
		Sphingomonas sp	High, dep	Water syst.hosp., decomposer soil
		Brevundimonas sp	High, dep	Water env.
		Massiliae timonae	Low, dep	Theatre, water, soil
		Burkholderia	High, dep	Hosp.env.water soil, plants, fruit, veget
O2	Other Gram Negative Rods	Haemophilus influenza, sp	High	Carrier airway
		Cardiobacterium	High	Airway
		Acinetobacter sp, non-fermmenter	Depends	Skin, Hospitals, env.
		Bordetella	High	Carrier airway
O2	Gram Negative Cocci	Neisseriae sp	Depends	Airway
		Neisseria meningitidis	High	Carrier nasopharynx
		Neisseria gonorrhoea	High	Carrier gen/oropharynx
		Veilonella sp	Low	Airway/gi
		Roseomonas sp	Low	Skin commensal

(continued)

Table 11.4 (continued)

	Genus	Species	Virulence	Comments/origin
O2	Staphylococcus Gram Pos Cocci	Staphylococcus aureus	High	Carrier(33%) nose human
		Staphylococcus epidermidis, capitis, sp	Low	Skin human
		Staphylococcus schleiferi	Depends	Carrier, probably animal
		Staphylococcus lugdunensis	High	Carrier perineal, skin
		Micrococcus	Low	Air, transient skin
	Streptococcus (Str.) Gram Pos Cocci	Streptococcus ABCDEFG Haemolytic	High	Carrier human all mucosal surfaces
		Str.pneumoniae, pneumococcus	High	Carrier airway human
		Str.viridans, indifferent, Str. species	Depends	Commensal airway, gastrointestinal
		Enterococcus	High	Intestinal commensal
		Granulicatella (Nutrient Deficient Str)	Low, dep	Mouth gi. genital.
	Other Gram Pos Cocci	Leuconostoc	Low	Env. pla frui veget
		Aerococcus	Low	Env./human skin
		Lactococcus	Low	Env. food vegetation
		Gemella	Low	Commensal oropharynx
		Pediococcus	Low	Env. plant fruit beer
		Rhotia (= stomatococcus)	Low	Carrier mouth human
		Kocuria rhizophila	Low	Children skin dust
	Corynebacterium Gram Pos Rods	Corynebacterium species diphtheroid rods	Low	Skin commensal
		Lactobacillus sp	Low	Comm.mouth gi genital
	Gram Pos Sporeformer	Bacillus species (and spores)	Low	Air, saprophyte.env.
		Paenibacillus sp (and spores)	Low	Saprophyte.env.
AN	Gram Pos Sporeformer	Clostridium perfringens/difficile/septicum	High	Gastrointestinal comm./carrier
		Clostridium sp	Depends	Commensal gi
	Gram Pos Rods	Eubacterium	Low	Commensal gi
		Propionibacterium acnes, sp	Low	Commensal skin
		Bifidobacterium sp	Low	Commensal gi
		Actinomyces	Depends	Soil/oral/intestinal
AN	Gram Neg Rods	Bacteroides fragilis, sp	High	Commensal gi
		Fusobacterium sp	Low	Commensal gi

(continued)

Table 11.4 (continued)

	Genus	Species	Virulence	Comments/origin
AN	Gram Pos Cocci	Peptostreptococcus sp	Low	Comm. skin air uro gi
		Peptococcus sp	Low	Comm. skin air uro gi
	FUNGI	Yeast sp	High	Carrier, fruit, veget
		Candida albicans, sp	High	Carrier, fruit, veget
	Fungi plus spores	Aspergillus fumigatus, sp	High	Environmental
		Fungi sp	High	Environmental
		Acremonium/Vertallium	High	Soil/Wet/Work
Important descriptions				
		Spore forming	Depends	
		Aerobic Gram Pos Rod&Coc	Low	
		Aerobic Gram Neg Rod	High	
		Non Fermenting Gram Neg Rod	Depends	Moist/water environment
		Aerobic Gram Pos &Neg Coc	Low	
		Anaerobic Gram Pos Rod&Coc	Low	Not clostridium
		Anaerobic Gram Neg Rod&Coc	Depends	

The bacteriology Reference List describes/classifies the pathogenic risk (degree of virulence) of microorganisms and can be a guide for making decisions in Tissue Banks
*carrier = only with human for short times; O2= aerobic; AN= anaerobic; comm.= commensal; syst.= system; hosp.= hospital; dep = depends: discuss with the medical microbiologist regarding donor and recipient; gi = gastro-intestinal; env. = environment; nasoph = nasopharynx; air = airway; pla = plants; frui = fruits; veget = vegetation; sap = saprophytes; sp= species; uro = urogenital; gen = genital [103, 104]

well as any antibiotic residues to allow better identification of any contaminants that may be present (this is not yet validated). The best way to identify the presence of any contaminants is to culture the filtered transport fluid in blood culture medium as well as another medium (broth) or agar plate including a medium that supports fungal growth using different temperatures to maximise the identification of all contaminants (environmental fungi need to be incubated at 20 °C). Similar comments are relevant for the processing of culture samples taken post-decontamination of the final product. Both methods, antibiotic neutralisation and rinsing, must be validated before use. Final product culture samples must also be cultured on enriched media to ensure that all contaminants that may be present are identified.

4. Bacterial bioburden: as previously discussed there is usually a delay between taking samples for culture and processing the samples. This means that the

bioburden at the point of culturing will be different to that at the point of sampling. Bacteria will die (usually because the optimal temperature for bacterial growth is 35C rather than 4–8C or even room temperatures) but other bacteria will grow at these lower temperatures. Trying to estimate the bioburden is therefore unreliable. Estimating the bioburden would only be informative if the tissue sample from the donor is placed immediately on to agar media, at the point of sampling. In all other situations the bioburden estimates will be unreliable. As such the bioburden is not as important as the identity (and therefore pathogenicity) of any contaminants that are present.

d. Environmental

Environmental monitoring is not normally done in a Microbiology Laboratory. However, microbiology laboratories carry out many activities that need control of air movements and staff at all times during the handling of human material. For patient cultures the incidental contamination is not problematic (patient samples can be repeated if necessary) but for tissue processing it is of utmost importance.

The Microbiology Laboratory should process tissue sample cultures in a clean room and preferably also in a Laminar Air Flow cabinet if checking for sterility is important. On an open bench this is not possible, and the contamination rate is very high. Regular environmental monitoring of the room, environmental air and surfaces is important. Work should be carried out in a very disciplined manner, avoiding processing samples from different donors at the same time to avoid cross-contamination. Before processing samples all surfaces should be cleaned and during sample processing settle plates and contact plates should be taken in a similar process followed by TEs. In the eventuality of positive environmental monitoring results, comparison of these results with the cultures from the donor material would assess whether the contamination was introduced by the microbiology laboratory or was of donor origin. Sending a repeat sample to the microbiology laboratory would help prove the source of contamination (reason why the TE must keep at least a biopsy or fluid in reserve until the last results of cultures become available).

11.3 Specific Items

1. Viability: Long-term durability of cardiovascular tissue is not dependent upon presence of viable cells [105–107]. Low dose antibiotics, for 24 h at 37C [52, 108] seems not to influence viability. In general, the allograft cell survival in the recipient is poor. The performance of cryopreserved homografts is mainly a result of the preservation of the collagenous skeleton and the compounds of the

extracellular matrix. Viable endothelial cells evoke a cellular and humoral immune response and donor endothelial cells do not regenerate [105–107]. There is an immune response against viable human cardiac valve allografts which is not shown with decellularization of human donor aortic and pulmonary valves. Further testing should be done for the rate of sterility caused by the decellurising of tissue. HBD have 92% and NHBD 66% viable cells [108, 109].

2. Mycobacteria: there are three considerations with respect to the importance of Mycobacteria tuberculosis:

a. The incidence of cardiac tuberculosis is very low, especially in Western countries. Extra-pulmonary tuberculosis is <40% of the total, and cardiovascular tuberculosis averages only 2% of these 40%. Cardiovascular tuberculosis is always secondary to a pulmonary focus.
b. Sampling error in tuberculosis is high, especially in the cardiac diagnosis: cardiac biopsies are positive in <10%, and cardiac/pericardial fluid in 20% in donors with cardiac tuberculosis.
c. there is always inspection and investigation macroscopically and microscopically (if needed) by pathologist/banking staff. The best result to rule out tuberculosis is given by macroscopic inspection. For tuberculosis to be a risk there must be a nodule or a focus.

Conclusion is that tuberculosis culture should not be done unless the history is suspicious or any contact with tuberculosis was discovered in the donor. If suspect then it needs modern technics like MGIT cultures with longer durations (even months) [110–112] to be successful.

3. Spore Formers: There is a significant risk of contamination by spore forming bacteria in tissue banking. Mostly Clostridium spores but periodically also by Bacillus and fungal spores. Infections caused by spore-infected tissue may be serious [113] since there are no antibiotics that are effective against spores or against dormant bacteria of any type. Spores are universally present in the environment, especially fungal spores and Bacillus spores, surviving in any environment until they encounter favourable conditions in which they can germinate. To identify the presence of spores, samples need to be cultured in proper media under proper conditions for at least 14 days. Clean areas with sterile air flow will have no Bacillus or fungi present. On the other hand, such micro-organisms will be identified in dusty and non-sterile places or in certain seasons (especially for fungi). Clostridium will only be seen if the area contains decaying material or is contaminated with gastrointestinal/faecal material.

   There are only two points during tissue processing where this is likely: in the mortuary or in the operating theatre during and after retrieval if there was a perforation of the intestines, and in the microbiology laboratory where faecal material is tested on “open benches”.

4. Antibiotics: Antibiotics interfere with processes such as cell wall building and protein synthesis or DNA-generation that are only present if bacteria are growing. If growth slows down, the effect of antibiotics will be less and depending on the temperature even reach zero effect in when bacteria are not growing at 0–4°C. Decreasing temperatures will kill bacteria, because the optimal temperature for human bacteria is body-temperatures. Most environmental bacteria that need to live at lower temperatures don't survive on skin (temperature is too high) but also don't survive at 0–4 °C (temperature too low), except for spores. Antibiotic molecules have a positive or negative charge which allows them to bind to other charged cells or chemicals and so can neutralize them. Possibly the antibiotic acts as a charged chemical agent (especially at the huge doses most TE use for tissue decontamination, often 20–30 times or higher than the dose used in clinical practice) together with the low temperature may cause ischaemia and toxaemia for cells including bacterial cells. There is only one antibiotic that has been proven to work at 0–4°C: polymixin.

There are two classes of antibiotics: those that work well if the concentration is above the Minimal Inhibitory Concentration (MIC) of the bacteria and increasing the concentration of antibiotics in this class does not improve their effectiveness (all beta lactam i.e. penicillins and cephalosporins). The other class of antibiotics do work better at higher doses up to a certain maximum effective dose, often 8 × above the MIC of the bacteria; these antibiotics are often toxic in general, but even more so in high doses (e.g. Aminoglycosides) [114]. There is good evidence from clinical work that antibiotics used at the "low" clinical doses are effective. It is unclear why Tissue Banks do not use similar doses? [65, 111]. The antibiotic doses used in TEs would be toxic for humans [115]. There is also evidence from previous studies carried out in Rotterdam that demonstrated that antibiotics have an optimal effect at 37 °C with an incubation period of 6 h for all combinations of antibiotics tested. The same results were achieved when cardiac valve tissue contaminated with Pseudomonas and Coagulase Negative Staphylococci was decontaminated within 6 h at 37 °C. On the other hand, there was a residual low level contamination still present if decontamination with antibiotics was carried out at 4 °C.

Why do we see effects on bacteria at 4°C?

a. The low temperature will kill bacteria (human bacteria are acclimatised to 30–35 °C, all other temperatures will slow them down or kill them).
b. In solutions with high antibiotic concentration there are chemical and physical mechanisms working (a lot of antibiotics have a negative or strong positive charge and will bind to other molecules with chemical effects) which will have detrimental effects on bacteria.
c. It is also worth considering the toxic effects of a cold ischaemic episode on tissue or cells. Decontamination at 30–35 °C will not harm tissue and cells, but 0–4°C will do so, disturbing their integrity. A further reason to carry out tissue decontamination at the usual human body temperature!

5. USE of Antibiotic concentrations in Tissue Banks: general concentrations used by TEs are >20 × or even higher when compared to clinical doses.

Vancomycin MIC 0,12-4,0 mg/l. Concentration used in patients 15–20 mg/l. Concentration used in TEs 60–600 > mg/l.

Gentamicin MIC 0,03–0.5 mg/l. Concentration used in patients 10–15 mg/l. Concentration used in TEs 20- >300 mg/l.

Ciprofloxacin MIC 0.06–2 mg/l. Concentration used in patients 7 mg/l. Concentration used in TEs 50- >200 mg/l.

The above information is sourced from the European Quality Round 2016 [21] and Kohneman [116].

6. What antibiotics should be used in decontamination protocols? To cover all possible contaminants that may be present an antibiotic cocktail is needed with a spectrum against penicillin resistant gram positive bacteria (Coagulase Negative Staphylococci), general gram negatives including environmental strains (Pseudomonas like) which can only be covered with a combination of two antibiotics, and an antibiotic against anaerobes plus one against fungi and yeasts. There are many combinations possible but as was demonstrated in the Quality Round carried out in 2016 many Tissue Banks use combinations which do not give adequate cover against anaerobes or fungi or environmental gram-negative micro-organisms and even not enough coverage against skin staphylococci resulting in failure to decontaminate properly.
7. Other methods of sterilizing tissue: superoxide [117], super critical carbon oxide and other processes are not validated yet, but proof of principle has been given for superoxide. Sodium hypochlorite as a chemical is with some limited success used in TEs but not as the sole disinfectant in treating the tissue [40].

11.4 Future

The ideal would be that as long as a 100% sterility of the process is not possible:

A donor is transferred to an operating theatre and tissue is retrieved and explanted within 6 h of death. Otherwise the donor must be cooled within 6 h of death and retrieval carried out within the next 66 h (i.e. within 72 h of death).

At retrieval, before starting the process a blood culture is taken from the donor and immediately injected into closed blood culture bottles). From all retrieved tissues a biopsy is taken and placed straight on CO_2 ice in a closed styrofoam box and transported to the Tissue Bank. Further environmental monitoring settle plates and fingerprints from the gloves of the retrieval staff are taken on a plate and also sent to the TE.

The TE stores all samples in a separate refrigerator (cleaned) and handles everything in a clean room within a Laminar Air Flow cabinet. In the cabinet the tissue is tested for the presence of antibiotic residues and the transport fluid as well

as the tissue biopsy is placed in a blood culture medium that neutralizes all toxic substances and antibiotics within 1–2 h. After that the tissue is cleaned, dissected, biopsies are taken and placed on agar and in enriched media for pre-decontamination testing. The tissue is then decontaminated in an antibiotic cocktail for 6 h at 35 °C (or different if that is validated) and after that again in neutralizing medium for 1–2 h, a further biopsy is taken and cultured in enriched media, and the tissue product is stored according to protocol.

All cultures are placed in an incubator for 1–2 weeks at 35 °C and some agar plates at 20 °C for fungal growth. All environmental monitoring samples are collected and placed in the incubator.

All environmental monitoring results are compared with the donor results. If all cultures are negative after 1–2 weeks the tissue is safe to release for clinical use.

If the cultures are positive, those are sent to the microbiology laboratory together with a negative control culture (as a check for contamination that may be introduced by the microbiology laboratory. Any contaminants present must be identified to species level. Once the results are available discussion with the microbiologist and the retrieval staff will allow a decision to be taken as to whether or not intervention is required and whether the tissue product is safe for clinical use or not.

Ensure a Clean Retrieval and a Clean Microbiology Laboratory and carry out environmental monitoring
Check Tissue for Antibiotic Residues and Toxicity in Donor and after Decontamination
Use high Sensitive Culture Media and only send Positive samples to the Microbiology Laboratory for further identification
Keep Sampling Error Small by using as much Fluid as possible or more Biopsies, and include negative controls

The Brussels Cardiovascular Tissue Bank (European Homograft Bank EHB) has validated the sterility testing of tissue samples together with transport in preservation fluid. The tissue is homogenized in a disposable system (Gentle Macs dissociator) and injected in BD BACTEC Fx bloodculture bottles (neutralizing toxic and antibiotic components in the donated tissue). These are incubated and continuously registrated in a Bactec system for potential growth of any contaminants for a period of 2 weeks. This proces represents a closed automated system without external manipulation in the Tissue Bank or in the clinical microbiology laboratory [118].

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Establishing a Quality System in a Tissue Establishment

12

Alex Aquilina and Fewzi Teskrat

"*Quality is everyone's responsibility*"
William Edwards Deming

Abstract

The core concepts of quality are applicable in all manufacturing activities. In the field of tissues and cells, this requires a high degree of knowledge and expertise, not only of quality systems but also of the actual processes since the risks and their mitigation can be quite complicated. The implementation of a quality system is not a 'one size fits all' process. Very often people are familiar with what should be done, but less so with how it is done. The starting point is understanding the concepts of quality and even the historical perspective. Quality should not be seen as a burden but as a tool which all the members of the tissue establishment are responsible for. The key constituents of a quality system as applied to a tissue establishment are provided in this chapter. Quality has to be considered in its context, also taking into account the various other activities which may have an impact on the product or service. Activities and services such as Biovigilance, IT, Data Protection, and customer expectations may all have a reciprocal impact on the Quality system. Another context which may also be significant is the cost of quality or the hidden costs of the lack of it. Ultimately quality is 'getting it right' first time.

A. Aquilina (✉)
National Blood Transfusion Service, Tal-Pietà, Malta
e-mail: alex.aquilina@gov.mt

F. Teskrat
ART Clinic, Mater Dei Hospital, Msida, Malta

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12.1 Background

Scope: The aim of this chapter is not to provide a comprehensive detailed account of Quality assurance/management/control within a Tissue establishment as there are various excellent references which deal with the topic. It is rather a step wise approach to the implementation of essential components for a quality system as relevant to a Tissue establishment.

Tissue banking professionals should understand that they must be advocates of quality because their establishment invariably offers multiple services and has customers and stakeholders at many levels; from donors and donor families to tissue banking co-workers and colleagues, as well as suppliers of equipment and reagents, and transplant professionals and tissue recipients. A tissue establishment today can provide services that are focused and limited (i.e., tissue recovery) or it can be a very complex operation that performs work encompassing an array of functions (i.e., obtaining donation consent/authorization; donor screening; infectious disease testing; tissue recovery; tissue processing and testing, storage and distribution) and this may involve various types of cells and/or tissue.

12.2 The History of Quality Management

The history of quality management can be traced all the way back to The Middle Ages. Work completed by journeymen and apprentices were evaluated and inspected by the skilled worker to ensure that quality standards were met in all aspects of the finished product, ensuring satisfaction of the buyer. And while the history of quality management has gone through a number of changes since that time, the end goal is still the same.

It was during the 1920s when quality management systems, as we know them today, started to surface. While the focus of quality management was still on the end product, it was the first time that statistical theory was applied to product quality control.

Product quality control was determined through inspections. This involved measuring, examining and testing the products, processes and services against specific requirements to ensure that each element adhered to set standards and guidelines.

This algorithm worked for quite some time. Over time, however, businesses began to grow and expand. More and more products were manufactured and companies started to experience difficulties in following through with quality control standards. It became evident that there was a great need for change and development.

Change and development were brought forth during the 1940s by industry leaders and experts like Deming, Dodge, Juran and Roming. This would be the beginning of Total Quality Management as we know it today.

At the time inspections were now carried out by production personnel. They were responsible for inspections during specific production intervals. This would change the focus from simply inspecting the end product to actually preventing end product problems through early detection on the production line.

It was also during the late 1940s that Japan caught wind of Total Quality Management (TQM). At that time, Japanese products were considered poor quality imitations. Hearing about the success of quality management in the west, Japan employed the assistance of quality management experts like Deming and Juran. Little did the Western culture know at that time, Japan would soon push the envelope and set new standards in TQM.

During the first international quality management conference in 1969, Feigenbaum would first use the phrase Total Quality Management. Feigenbaum, however, would not meet the depth of understanding of the term that Japanese attendee and speaker, Ishikawa would. Ishikawa would indicate during the conference that TQM should apply to all employees within the organization—from the workers to the head management [1].

12.3 Why a Quality Management System

Quality management field includes management methods and analytical techniques, whose subject is quality management. Concepts of quality in organizations are based, in principle, either on norms and standards (international, national or corporate) or on the concept of TQM (Total Quality Management).

The ISO 9000:2015 and ISO 9001:2015 standards are based on seven quality management principles that senior management can apply for organizational improvement:

- Customer focus.
- Leadership.
- Engagement of people.
- Process approach.
- Improvement.
- Evidence-based decision-making.
- Relationship management.

12.4 Why Quality?

The introduction of the 4th Edition of the Council of Europe's Guide to Quality and Safety of Tissues and Cells for transplantation when referring to tissues and cells states: 'However, as with all material of human origin, they carry risks of disease transmission that must be controlled by application of scrupulous donor selection

and testing criteria, and also by ensuring that **comprehensive quality systems are in place**' [2].

In specific terms, **Quality** and **Safety** are intrinsically interlinked.

12.5 What is Quality?

This may sound as an obvious answer, however there are many definitions of quality, and it very much depends on the context and perspective.

Quality is a measure of excellence or a state of being free from defects, deficiencies, and significant variations.

The term quality is used in all areas of the organization, manufacturing, engineering, business and the services sector. There are numerous definitions of quality:

- Business dictionary defines quality as "a measure of excellence or a state of being free from defects, deficiencies, and significant variation".
- Joseph M. Juran defines quality as "fitness for purpose" [3].
- Philip B. Crosby defines quality as "compliance to requirements" [4].
- Armand Vallin Feigenbaum defines quality as follows: "The quality of the product is the sum of all its production design and technical characteristics that determine the level at which the product will fill the customer's expectations".
- ISO 9001 defines quality as "a degree to which a set of inherent characteristics fulfils requirements. While according to the standard, requirements are expected (e.g. customers) or mandatory (e.g. according to standards)" [5].

Quality can also be defined from different perspectives:

- **From the customer point of view**: quality means fitness for use and meeting customer satisfaction.
- **From the process point of view**: quality means conformance with the process design, standards, and specifications.
- **From the product point of view**: quality means the degree of excellence at an acceptable price.
- **From the cost point of view**: quality means the best combination of costs and features.

In the context of tissues and cells all these perspectives may be to some or significant extent relevant: definitely the customer (patient, surgeon, hospital) needs to be satisfied, the process and product must conform to accepted standards, and the cost should be controlled, reasonable and sustainable.

Quality of tissues and cells is achieved through compliance with requirements at four levels [6]:

a. The legal framework that provides the overall context in which the donation, procurement, testing, processing, storage, distribution and import/export activities for tissues and cells are performed;
b. The QMS, which is a tool to ensure that tissues and cells consistently comply with technical and legal requirements;
c. The technical requirements specific to each type of tissue or cell, which ensure quality, safety and efficacy (as detailed in Part B of the EDQM 'Guide to the Quality of Tissue and Cells for Human Application' [7]);
d. The authorisations in place for the specific activities, from specific competent authorities.

12.6 Examples of Methods to Be Used in Quality

Deming Cycle (PDCA Cycle)

Deming Cycle (PDCA Cycle) is a method of a gradual improvement of the quality of the products, services, processes, applications, data, going through repeated implementation of four basic activities: plan, do, check, act.

- P—Plan—planning proposed improvement
- D—Do—plan implementation
- C—Check—check the result from the original plan
- A—Act—plan adjustments and the actual implementation based on the verification, and space implementation of the improvements in practice.

DMAIC—Improvement Cycle [8]

DMAIC—Improvement Cycle is a universally applicable method of gradual improvement that is an integral part of the method Six Sigma. It is used for any improvement—for example, quality of products, services, processes, applications, data. Each stage in the cycle helps achieve a real improvement. It is an improved PDCA cycle.

- Improvement cycle phases are:
- **D (Define)**—objectives are defined, it is described subject and improvement objectives (product, service, process, data, etc.)
- **M (Measure**)—measurement of initial conditions in term of the principle "what I don't measure, that I don't control"
- **A (Analyse)**—analysis of the facts, causes of deficiencies
- **I (Improve)**—key phase of the whole cycle, in which the improvement is based on analysed and measured facts

- **C (Control)**—improved deficiency should be introduced—to manage and maintain the improvements in life.

European Foundation for Quality Management (EFQM) Excellence Model [9]

EFQM Model was developed by the EFQM Foundation as a framework for the implementation of quality management methods in the organization. These are:

- Practical tool for self-evaluation
- Guidelines for improving
- Framework for the management system of the organization
- Way of the terminology unification.

12.7 Available Standards and International Legal Documents

This is a non-comprehensive list of documents available with relevance to Quality issues in Tissue and Cell banking

- The Int**ernational Organization for Standardization (ISO)** requirements, as addressed in the ISO 9000 Quality Management System family of standards. ISO standards have been developed to assist organisations of all types and sizes to implement and operate effective QMS. **ISO 9001:2015** standard is one such set of requirements (for other examples, see Quality Certification) that defines and outlines all the typical policies, processes, documented procedures and records that are needed for a successful QMS, and can be used and tailored for the needs of any organization [10].

(*The concept of risk occur occurs forty-eight times in ISO 9001:2015, compared with only three times in ISO 9001:2008 and the quality system is no longer a stand-alone process but should be linked with the strategy of the Establishment).*

- **Directive 2004/23/EC** and—which sets the standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells—and its associated technical directives **(2006/17/EC and 2006/86/EC)** provide key elements to be included in a tissue establishment QMS; these requirements are legally binding in EU member states [11–16].
- **The EU Guidelines for Good Manufacturing Practices (GMP)** [1] provide specific guidance for the preparation of medicinal products. However, much of their content is also relevant for the procurement, processing, storage and distribution of tissues and cells. Wherever (in the EU) products containing tissues or cells are classified as advanced therapy medicinal products (ATMP), then the full requirements of GMP must be applied [17].
- **European Directorate for Quality of Medicine (EDQM)** Guide to the quality and safety of Tissue and Cells for human application 4th Edition [18].
- **Good Tissue Practices** for European tissue banks were developed by the EU-funded project EuroGTP, which aimed to agree harmonised practices and techniques across Europe and to increase the know-how and level of competence of tissue establishment personnel. Much of the guidance developed in that project has been incorporated in the chapters of this Guide [19].
- **FACT–JACIE** International Standards for cellular therapy product collection, processing and administration, published by the Foundation for the Accreditation of Cellular Therapy and the Joint Accreditation Committee of the International Society for Cellular Therapy and the European Society for Blood and Marrow Transplantation [20].
- **NetCord-FACT** International Standards for cord blood collection, processing and release for administration [21].
- **Pharmaceutical Inspection Co-operation Scheme (PIC/s)**: This scheme has a number of relevant aide memoirs and documents relevant to tissue establishments [22].

12.8 Setting Up a Quality System

When a quality system needs to be developed or improved a number of questions crop up:

1. What are the topics that need to be covered?
2. What is the starting point, i.e. what comes first and what follows?
3. What resources are required?

It is also important to identify the differences between **Quality Control**, **Quality Assurance** and **Quality Management**, and how the three interact to establish the quality of the final product.

Quality assurance (QA) is a broad concept that focuses on the entire quality system including suppliers and ultimate consumers of the product or service. It includes all activities designed to produce products and services of appropriate quality.

Quality control (QC) has a narrower focus than quality assurance. Quality control focuses on the process of producing the product or service with the intent of eliminating problems that might result in defects. Quality control refers to those activities, such as verification steps, sampling and testing, which are used to ensure that materials, processes and the final product meet the required specifications. Internal quality control in a testing laboratory includes use of positive, weakly positive or negative control samples as appropriate. External quality assessment (sometimes also called 'proficiency testing') involves analysis of unknown samples and evaluation of the results by a third party. Quality control of critical functions can be undertaken using audit techniques that include a sampling plan.

Quality management (QM) is the totality of functions involved in the determination and achievement of quality (includes quality assurance and quality control).

Quality control	Quality assurance
Focused on product	Focused on process
Reactive	Proactive
Line function	Staff function
Finds defect	Prevents defect
Testing	Quality audits

Dr. Joseph Juran—"The Juran Trilogy" [23]

Quality planning

- Determine who the customers are,
- Determine the needs of the customers,
- Develop product features that respond to customer's needs,
- Develop processes that are able to produce those product features.

Quality control

- Evaluate actual Quality Performance,
- Compare actual performance to quality goals,
- Act on the differences,
- Documents defining how to control products or services that do not meet requirements.

Quality improvement

- Establish the infrastructure needed to secure annual quality improvement,
- Identify specific needs for improvement—the improvement projects,
- Provide the resources, motivation, and training to teams.

12.9 Risk Management

Before answering the questions referred to in Sect. 12.8 it is best to consider the topic of **risk management**. Again, there are many documents which refer to risk management [24–28] and perhaps the assessment of risk will impact especially the answer to questions 2 What is the starting point, i.e. what comes first and what follows? And 3 What resources are required. In the context of tissues and cells the risks relate to multiple factors, such as which tissue/cells are being considered and their procurement, the number and competence of staff, the available premises, procurement of the material, transport and storage, costs and sustainability, outsourcing and many more aspects including:

1. the fact that some tissues are procured from dead donors (skin, tendons heart valves) whilst others from live ones (stem cells, cord blood and bone)
2. many third-party agreements with hospitals, suppliers, other institutions etc.

Some tissues can be processed and sterilised (bone) whilst others have to be viable (stem cells, cord blood) and therefore not amenable to end sterilisation steps. Getting risk management right greatly facilitates the implementation of the quality system in a cost-effective manner. Again, it is not the scope of this chapter to delve into the details of Quality risk management, and the implementation of a system can be found in the excellent documents referenced above.

12.10 Applying a Quality System to Your Activities

"The QMS needs to be specific to the product or service the tissue establishment provides, so it is important to tailor it to your needs".

Applying quality management in donation and banking of tissues and cells Quality is the responsibility of all personnel involved in the process of providing tissues and cells for clinical application. A systematic approach to quality management must be implemented and maintained throughout the entire process. A good system addresses quality management under the following headings:

a. *Personnel and organisation;*
b. *Premises, equipment and materials;*
c. *Contractual arrangements;*
d. *Documentation and record-keeping;*
e. *Quality control;*
f. *Quarantine and release;*
g. *Process validation;*
h. *Traceability;*
i. *Complaints;*
j. *Investigation and reporting of non-conformance, adverse events and reactions;*
k. *Recall;*
l. *Self-assessment, internal and external audit;*
m. *Quality risk management;*
n. *Fiscal and continuity planning;*
o. *Tools for continuous quality improvement.*

12.10.1 Where Do I Start?

This depends on the starting point.

Very often a quality system, even though not formalised and optimal would exist in a functioning tissue establishment. The issue here would be to identify what is being done, what is the required level of quality (considering legal requirements and risk assessment) and addressing the gap. The priority would be to make a risk assessment and tackle the higher risk areas first.

For a new establishment that is to be set up, again this depends on the risk parameters. The basic concepts are always the same as above (from a to o), however the emphasis and resources may change according to the risks involved, e.g. Doing autologous stem cell banking within a closed system is very different from processing Pancreatic islet cells. In the former the requirements for premises, competence, equipment, process validation etc. although all present are very different from what is required for islet cell processing.

In simplistic terms a sequence of events needs to be established. The QS must be applicable to the process/product involved. Below are the core issues to consider in a non-comprehensive list.

a. Are there the right people with the right training and competence to the job?

All staff should understand the importance of quality and their role in achieving it consistently.

Are the roles and responsibilities clearly delineated and key personnel identified?

Is training defined (initial and ongoing), documented and certified? Is competence assessed?

b. Do I have the right premises which are appropriately qualified for the job (product)? Are the appropriate standards (e.g. air flow, cleanliness) maintained (according to the activity/product). Are storage areas adequately qualified and monitored? Is there a clear segregation of quarantine and non-quarantine material?
c. Is there a list of critical equipment? Is the equipment qualified? Is there a defined maintenance/calibration plan? Is there a validation plan?
d. Is there a controlled list of all critical material which is fully traceable for every activity?
e. For outsourced activities (e.g. testing) are there the right contracts and relevant audits in place?
f. Are third party agreements in place which clearly define roles and responsibilities (e.g. with hospitals or tissue procurement facility)?
g. Are contracts with suppliers of materials clearly defined including supplier audits?
h. Is a document management system established, with clear document hierarchy, authorisation, policies/procedures, accessibility, confidentiality, document change control, identification, archiving?
i. If an IT system is in use, is the system validated, with robust back up and down time procedures?
j. Have the Quality Control parameters been set up? Including Sampling plan, product characteristics, and non-conforming products.
k. Is there a process of clearly identifying quarantine from released material? Is there a clear procedure for release for each product and including authorised person?
l. Is each process validated, whether this is directly linked to the tissue/cell or support process (e.g. testing)? Is the validation process acceptable and authorised?
m. Are all the critical events traceable? Who has done what using which materials and end product/result can be traced?
n. Is there a system to identify, analyse and respond to complaints from clients, staff and suppliers?
o. Is there a system of capturing, analysing (root cause analysis) and reporting out of specification products, adverse events and reactions?
p. Is there a system of recall of released products (whether internal or to 3rd parties)?
q. Is there a system of material in use or storage recall?
r. Are all of the above auditable? Is there an organised system of internal audits which covers all the critical processes, including core activities (e.g. product processing), support processes (e.g. IT system) and management processes (e.g. management review)?
s. Are external audits performed?

t. Are audits (internal and external) effectively followed up?
u. Are the concepts of risk management understood and implemented? Is there a risk register? Are all relevant processes (validation/qualification, non-conformances/non compliances/incidents, change control, complaints) risk assessed and followed up?
v. Is the system sustainable? Are there contingency plans for business continuity?
w. Continuous Improvement: *Management commitment and support are essential for the development, implementation and monitoring of a quality system to ensure continuous improvement*. (CoE 4th Edition 1.3)
x. Does the system promote continuous improvement? How is this implemented?

12.11 Biovigilance

Biovigilance is the systematic monitoring of serious adverse reactions and events (SAREs) from the selection of the donor to the follow-up of the recipient, with the objective of making the application of tissues, cells, MAR and organs safer and more effective [29].

It includes all organized processes concerning detection, evaluation and monitoring, which are associated with adverse events and reactions observed at donors or recipients, as well as with epidemiological follow-up of donors/recipients.

Biovigilance should not be only at the local level of the tissue establishment/clinical centre but at a regional and National Level. Most countries impose a legal obligation to report SAE's and SAR's to a centralised competent authority. There should be a National organisation (Competent authority) with clear policies and procedures for the management of SAE's and SAR's.

A quality system is not an end in itself; it is a means to a safe and sustainable product and customer satisfaction. The implementation of an effective biovigilance system covering all aspects from procurement of tissues/cells to processing, testing, clinical application and follow up provides essential information on the products and processes/procedures performed. Basically, it is a system of 'looking at the past' to 'predict and improve the future'.

The tissue establishment is responsible for providing clear guidelines, forms and instructions to clinical users, removal organizations, as well as to important third institutions on how to notify SAREs in accordance to national or local requirements. The report and management of a SARE should be integrated in the quality system of the tissue establishment, with one or more SOPs describing the report recognition process, research, follow-up of corrective and preventive actions and report to the Competent Authority.

12.12 Quality Systems and IT

IT systems today often provide an essential platform in tissue and cell banking. They play a critical role in the functioning of the system. Hence it is not surprising that Quality and IT are often intrinsically linked. It can be stated that a sound quality system may significantly depend on a proper IT system, however definitely there will not be a robust IT system without a good Quality System.

The characteristics mentioned for a quality system apply to software and hardware. The concepts of qualification, validation, risk management, change management, documentation, contingency plans, traceability etc. all apply.

In cell and tissue banking where, multiple tissues are taken from the same donor and transplanted to multiple patients, and where the same tissue is often transplanted to multiple recipients, clearly a robust IT system is necessary. (This is covered in the chapter on IT).

12.13 Data Protection

Although perhaps not directly related to quality, the concepts of data protection impinge on certain aspects of a QMS and vice versa. The General Data Protection Regulation—GDRP legislation in the EU Members states [30] imposes obligations on establishments which have to be taken into account.

12.14 The Cost of Quality

There is a cost to quality, however this has to be taken in the context of the cost of 'no quality'. Very often authorities need convincing to invest in Quality systems and quality development. A risk assessment for the lack of investment in quality puts the issue in perspective. The concept of the cost of poor quality was first described by Armand V. Feigenbaum in a 1956 Harvard Business Review article [31].

12.14.1 The Cost of Quality Can Be Categorized into Four Categories

A quality system has prevention and appraisal costs.

1. **Prevention Cost**: Prevention costs are the costs of all activities that are designed to prevent poor quality from arising in products or services. The example of the prevention costs includes: Education and training, human resources in auditing.

2. **Appraisal Cost**: Appraisal costs are costs that are incurred to ensure the conformance to quality standards and performance requirements. Examples of the appraisal costs include: Supplier acceptance, sampling (Quality Control) and calibration.
3. **Internal Failure Cost**: Internal Failure Costs are the costs that are associated with defects found within the organization before the customer receives the product or service. The example of the internal failure costs includes in process product failures-discard.
4. **External Failure Cost**: External Failure Costs are the costs that are associated with defects found after the customer receives the product or service. The example of the external failure costs includes: Service level agreement penalties, lost customers and opportunities, loss of credibility.

Lack of quality will lead to failures, and these may have significant costs and potentially catastrophic results (e.g. failure in storage with product deterioration of autologous stem cells needed post Bone marrow suppression).

Cost of lack of quality can also be classified as visible and invisible costs. The below graphics explains both types of costs.

Visible costs: product rejection, reprocessing, cost of inspection and discard etc.

Invisible costs: excess inventory, additional controls, fines, litigation, complaints, loss of credibility etc.

It is often the case that invisible costs significantly outweigh the visible costs (Fig. 12.1).

Fig. 12.1 The 'iceberg diagram' illustrates that it is often the case that invisible costs significantly outweigh the visible costs

12.15 Role of Management and Quality Management Review

Although quality is the responsibility of all personnel, the role of management cannot be overemphasised.

Management has the ultimate responsibility to determine and provide adequate and appropriate resources (human, financial, materials, facilities and equipment) to implement and maintain the QMS and continually improve its suitability and effectiveness through participation in management reviews [32].

A QMS which is not well lead, resourced and actively supported is bound to fail.

The management review process requires Top management to periodically review the QMS to ensure its continuing suitability, adequacy, effectiveness and alignment with the strategic direction of the organization.

Why undertake management reviews?

1. Determine and evaluate QMS performance
2. Determine the need for change and improvement
3. Determine the suitability of the policies and the objectives.

The purpose and final outcome of the management review should be continual improvement of the QMS. As the organization's QMS increases in its effectiveness and efficiency, the performance will likewise increase. (ISO 9001/2015).

12.16 Conclusion

Quality is doing the right things; Doing things right; Doing things at the right time; Meeting or exceeding client and communities' expectations; Expressed satisfaction of clients; Meeting needs not wants; Taking into account effective use of resources; Compliance with standards; Continuous Improvement.

And importantly doing all the above consistently and in an auditable manner. A QMS ensures that Quality as described above is delivered.

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IT System Requirements for Tissue and Cell Banking

13

Linda Lodge

Abstract

It has been acknowledged for some time that using IT systems in tissue banking is invaluable, supporting consistent data recording, process management and traceability. It is also recognised however that the process of IT system implementation is difficult and does not always result in an optimal deployment. The considerations and tasks required to successfully implement are often not well understood in the tissue banking environment. There is no single point of reference on how to select, implement and manage such a system. The subject of IT System implementation is extensive and while an abundance of literature exists, it is difficult to provide a suitably summarised source that is absolute in the precise user context. Therefore guidance has to be reasonably generic and open to interpretation which can be overwhelming to a non-technical reader. This chapter attempts to highlight and summarise areas that should be considered significant, providing indicators as to what should be deliberated and carefully managed. The observations and suggestions here have been gathered over the course of 30 plus years' experience implementing IT Systems in a healthcare setting (including tissue banking) and draws from examples where the same mistakes are repeated primarily due to individuals being unaware of the importance of the issue. This is still not a definitive guide, more of an aide-memoire; however, it is hoped the information provided will usefully inform the IT System implementation process and be of assistance to the tissue bank.

L. Lodge (✉)
Scottish National Blood Transfusion Service (SNBTS), Edinburgh EH17 7QT, Scotland
e-mail: linda.lodge@nhs.scot

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13.1 Introduction

An IT system can be defined as a technological solution, comprising of the hardware, software and documentation components, essential to the delivery of an IT service in support of a predefined set of business processes. Business functions and procedures will need to work in tandem with an IT system to deliver the requisite functionality and benefits from an IT system implementation.

To ensure an appropriate technology solution is implemented, operational necessities for all elements of the system need to be thought through and clearly defined.

Lack of adequate specification of requirements can lead to issues with system usability, safety, quality, maintainability and implementation. Instead of solving an operational problem an inadequately specified IT system may simply change the problem or indeed make it worst. Attention at all stages of an implementation is important but particularly important in the specification stage to ensure a beneficial fit for purpose IT system is implemented.

13.2 Why Introduce an IT System?

Before deciding that an IT system is essential, consideration should be given to the problem(s) to be solved. Consider the following questions:

1. Are there actions that require to be continually repeated in a standard way e.g. product processing steps, test requests and result entry, recording of environmental monitoring information?
2. Does a process require completion of standard but complex calculations e.g. dilution formulas?
3. Does data have to be recorded and retrieved to be used later in a process e.g. donor/patient demographics, donation consent, medical history?
4. Are processes spread between departments or sites e.g. consent, collection, testing, storage?
5. Are decisions dependent on data from multiple processes and/or prone to error e.g. product release dependent on consent, medical history, test results and process outcomes?

If any of the above is considered relevant to your operational environment an IT system, to help manage the issue(s), should be considered as part of a fit for purpose operational solution.

13.3 The Benefits of an IT System

IT systems have the ability to release genuine operational business benefit if they are implemented and used correctly. Thought should be given to the improvements wanted and the organizations' operational objectives e.g. increased throughputs or product quality or traceability, all of these? Understanding the operational objectives and priorities will give a good indication of what is required from an IT system. Some of the benefits a Tissue Bank could expect from a Tissue Bank Management IT system implementation would include:

- Reduction in the number of paper records which are susceptible to loss or damage that have to be maintained
- Rapid, easy access to structured donor and patient information at point of need
- Standard definition and execution of process e.g. multiple sites, same products/donors/process, only need to define process once
- Reduction in transcription or translation errors e.g. electronic reading of coding systems and management of data eliminating manual error
- Data security, using features like access control to functionality or sensitive data e.g. access levels associated with user system login and operational role preventing unauthorized access
- Full traceability at each stage in the process e.g. donor to tissue to patient logging of transactions
- Ability to collect, index, search, analyse and visualize large and complex data sets e.g. operational and management reports, trend analysis, problem analysis
- Execution of complex protocols and algorithms e.g. test result management, process calculations, event triggered consequences environmental monitoring and management.

Operation of a tissue establishment in keeping with the requirements laid down in the EU Directive 2004/23/EC [1], is required to consider how each of the above can be effectively achieved. While the directive does not specifically mention an "IT system" it does talk of coding systems to identify donor, product and recipient and "an adequate system to ensure traceability". This "adequate system" may be achievable using a paper based system if the number of products is few and the volume of transactions managed small however as operations develop, possibly growing and becoming more complex, introduction of an IT system is worth considering and may become essential as operations grow.

Furthermore Article 25 of the EU Tissue and Cells Directive [1], describes the intention to design a Single European Coding (SEC) system [2], to support traceability and maintenance of data for a minimum of 30 years. This coding system has been developed and transcribed into UK law. It requires that a code 40 characters in length be applied to all in scope products and maintained to aid product traceability. Management of such a code is infinitely easier with the help of an electronic system (Fig. 13.1).

DONATION IDENTIFICATION

ISO Country Identifier	TE Code (TEC)	Unique Donation Number
2 characters (alphabetic)	6 characters (alpha/numeric)	13 characters (alpha/numeric)

PRODUCT IDENTIFICATION

Coding System Identifier	Product Code	Split Number	Expiry Date
1 character (alphabetic)	7 characters (alpha/numeric)	3 characters (alpha/numeric)	8 characters (numeric)

Fig. 13.1 Single European code structure

13.4 What Are the Operational Requirements?

Operational requirements describe what the IT system is being asked to provide and support e.g. donor management, process control, stock control, patient management, traceability, test management, data analysis, reduction of paper and so on.

Defining the operational requirements should begin with an upfront agreed statement clearly describing the scope of what has to be achieved, it should identify what is included but equally what is not included. It is important to get agreement of this statement at an executive level. It is also helpful to identify at this point any known constraints. For example if time or money is an issue the scope may already be limited. What problem(s) are to be solved? What operating improvements are needed? What benefits are wanted? All of these should be prioritized with the criticality being understood and agreed by all. If this is not done a difference of opinion or understanding may well lead to problems during an IT system implementation. These discussions and agreements begin to shape the context, extent and complexity of the operational requirements to be satisfied by an IT system.

These operational requirements should be described in a structured document that can be issued to potential IT system vendors. System vendors should be able to respond to each requirement, providing a statement of compliance confirming their system solution will satisfy the operational requirement. If a vendor cannot offer compliance with a particular requirement, at that point in time, they may offer to develop functionality to suit at some point in the future. Some requirements may not ever be able to be satisfied by the solution offered. Defining the criticality of each requirement will help determine whether or not this is a problem.

When writing operational requirements the principles of SMART goal/objective setting [3], should be applied:

S—requirements should be specific, ensure they are well defined and clear
M—measurable, requirement should be testable to show it has been achieved
A—agreed, everyone agrees this is a requirement
R—realistic, can be achieved with the resource, knowledge, skills available
T—Time-based, can be done in the time available.

Defining understandable, realistic and testable operational requirements is an essential step towards finding an IT system that will be fit for purpose.

13.5 Developing the Operational Requirements Document (ORD)

To develop the operational requirements it is recommended that people from all levels of the organization are consulted but vital that there is representation from those who actually do each process. People who are not directly involved in a process may have a valid view or opinion concerning what is needed however beware of making assumptions, facts are very important at this stage. Be equally aware of being influenced by people who are resistant to change and are eager to recreate things as they already are. Aim to create a team that allows ideas and differences to be constructively discussed. Encourage the status quo to be challenged. This will hopefully lead to a balanced set of requirements being agreed which will supportively take the organisation forward.

There are many methodologies and sophisticated tools to help document the business process and define operational requirements. The main objective at this stage is to do what it takes to understand your process and identify desired improvements using whatever methodology, or adaptation thereof, that works for your team and to then clearly define what is considered necessary from an IT System. Countless books and articles are available relating to the topic and can be an invaluable aid to understanding business analysis methodology and requirement writing.

In its simplest form business process analysis requires a step through of every task and action performed, noting who does what, when, where and why and if improvements could/should be made. It can be an enlightening experience.

Figure 13.2 shows an example of a high level Living Donor Registration Process, using a swim lane approach to define responsibility and points of handover or interface, could be represented. Each process can then be "exploded" to produce lower level process or step maps if needed, allowing more detail drawn out until the necessary level of understanding is reached. It is not always necessary to go as far as single step level definition but two to three levels of detail is not unusual depending on the complexity of process.

Integrated Definitions Methods IDEF0 [4], is another analysis method of identifying inputs, outputs, mechanisms used and influencing controls for each process. See Fig. 13.3 IDEF0 Diagram. This method is helpful when trying to understand inbound and outbound data flows and dependencies of each process, external factors that influence or constrain process behaviors and equipment that may be necessary to complete the process e.g. a label printer. As before two to three levels of detail may be needed to reveal the true nuances of each process and how they interconnect.

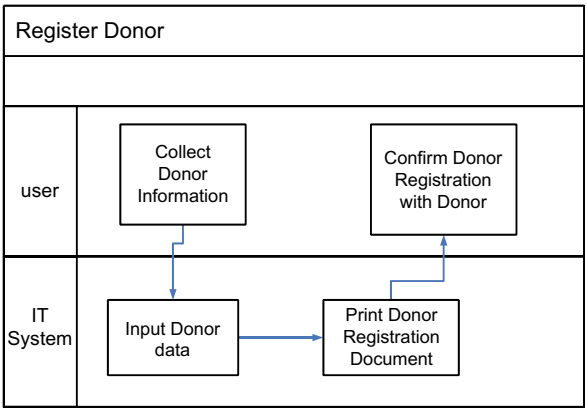

Fig. 13.2 High level process diagram

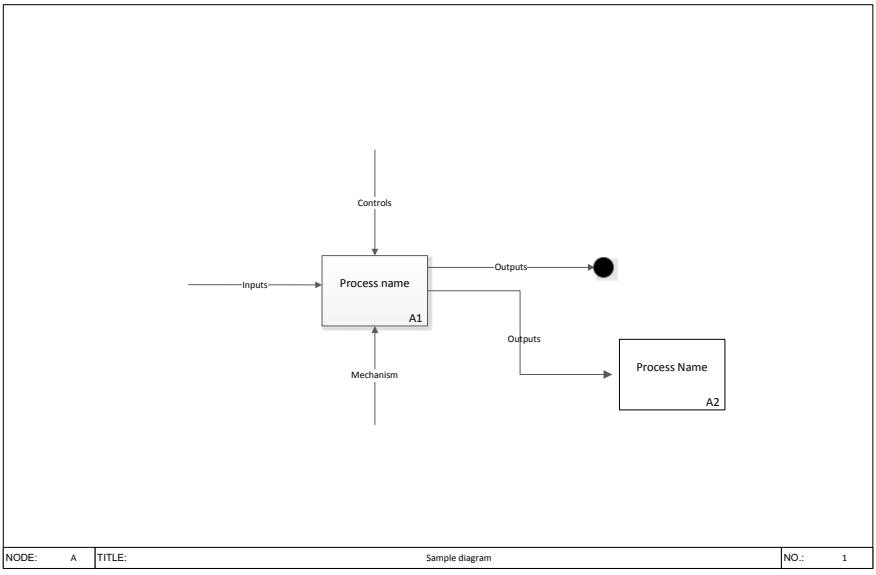

Fig. 13.3 IDEF0 diagram

The method chosen is often determined by the complexity of the business processes being examined and the analysis method the team are familiar with. What is important here is sufficient understanding of process detail in order to identify a comprehensive set of operational user requirements.

An exercise of this nature can be very useful even if there is no intention of introducing a new IT System. Failings in the existing process can be highlighted and an insight into what improvements may be made provided. For some problems the solution may lie in a relatively straightforward change to operational procedure. Be aware however that an IT System may not always be the ultimate solution and implementing an IT system to simply mirror a manual process which does not work particularly well will not improve the process. If there are fundamental issues with the operational procedure these must be addressed, electronic replication of a flawed procedure will not solve the issues.

Once the business processes in scope are understood and the need for an IT system established, writing the operational requirements specification for issue to potential vendors can begin. Requirements can be written at different levels of detail. For the Operational Requirement Document (ORD) the level should remain fairly high and generic, the important factor being to ensure that key functionality required is noted and understood by all parties. For a large and complex set of requirements the ORD may be further refined to ensure where necessary more specific details of a requirement is understood and agreed. This level of requirement specification is often referred to as a User Requirement Specification (URS) although ORD and URS are regularly used interchangeably. The key here is to provide sufficient information at the appropriate time to ensure all parties understand what is required to deliver a system fit for purpose. Keep in mind that the vendor does not know the specifics of your business as well as you do, it is your responsibility to give sufficient insight in order that requirements can be understood. Vendors can however frequently offer valuable insight into how others may have solved a particular problem, so keep an open mind and be prepared to consider other ways of doing things.

Requirements should be specified in terms of functional and non-functional requirements. Each requirement should be considered mandatory or non-mandatory [5]. Careful consideration should be given to mandatory requirements and how they are written. An overly rigid specification may exclude excellent solutions from being proposed as the system cannot meet the precise requirement at time of proposal in some small way. It may be that in reality the requirement does not have to be met in the exacting way it is written or it could be allowed to be developed at a future date but wording of the requirement prevents either course of action from being considered. An ORD written as a very generic description of issues which invites the vendor to propose solutions is easier to write however the solutions proposed could vary considerably and evaluation to ensure requirements are fully supported can be a lot more difficult. The chosen approach is likely to be somewhere in the middle ground influenced by complexity of requirement, resource availability and operational ability to consider a wide range of options.

13.5.1 Functional Requirements

Functional requirements are statements describing "what" a system is required to do in order to satisfy organizational needs. Stating "how" a function is to be carried out should be avoided. By concentrating on "what" a system needs to do, rather than how to do it, gives suppliers more freedom to offer different ways in which requirements might be realised. This allows new ways of working to be considered. An example of a generic requirement for a Tissue Bank might be "The system shall allow donor information to be recorded in the system". Written at this level the requirement is very generic. Information that must be recorded is not mentioned therefore simply recording the donor's name would make a system compliant with this requirement. In reality written exactly in this manner is probably too extreme and taken this literally the actual requirement is unlikely to be met in a way that is fit for purpose. To overcome the issue, if there are mandatory data items that must be recorded it would be acceptable to add a list e.g. "… donor information consisting of First name, Last name, DOB, Gender etc. There is still no mention of "how" the data items should be entered or in what order. This affords the vendor the freedom to suggest a variety of technologies and formats to enable data entry. By way of example, manual keying, document scanning or barcode reading could all be offered and options could be given for where and when in the system data entry takes place, provided the full list of "donor information" is catered for. This approach could present an opportunity to explore different options. It may offer a chance to look into technologies not previously considered possible. For some requirements there may be a wish to streamline or replace the way a process is done. For example recording donor consent may be currently done by filling in a paper form. The consent information may be kept only on paper form or the information may be transcribed into a system and the original form stored. If the wish is to minimize manual data entry and reduce paper storage the requirement should be written to describe what is required consider writing something like "The system should provide the user with a way to consent donors that reduces/eliminates the need for paper forms and manual data entry".

13.5.2 Non-functional Requirements

Non-functional requirements define how the system should operate. These are often forgotten as people get carried away with the possibilities of new functionality. Non-functional requirements are however critical to a requirements specification if the system is to be used in the Tissue Establishment environment. Typically non-functional requirements are concerned with areas such as system accessibility, availability, scalability and performance. Consider who is allowed to access the system and from where. When has the system to be available; when would it be acceptable for the system not to be available? How much growth in volumes and throughput is expected in the next 5–7 years? An example of a non-functional performance requirement is "… a label must be produced from the selected printer

within 2 s of making the request". A product release label may be beautifully designed however if it takes 5 min from the time the label is requested to it being produced by the printer it is unlikely that the system performance for this function will be acceptable for deployment to the live environment. These non-functional requirements should be considered in the same way as functional requirements as they are as likely to determine whether or not your system will ever be fit for purpose.

In addition to supporting requirement specification as system implementation progresses business process maps can be used to inform the development of process and system documentation such as standard operating procedures, training materials and system user guides for the end users. Using the process maps as a guide the business processes can be organized into sections and key activities documented to support correct use of the system functions. When the system is built consider taking screen shots from the system and inserting them into the user guide document to create visual system guide. Together process maps and user guides can provide a comprehensive support tool for anyone using the system.

13.6 Technology Solutions

In response to the ORD, vendor proposed solutions may encompass hardware and software elements to deliver the complete system. However hardware and software elements may be provided by different suppliers. It is quite common for a software supplier to provide only the application software however they should always provide a technical specification for the hardware and operating systems that their application is compatible with. It is advised to engage the support of an IT department/expert to help in this scenario.

Hardware comprises of the physical equipment necessary to make the solution operational e.g. servers, printers, scanners, communication connections etc. The physical system design will describe how hardware elements should be arranged to support system operation and where it will be physically located. There may well be different system design choices and final selection could be influenced by a combination of factors including existing IT infrastructure and available support skills and capacity. Cost is often a critical factor in this area and can rise quite rapidly depending on choices made. Requirements should take account the number of system environments needed for production, testing, training and disaster recovery as well as the level of business reliance required and this will dictate the amount of hardware required for the full implementation. For the more complex systems and requirements seeking specialist IT advice in this area would be a sound investment.

The software element of the solution comprises the instruction sets or programs and operating information, used by the computer hardware, to make available the IT application required to deliver the functionality stated in the ORD. There are various layers in an IT application e.g. user input interface, database management

system, communication protocols etc. all of which must work in tandem to make a system usable, safe and reliable.

The objectives of the tissue bank and its' operating environment should drive the selection of technologies to be introduced with an IT system. For example if objectives include reducing manual keying and associated errors, data carrier technologies like barcode systems, Radio Frequency Identification (RFID) may be considered. Where paper forms continue to be a necessity scanning technologies and character recognition (OCR, ICR) systems could be a considered part of the solution. Management of donor and patient records, processing of product, requesting and resulting of tests are processes which will be made easier with the support of a transactional relational database to manage and store records. Where multiple sites are involved then a web based system may be the preferred option. For small organisations, a third party hosting or cloud based system arrangement may be the most cost effective solution as the cost of procuring and on-going maintenance of the physical hardware can be off set against the cost of a managed service where a 3rd party takes responsibility for the well being of the hardware and software. The tissue bank should consider the environment in which they operate along with the funding and skills available to them when evaluating available solutions.

13.7 Managing the Data

In the UK and many other countries, the international ISBT128 standard [6], for terminology, identification, coding and labeling of medical products of human origin is the coding standard of preference. The standard supports consistency in identification, data transfer and traceability for transfusion/infusion and transplantation across the world and has professional community endorsement. The ISBT128 standard data structures representing donor, donation and product information can be simply presented in a barcode with both linear and 2D barcode representations supported. Besides barcode the same data structures can be carried in other electronic data carriers such as radio frequency identification (RFID) tags or electronic data interchange (EDI) messages.

13.7.1 Barcodes

Barcodes provide a secure method of data transfer. Data is represented in a machine readable format which can be decoded by a bar code scanner. The barcode scanner electronically transfers the data to the IT system where it is processed and recorded.

Linear Barcodes hold 1 data item per barcode and are used in the labeling of items like donations, samples or products. Multiple linear barcodes can be placed on a product label allowing the label to carry coded information about the donor, product, expiry date, etc. Using appropriate scanning devices these barcodes can be

ISBT128 linear barcode containing an ISBT 128 donation identification number. The eye readable code is displayed above the barcode

Fig. 13.4 ISBT128 donation identification linear barcode

This 2D barcode carries all the data shown below related to the donation number, blood group, product code, expiry date and test results
=+08042=G17031343009590=%2800=<S1384000&>0173202359&(00000=\9300000000000014300&{000000000000000001&"000000000300000000=>017320

Fig. 13.5 2D data matrix barcode

read. Laser scanners read using a single sweep of a beam of light from side to side across the barcode. Image scanners optically scan the barcode and convert it to a digital image. Data is electronically transferred to the IT system without the user having to manually key in the data and risking transcription errors. A basic disadvantage of the linear barcode however is the limitation in the amount of data each barcode can carry. Linear barcodes grow in size depending on the number of vertical lines and white space needed to represent the data item and therefore the amount of label space needed to accommodate the barcodes increases accordingly. This causes a serious limitation in the amount of information that can be coded on a small label using linear barcodes (Fig. 13.4).

2D data matrix barcodes can be used to carry 1 or more data items in a single barcode. The data structures used are the same as those used in linear barcodes however the multiple data structures are carried in a compound message within the 2D barcode. The barcode presentation is markedly different from the linear barcode. Using less label space but having the ability to carry a lot more information the 2D barcode makes labeling small containers a lot easier however like all electronic data carrier solutions the recipient must have the ability to decode the information in the 2D barcode (Fig. 13.5).

13.7.2 Radio Frequency Identification (RFID)

RFID is a technology used extensively for tracking the movement of packages with in the courier and retail industries. In healthcare this technology is becoming more widely used to manage inventory, identify patients and record environmental

RFID tag can be encoded with product information and applied to the product container

Fig. 13.6 RFID tag

RFID tag is passed over the reader, the information carried in the tag is decoded and passed to the IT system

Fig. 13.7 RFID desktop reader

conditions. A RFID tag containing a programmable chip is attached to the item of interest and the IT system writes/reads data as required to and from the tag. The data carried will be determined by what the tag is being used for e.g. temperature monitoring may have the donation number and time stamped temperature readings, for inventory management the tag may carry the donation number, the product code, the location. The equipment needed to support this technology will depend on how it is to be used but includes suitable tags and readers. Fridges and freezers can be adapted to work with this technology and there are now tags available that will survive a spin in the centrifuge (Figs. 13.6 and 13.7).

13.7.3 OCR/ICR/Touch Screens

In places where data collection involves filling in paper forms e.g. donor consent forms, transferring the data to the IT system and storing the paper copy for the regulated number of years can be tedious, risk prone and expensive. It might be advantageous to consider other ways of collecting and entering this data.

It is possible to design electronic forms. The person completing the form is shown the electronic display where the data can be entered directly in to a system removing the need for paper. Entry can be by data keying, option selection using a mouse, touch screen or a mixture of the options. Asking a donation consenter to fill in the full consent form may not be feasible or desirable however forms can be designed to allow multiple user entry allowing both the consenter and the nurse to enter data. Electronic forms can be designed to be filled in on multiple devices such as a PC, tablet or mobile phone offering flexible and portable options for data collection.

If however the paper form remains the preferred data collection method it can still be electronically managed to reduce the overheads of data keying, the risk of

Fig. 13.8 OCR/ICR form

transcription errors and the cost of storing paper. The paper form can be scanned using a desktop scanner to take an image of the completed form. This image is then stored in the system database and can be accessed and viewed in the IT system when required allowing the paper form to be destroyed. This works well when there is minimal data on the form that is needed for actual system transactions e.g. for cases where it is simply the appropriately filled form that has to exist in order for processing to continue. This method has the advantage of being quick and straightforward to implement.

A more complex system would involve the IT system reading the form and making an interpretation of the data. The technologies involved here are known as Optical Character Recognition (OCR) and Intelligent Character Recognition (ICR). OCR converts handwritten images into machine readable text; ICR allows the data to be read into the IT system database. This type of system works well for forms where data is structured and limited with the data entered onto the form via pre-defined boxes e.g. a prescription form or survey return. It is less suited to forms where there is a great deal of free form unstructured text (Fig. 13.8).

13.7.4 Electronic System Interfaces

Where there are multiple IT systems used in the supply chain e.g. different systems used by different organizations, data can be passed from one IT system to another via a system interface. Electronic system interfaces should be considered where there is a regular need to pass large quantities or complex data or where it is essential that transcription errors are not introduced e.g. test result entry. System interfaces can be inbound (from another system) or outbound (to another system). Once developed and validated an automated interface can be run as required and reduces the risk of manual data entry. This type of data entry is often used when receiving results from test analysers allowing the results to be updated directly into the donor/patient record without manual intervention. Unfortunately all systems do not hold data in the same format or use the same coding systems therefore system interfacing often involves some form of extract, transform and load (ETL) process. The ETL process is developed to map/format data from the sending system to meet the requirements of the receiving system during the interface process.

13.8 Database Management Technology

Once data is entered into an IT system it must be managed in a way that maintains security and integrity. IT systems utilise database management systems for this purpose and they are now an essential element in effectively managing data. A number of relational database management systems are available e.g. Oracle [3], Microsoft SQL [4] and specifically designed to manage the receipt, storage and retrieval of vast amounts of related data. Properly used this technology has the ability to ensure data integrity is maintained during operational use. It also has the ability to optimize system performance while retrieving large volumes of data. Some IT applications will only run on specific database technology while others can be run on a variety by manipulating configuration parameters in the application or hardware. It is also fair to say that the costs involved vary significantly between technologies, implementation and support. Choice will often depend on what is already in use, the knowledge and skill of the support staff and the available budget within the organization.

13.9 Selecting an IT System

In an ideal world there would be no constraints and selection of a system could be justified solely in terms of the "best fit" for the requirements. Unfortunately very few establishments are afforded that luxury. Considerations of time, effort and cost must be justified. Therefore a well understood method of selection should be employed and agreed prior to operational requirements being issued to prospective vendors. There is no absolute set method for doing this however it is important that the process can be audited and justified. A common selection method used is to set selection criteria in areas such as cost, time and functionality and apply a weighted score to each, based on the relative importance to the business. Proposals are then scored on their ability to meet the operational requirement and points attributed against each of the selection criteria. Once evaluation and scoring has taken place the proposal with the highest score is deemed most suited to the needs of the business and therefore considered the preferred solution at this stage. This system is not infallible, how well have vendors understood the requirements, how well have the proposals been understood and what relevant information has not been factored into the selection criteria? For a complex set of requirements it is not unusual for the scoring process to be run in phases, where points of clarity are issued and received by the tissue establishment and vendor alike. Discussion with vendors to clarify requirements and responses can lead to positive and negative adjustments in the initial proposal. While these discussions can be time consuming and require additional time to evaluate outcomes it is not a stage that should be cut short unnecessarily as misunderstanding here can be very costly further down the line.

During the assessment of a proposed system continually refer back to mandatory requirements. It is very easy to get carried away by slick demonstrations of "nice to have" functionality, the implication being that "you too could have all this with the press of a button", nothing is that simple! Producing usable, meaningful results takes thought and effort to define the requirement, develop the functionality and process the data.

While known future requirements should be considered at this stage beware of adding additional functionality now that you don't need, you don't want to be paying now for something that won't be used for 5 years. Where future requirements are certain at this stage it may be possible to discuss phased implementation and therefore phased payment with the vendor.

A key objective of this phase over and above selecting the most appropriate system is to ensure the procurement process can be demonstrated and the outcome justified should the need arise.

13.10 Cost of an IT System

When calculating and considering the cost of an IT system all elements must be accounted for. It is easy to miss significant costs that may in the future put strain on an already tight budget. It is also important to recognize all the potentially avoided costs a new IT solution may support. I have known of a £100,000 solution being discounted until it was recognized that there was a longer term saving of £2.2 m to be made.

When identifying system budgets people often think only of the application cost and completely forget the hardware costs or what it takes to support the system over its' lifetime. IT Systems may require different hardware and software elements depending on how the system is to be deployed. Some of these requirements may differ considerably from existing IT provision and could attract a whole range of new needs and associated cost. Hardware elements may also require to be changed should they go "end of supportable life" during the time the application is in use. While hardware can often be used beyond this time, the cost of maintenance and support climbs accordingly. Replacement is usually a more cost effective option. An appreciation of how, where and when cost may be incurred is vital for future planning to ensure a clear upgrade path and indication of when a replacement application and related hardware should be considered.

On-going support and maintenance costs should be fully explored. All too often organizations consider "go-live" of the IT system to be the end of the journey when in fact it is just the beginning. Over time operational requirements will change e.g. new tissues to be supplied, a new site opened, legislation changes, all of which have the potential to force change in the IT system.

Expected change in the operational footprint should be taken into account. Ideally the IT system should be sized at implementation to accommodate expected growth with little or no additional cost implications, this is not always feasible. The

following factors are examples of what can affect system growth and have the potential to impact licensing or hardware cost:

- Additional sites added which increase the user base.
- New product or processes developed.
- Increase activity, more donors, patients, products, testing, issues etc.

A system which reaches capacity, will fail in some way, disrupting or stopping operations until additional capacity is introduced. Better to know and ensure additional capacity can be introduced before the point of failure is reached. Conversely a reduction in operations should also be considered. If reductions mean there is less sites, less users, less throughput can licensing fees be reduced, hardware be retired, will support and maintenance cost decrease.

It is strongly advised that professional IT guidance is sought to aid with understanding of the non-functional requirements and to support selection of IT infrastructure components.

All of this should be fed into the total cost of system ownership. Work done up front to ensure transparency and understanding will reduce the risk of a nasty shock further down the line. Total cost should incorporate, the system licensing, installation and development costs, hardware and operating system costs as well as on-going support and maintenance estimated over a 5–7 year period. The 5–7 year period is considered a reasonable lifecycle for an IT solution although in reality systems may continue to support operations for considerably longer if a system upgrade path is followed.

Beware of vendors who give a pricing strategy that is very attractive for the initial purchase but then relatively minor changes carry a large price tag once the IT application has been implemented. If this is considered a risk, mitigation might involve negotiating with the vendor for prepaid discounted change days as part of the support and maintenance contract. The downside is that they may be lost if not used in year. Regardless of the pricing structure consideration of these types of scenarios can help minimize the budget impact of unexpected cost appearing.

During system selection if may also be worth considering how much change can be end user managed e.g. addition of new products or tests,, report development versus what has to be provided by the vendor. The more ownership the organization has of how system change can be applied the more control there is of time and cost to deliver. Bear in mind however more user responsibility places a greater reliance on the availability of sufficient capable staff to effectively manage the system.

13.11 Implementing an IT System

The effort required to implement a new IT system is all too often underestimated. This is an area where both vendors and users tend to be, at best, optimistic, at worst, totally unrealistic. One of the reasons that so many IT implementations are reported

to overrun is that expectations were unachievable from the outset. It is unfortunately very common for the expected implementation date to be set before the requirements or solution is known. System implementation effort can be considerable and not necessarily directly related to size or complexity of the system to be implemented.

There is no shortage of advice available on how to run a successful implementation in a variety of settings. Michael Hawksworth [7] however describes 6 fundamental steps that I think can be applied to any environment and fits nicely with the Tissue Bank environment.

13.11.1 Define Success

What does success look and feel like? What are the deliverables from the implementation that must be achieved? What expectations must be met? Remember the operational requirement statements do they reflect success if met. Does everyone share the same vision and have the same understanding of what is needed to get there?

If only 80% of tissues can be managed via the new system on implementation is that success? If a tissue from another establishment has to be re-labelled to be accepted into your inventory does that still constitute success? If a stock report is not in your preferred format is implementation still a success. It can be surprising how different views can change the perception of success. An up-front awareness of these different views can make issues easier to manage should they occur.

It is very important to identify factors out with the system e.g. adoption of an international coding method, amendment of a delivery note format, resource needed to configure and system test, that may have to be resolved or delivered in order to enable successful implementation. Establishing the critical success factors and dependencies at the start will engender common understanding in the scope of the implementation.

13.11.2 Set Priorities

There is rarely one goal or a simple problem to be solved. The solution will be required to solve a number of problems e.g. print labels, register donors, electronically accept test results. While different individuals may see different priorities it is wise to have overall agreement before issues arise and difficult conversations need to be undertaken. Compromise may be needed, for example if a target of 2 s to produce a label is proving difficult to achieve but label production consistently completes within 4 s, should additional time, money and effort be spent trying to reach the 2 s target in preference to attending to other outstanding tasks? Does the 4 s production time really adversely affect overall performance to the point that performance is unacceptable?

By setting realistic priorities implementation can stay focused on what is important and not waste time and money gaining an odd second in performance while compromising efforts to ensure critical functionality is safely delivered.

If finalizing implementation is becoming challenging be prepared to critically assess the priority of outstanding issues for an initial implementation. This approach may provide opportunities for phased implementation where more than one implementation of the system will take place over a set period of time. The functionality available builds as each implementation takes place. If implementation can be phased there may be an opportunity to release some system benefits sooner and may buy time to work on more difficult achievements. This type of iterative implementation can be described as using agile methodology with output for each phase consisting of specific functionality and referred to as the Minimum Viable Product. Additional functionality can be added in future phases or sprints. This approach can help reduce the pressure on operational staff trying to manage the change a new system brings while maintaining operations. Where the agile approach to functionality release is less applicable phasing may still be possible across sites, departments or processes.

13.11.3 Avoid Modifications

There are different levels of modification that may be requested during an implementation. While some low level modification e.g. parameter configuration is inevitable, modification requiring major system redesign should be avoided. If a system can't link a tissue to a donor in a few steps, is it the system you should start with? If the right application has been selected amendments should be primarily cosmetic e.g. changing an item description in a system menu. But even cosmetic changes have associated cost and should be carefully considered. If the screen background is grey and you prefer blue what real value will be added by making the change, is it worth the cost?

All change attracts cost however changes at a fundamental design level are particularly costly and can be dangerous, with potential to adversely affect the overall integrity of the system. Asking a system to do something it was never designed or intended to do should be avoided. Reputable vendors will quite rightly refuse this type of change if they believe it will affect the integrity of the system functionality. Even when a change of this magnitude is accepted beware of unintended consequence; a change in process may be by far the better option.

While changes to system configuration e.g. defining test list, defining the equipment list, during the development stage are quite normal once the system has entered formal validation even minor change can have real negative impact on project time, effort and cost because of the potential for error and rework required to change and retest. It is good practice to get an understanding of what has to be configured e.g. tests, consumables, reports, formulations etc. and prepare and validate the relevant information in advance wherever possible.

13.11.4 Prepare for Change

While some people embrace change others do not. It is good practice to involve all staff required to use the system in some capacity from an early stage. If business process has to change to accommodate a new system the earlier this is identified and communicated the better.

Involve as many people as possible from across the organisational hierarchy when defining requirements, validating the system and planning the implementation thereby giving as much opportunity to participate as possible. Let people see what is coming as soon as is practicable even if it is not the finished product, they will be more likely to engage positively and the monster becomes a bit less scary!

13.11.5 Gain Executive Support

If the people at the top of the organization are on board supporting the implementation it will be easier to agree success and obtain constructive support should difficulties arise. It will be to your advantage if you have supporters at this level who know and understand what the team are going through and what they actually have to do. Implementation delays causes frustration at all levels but the further removed a person is the more problematic it becomes when they don't understand what is happening and what is involved in putting things right.

13.11.6 People

Very often the amount of time and effort required from operational managers and users to implement is grossly underestimated. Those who carry out specific operational tasks e.g. manage donor consent, process tissue, distribute tissue are best suited to define requirements and validate solutions in their area. They should be consulted and involved throughout from requirement specification to go-live implementation. This can require a momentous effort especially if day to day duties require to be maintained at the same time. Where people resources are limited it may not be feasible to assign people 100% of their time to the implementation however for key staff it is important that there is dedicated time set aside for implementation tasks in a location away from the routine work area if possible. Where there are part time assignments the overall time to complete a task must be increased accordingly, unfortunately optimistic planning often forgets to make this adjustment. Dedicating uninterrupted time to the implementation will be more productive and deliver a quicker, smoother implementation.

Serious consideration should be given to finding a full time dedicated project manager, someone who will plan, monitor and control the project activities. It could prove to be money well spent to bring in an external resource for the duration of the implementation if these skills are not available internally.

13.12 Implementation Approach

It is recognized as good practice to employ a structured approach to project management and implementation. In Tissue Banking a risk based approach to validation and implementation is necessary to provide a GxP delivery. Traditionally this has been delivered using relevant PRojects IN Controlled Environments II (PRINCE2 [8]) methodology following Good Automated Manufacturing Practice (GAMP5 [9]) Guidelines. Competent authorities expect to see evidence of this level of control and structure demonstrated during any pre/post implementation inspection.

As system design methodologies and technology changes the traditional approach to implementation is also being challenged and changed. Agile methodologies have been around for a number of years and are taking hold as the implementation methodology of choice. An agile implementation approach delivers a number of iterative and incremental system releases before the full version is delivered for use, giving the user an opportunity to identify necessary improvements earlier in the process. It must be recognized that this approach does not suit all implementations however that does not prevent general principles being applied.

The goal is to find and follow an implementation approach that supports delivery of the project objectives ensuring delivery of a fit for purpose system, on time, on budget.

The implementation approach should be documented, to ensure understanding of what will be delivered and the general principles of how it will be done. This will serve to manage the expectations of executive management, competent authorities and end users alike.

13.13 System Validation

A major part of system implementation is validation. When planning an implementation as a rule of thumb an estimated 40% of overall implementation effort should be allocated to validation. Unfortunately as implementation projects run late it is often an area that is squeezed therefore informally testing a system from the earliest opportunity is always beneficial.

Validation can be divided into different types of testing typically including functional testing, load and performance testing and user acceptance testing. Each phase can be run as informal or formal test runs. The informal stage is generally where the first run through of functionality takes place and is used to validate that processes behave as expected and that test scripts are representative of the scenarios to be tested. The formal stages may repeat some of what was done during the informal stage however a predetermined plan with approved test scripts will be followed and the outcomes evidenced and documented. Deviations will need to be managed and risk assessed to determine whether or not there is an issue to be addressed and a defect to be managed.

Functional testing considers the system requirements described in the ORD/URS and tests that the required functionality has been provided e.g. donors demographics can be registered, products will only be released if all issue criteria has been satisfied.

Load and performance testing applies operational load to the system in terms of number of users and volumes of transaction. As concurrent users increase the number of transactions the system has to perform and the amount of data it has to manage increases accordingly. Performance is measured to ensure that the system performs to a level that will support expected operational activity. Increased activity can highlight underlying design issues with the system. Usually these issues are relatively easy to fix once the cause is identified e.g. a specific query may need rewriting; additional memory may need added to a server. Regardless of the issue it is better to find and fix before the system is being used in live operations when a fix many be more problematic to apply.

User Acceptance Testing (UAT) should be conducted by representatives from operations who will use the system once it is implemented. UAT testing tries to create scenarios representative of real life which are run through the system as they would be in live operations. The outcomes are anticipated to confirm that the system not only produces the expected result in terms of data that may be created/modified/deleted and actions that are fulfilled by the system but that it does so in a way to facilitate genuine operational activity.

It is essential from a GxP point of view that the validated state of a system is maintained throughout its' lifetime. As the business operations evolve it is inevitable that system changes will be necessary. Each time change is applied it must be validated to ensure that system integrity has not been compromised. This may not involve a full system regression test but each change needs to be assessed to understand the impact it may have on system integrity. Testing scenarios applied should strive to ensure the change has had no unintentional impact and the system integrity remains intact.

Ideally test scripts should be written and maintained in a way that allows them to be reused though out the system lifecycle as needed. Creating the test scripts for the initial validation is a considerable overhead therefore ensuring they can be reused will be of benefit in the future. There are electronic tools that can assist with the creation and management of test scripts. For systems with any degree of complexity which are expected to be in operation for a number of years investment in some form of automated testing could be a worthwhile investment. There are also companies who specialize in testing and can provide valuable support in creating and executing system testing which again may prove advantageous.

13.14 User Training

A constructive approach and positive reception from the system user community is an important part of any system implementation. Introducing a new system can uncover all sorts of underlying prejudice usually fueled by fear of the unknown. Change takes time to be accepted and settle in and therefore giving end users confidence in the system and their ability to use it in live operation is essential. Confident, competent users who are able to embrace the new system will make a system implementation go more smoothly but importantly the whole experience will be more positive for staff in general.

Early exposure to a system is very beneficial, allowing staff to see a system as it develops into the final version helps with understanding and allows them to appreciate change is coming. Introduction to the system at an early stage does not have to be in the form of formal training, someone showing a run through of a specific function, a test system to "play" on will all help with understanding and acceptance.

Formal training can only realistically start once the system is stable and representative of what the final product will look like. It may however be possible and practical to train on various aspects of the system even if some areas are not complete e.g. donor registration and management may be complete but product release is still under construction. Operational considerations will dictate how best to manage.

Training materials and sessions need to be designed to help the user understand what the system can do and how to do it. Creating user guides using screen shots from the system is a good method of providing system instruction which can then be used in training sessions and to provide ongoing user support.

13.15 Managing an IT System

The requirements for ongoing IT system management should be considered at the procurement stage as this will have a bearing on the terms and conditions of the support and maintenance arrangements which need to be agreed between tissue bank, system vendor and any other 3rd party supplier that may need to be involved. For tissue banks that are part of a wider organization there may be in house skills and capacity to manage an operational IT system, for others this option may not exist and outside help will be required.

There are various support models available. Options range from internal hosting and support of the hardware and application through to a fully externally managed service.

Internal management is where maintaining the IT application and associated hardware is the responsibility of an in house team who manage the day to day running of the system along with all changes to the software and replacement of the

hardware. In this option there may still be a contractual agreement with the application vendor to provide upgrades or make configuration changes.

Externally managed agreements may involve and external party taking on the same roles as an internal IT provide would. Alternatively at the far end of the support spectrum the vendor may offer "software as a service" arrangement (often referred to as Cloud Computing [10]) where a license is granted to use a pre-configured 3rd party application and all maintenance activity is part of the service contract.

Regardless of the service management option implemented it is essential for the tissue bank that operational support requirements are fully thought through. As an example, consideration should be given to the operational support requirements needed out with normal office hours. Should system issues arise after 5 p.m. can resolution wait until after 9 a.m. the following day or Monday morning if the issue arises on a Friday night? What support might be required on a bank holiday? Decisions need to be made regarding criticality and the impact on service delivery should the system become unavailable for any reason. The tissue bank business continuity plan should be used to inform how operations will continue in the event of system unavailability and therefore how immediate the requirement is for system issues to be rectified. This will inform the type and level of support contract(s) needed to ensure continued operations.

It is worth stating that maintaining even a fully managed service requires considerable involvement and effort from the Tissue Bank if the system is to continue to meet objectives. There is not a "hands off" option. Someone must take responsibility for managing the supplier relationships and service contracts. Service level monitoring is an ongoing task regardless of how the service is provided or who the vendor is. Where evolving business needs demand system or service changes these must be clearly articulated. The supply of system amendments have to be negotiated and validated when received. It may beneficial to retain certain activities within the Tissue Bank control e.g. user account management, reference data updates, report creation, from both a time and cost perspective. It is important that a level of operational knowledge of the IT system is developed and retained because if the vendor or system changes for any reason the Tissue Bank may find that with little warning important business and system knowledge is lost.

13.16 Conclusions

The importance of giving adequate time for detailed consideration of system objectives and operational requirements cannot be over stated. Everything that happens from the IT system procurement through to system retirement relates back to the original system requirements. Understanding what the system has to achieve will inform system configuration, development, validation, support and maintenance requirements. The effort required to implement is sizable and should not be underestimated however understanding priorities, keeping control, gaining

executive support and user involvement should help deliver a usable system. The desire for the business benefit delivered by a successful implementation will drive the financial, resource and time commitment considered necessary to meet expectations. When successful implementation of a fit for purpose IT system is completed multiple operational benefits can finally be realised.

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Ethical and Regulatory Aspects of Cell and Tissue Banking

14

Bridget Ellul and George Galea

Abstract

Human tissue and cell transplant medicine raises ethical, legal and societal implications (ELSI), affecting all stakeholders in the transplant process. Informed consent, the central ethical issue, ensures voluntary altruistic donation but relies on adequate transparent communication of potential risks to the donor, including psychological effects, loss of property rights and commercialisation. Specific issues related to saviour siblings, cord blood banking and deceased donation are introduced. Recipients require just access and regulation of safe processing to secure healthy transplants and to guarantee confidentiality without compromising traceability. Society benefits from altruistic donation can be increased through education campaigns, cross-border exchange programmes and regulation of trafficking.

European regulatory instruments deal with consent and protect the vulnerable, e.g. minors, who lack capacity to consent. European directives lay down mandatory procedures for safe practices, for procurement organisations and tissue establishments, to ensure maintenance of quality control, traceability and data privacy. The European General Data Protection Regulation (GDPR) allows data sharing with third countries provided adequate safeguards are in place. However, stakeholders prefer guidelines, rather than legislation, as these offer up-to-date scientific information.

B. Ellul (✉)
Centre for Molecular Medicine & Biobanking, University of Malta, Msida, Malta
e-mail: bridget.ellul@um.edu.mt

G. Galea
Department of Pathology, University of Malta, Msida, Malta
e-mail: george.galea@um.edu.mt

National Blood Transfusion Service, St Luke's Hospital, G'Mangia, Malta

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Complex issues concerning the emerging technology of cellular therapies, including stem cells, are addressed, outlining the interrelationship of ethical and regulatory issues with ease of traceability for different products.

Future transplant medicine relies on potential donors being aware of new technologies, local policies, guidelines and legislation regarding transplantation practices, so education campaigns and ongoing public dialogue are essential.

14.1 Introduction

Tissue and cell transplant medicine has come a long way from the first successful skin transplant in 1869[1] and the first syngeneic haematopoietic stem cell transplants for leukaemia in 1956.[2] Advances in medical knowledge and technology now enable the safe and effective use of human cells and tissues, as autografts and less commonly as allografts, in a variety of medical treatments. Moreover, current experimental research has established the basis of regenerative medicine and offers potential for future new treatment modalities.

Today's successful transplant programme depends on a robust infrastructure that underlies an efficient service to link the donors to the recipients via transplant institutions that are responsible for procurement, processing, storage and distribution of cells and tissues.

Transplant practices are only sustainable if grounded on ethical principles and a regulatory framework to safeguard the interest of all stakeholders. This chapter is focussed on providing the basic but salient ethical, legal and societal implications (ELSI) in regard to the use of tissues and cells for human transplant medicine. It will not address specific issues related solely to reproductive, embryonic and foetal cells, nor does it discuss blood and its constituents available for blood transfusion.

14.2 Ethical Issues

14.2.1 Donor Consent

Consent is the backbone of all medical care, enforcing the ethical principle of autonomy. There has been much literature on consent practices in organ transplantation, which is broadly applicable to consent for cells and tissues to be banked for clinical treatment, though there are also specific issues to be addressed.

14.2.1.1 Types of Consent

Donation can occur directly from a living person, with the common tissues being those that can regenerate, such as bone marrow and stem cells, and skin, but in

addition, others, like heart valves, bone, connective tissue, and corneas, can be donated by a deceased donor since viability of the tissue is not essential.

In a living donor, consent is a voluntary wilful decision, which leads to the institution of donation procedures, only after formally signing, in writing, an appropriate Consent Form. This 'opt in' system works also for donation from the deceased, with the individual being pro-active in life and registering the wish to be a donor. There are three possibilities, either mandatory registration in an official national register, or a personal plan in the form of an Advance Directive or specific communication with the family to express the donor's wish after their death. The latter scenario is however the least secure since one can never be sure that it will be executed by the family, which may express doubt as to the exact wishes of their deceased relative. Since the removal of tissues is an invasive procedure, if no autopsy has been carried out, it is best to have a definite explicit consent registered in life. 'Opt in' legislation ensures that the wishes of the donor are honoured and the relatives cannot reverse that decision. Having said that, the cooperation of the family is still essential for any donation to be taken. Relatives are therefore always consulted and in particular when the deceased adult is not on the donor register.

A less favoured system that is present in some countries is the 'opt out' system, where it is assumed there is 'presumed consent', meaning that everyone is assumed to be a donor after their death, unless a specific objection is officially recorded in an official register during life. This system has been proposed on the grounds that transplant needs are not being met and that the societal good is paramount and overrides the wish of the individual or the relatives. However, Veatch[3] has claimed that 'opt out' laws are just acting to 'salvage' organs that would otherwise be unavailable for much needed transplants. He argues against the concept of 'presumed consent' unless there is 'a valid basis for making the presumption', which is not borne out by the refusal of relatives to donate their deceased's organs, 46% refusal in the US[4] and 41% in the UK.[5] Also, population surveys often provide high rates of individuals in favour of organ donation but much lower rates of official registration. A survey by the British Medical Association (BMA) in 2017, found that 66% of 2011 persons in England, Scotland and Northern Ireland were ready to donate their organs after death but only 39% had registered on the organ donor register.[6]

In Australia, it is legal to retain tissues from an autopsy for 'transplantation, therapeutic, education and research purposes' as the use of these tissues is considered a 'public benefit',[7] provided that consent for the autopsy is obtained from the next of kin or the Coroner in forensic cases. It is a form of soft presumed consent as the relatives are still consulted by the tissue establishments, in line with National Guidelines for Organ and Tissue Donation.

However now, there appears to be a shift in opinions favouring 'opt out' systems, possibly because more people are pro-active in actually registering an objection. In Wales where a soft 'opt out' law was introduced on 1st December 2015, a population survey recorded 73% support for the legislation, which had only been 49% in 2012[8] and only 6% had opted out by the end of March 2016/17.[9] The 2017 BMA survey found that 65% of the 2011 persons who responded favoured

introducing an opt-out system (see endnote 6). The law changed in England in May 2020 and in Scotland in March 2021.[10]

While explicit consent or objection cannot be over-ridden by relatives or close persons, in countries with soft 'opt out' systems, if there has not been such definite documentation, relatives or close friends, as in the Welsh law, are contacted with a view to finding the wishes of the deceased. In Spain relatives have to confirm in writing that the deceased had not objected while in Belgium, relatives may object unless contrary to the deceased's wishes. Whatever the opinions of the relatives are, the autonomy and welfare of the donor should remain paramount.

The World Medical Association (WMA) recommends 'informed donor choice including the patient's right not to donate'[11] emphasising the need to make sure that the general public is aware of the local policies, particularly the necessity of registering their choice. The Eurobarometer survey in 2009 on organ donation and transplantation[12] revealed that 28% (range 18–64%) of those who responded were not knowledgeable about the transplant legislation in their country. There is significant worry that many are unaware of the possibility of registering an objection in an 'opt out' system.

The mandatory consent to register as a potential donor also allows for more autonomy as the potential donor can specifically choose the type of organ to be donated.

14.2.1.2 Capacity to Consent

Consent is only possible if there is capacity to consent, and a medical examination may be necessary to assess if this is possible. Capacity depends on being competent, that is having skills to enable one to understand and retain the information provided, consider its consequences and voluntarily decide on a course of action regarding health care. Moreover, in the legal sense, a legally competent individual, is a person who can participate in legal transactions. For the living donor and recipient, when in doubt as to the capacity to consent, this 'is a matter for clinical judgement guided by professional practice and subject to legal requirements'.[13]

Legal representatives, such as a guardian, can give consent for donors who are in a state of incapacity to consent, either temporary, as in a coma following drug abuse, or permanent, such as adults with mental disabilities or following traumatic brain injury or a stroke. However, the acceptance of vulnerable individuals, including minors, as donors, should be limited to when there is no alternative donor, and only if the donor suffers minimal risks - the rights of the donor should be paramount to the medical needs of the recipient. The Convention for the protection of human rights and dignity of the human being with regard to the application of biology and medicine: Convention on human rights and biomedicine,[14] also referred to as the Bioethics Convention, only agrees, in Article 20, to vulnerable donors providing regenerative tissue to siblings and then when no other compatible donor is available and if the donation is life saving. Also, the donation must be approved by a competent body, such as an ethics committee, or even a court of law. However, the Additional Protocol to the Convention on human rights and biomedicine concerning transplantation of organs and tissues of human origin, which also

deals with tissue and cell transplantation, while reiterating these principles, acknowledges that there is minimal risk in donating cells and so approves donation to a wider pool of recipients.[15]

Most states have regulated for consent to medical intervention for vulnerable individuals, including minors, especially in their mental health laws but it is also recommended to include the vulnerable individual in the decision making process.

With regard to minors, the age of consent to medical treatment varies in different countries, usually between 16 and 21 years. However, in view of the generally accepted opinion expressed in the Convention on the Rights of the Child, that a mature child must be consulted and must give an opinion, (so called assent), from the age of 14 years upwards, depending on the maturity of the adolescent, so called 'Gillick' competence, they may even be legally allowed to consent from 16 years upwards. In some countries they may 'opt in' and register as a donor as well from age 16. There is a safeguard because the potential donor is free to change their mind at any time and amend the registers. Naturally if they are potential recipients, they should also be involved in decision making. A refusal to receive a transplant in minors, may be subject to a final ruling by the medical staff taking a decision in the best interests of the minor. This may have already been addressed in national legislation and may be subject to obtaining authority to proceed from the courts.

Saviour Siblings

One specific scenario which arises is the decision by the parents of a child with a genetic abnormality, or with a malignancy, generally a lymphoid malignancy, to have another child by medically assisted reproductive techniques (ART), using pre-implantation genetic diagnosis (PGD), and HLA typing, to choose the embryo that can provide healthy haemopoietic stem cells for treating the ill sibling. Ethical issues are complex. The parents are motivated by the distress of their child and aim only for benefits of treatment but in the process a number of embryos will be discarded in the ART process. There are also potential psychological effects on the saviour sibling, once they are old enough to be informed what has happened; they might consider themselves a commodity.

Cord Blood

This is a form of directed donation where umbilical cord blood is collected for future use of haematopoietic stem cells (HSCs) when the transplant is less likely to be complicated by graft versus host disease[16] than with use of peripheral blood or bone marrow HSCs. There are private and public collections, respectively banking cord blood for private use by a family, including the donor, and for general public use. Ethical reviews and international bodies favour the public institution because of the fear that family banking 'could endanger altruistic and voluntary'[17] donations. Public institutions are rigorously regulated[18,19] and follow international Guidelines to obtain laboratory accreditation (see endnote 19) but it is 'not always mandatory for private banks to adhere to international standards'[20] and poor-quality samples have been reported in private banks.[21] Unless there is a known family condition amenable to HSC treatment, there is a low probability of the family ever

using the stored blood (see endnote 17) while a disproportionate amount of financial investment is necessary.[22] There is a move to have hybrid banks, with the possibility of private storage provided allogeneic matching can still occur (see endnote 22). Currently 'no therapies for non-blood-related diseases have yet been developed using HSCs from either cord blood or adult bone marrow'.[23] It is now accepted that consent is necessary from the mother before collection of cord blood, provided adequate information is provided, such as the Guidelines by the Council of Europe (see endnote 19), ideally during ante-natal care.

14.2.1.3 Informed Consent

What is the meaning of 'informed'? What should be communicated to the potential donor? Primarily one must make sure that there is enough dedicated time for the information session to allow time for questions and clarifications and the language used must be appropriate for the level of maturity and be well understood by the potential donor. It 'must cover retrieval, testing, storage, discard and access to medical records'.[24]

Significant risks that must be discussed concern the surgical retrieval procedure, with consideration of any possible side effects, the testing regime, which if positive may result in discard of the donation, as well as the need for follow up of the donor if the testing shows a condition that is relevant to the donor's health and family.

Donors need to be aware of their rights, depending on the local legislation, that they can withdraw their sample at any time, unless it has been already anonymised. An important aspect of transparency on a national level is strong campaigns to remind individuals of the registries that exist in that particular country.

There must be transparency as to where the harvested tissues and cells are to be banked and for what purpose they are most likely to be used. Some have argued that donors might object to the use of skin for cosmetic reasons, such as enhancement of genitalia[25] or for as yet unavailable uses, such as genetic engineering. In the UK it is recommended to obtain 'specific consent' if the tissue establishment wants to use the tissues and cells for non therapeutic use (see endnote 24). Any possible commercialisation must be stated openly, together with the possible benefits to the company and to any researchers, especially if substantial profits are likely, particularly with products developed from cells.

Informed consent from the recipient will also depend on adequate information about the expected outcomes, including an assurance of matched HLA typing and of safety with no risk of infections. The possible complications arising from the surgery, the graft itself and the long-term medication should be discussed. The potential recipients should be informed if there are alternatives to transplantation and any other treatment options should be clearly described.

One particular area of interest is the possibility of receiving unmatched marginal donation, that is suboptimal tissues, for example in terms of the age of the donor vis-à-vis the recipient's and for infected material to be transferred to a similarly infected recipient. Therefore, information should include possible alternatives to the transplant.

Counselling should always be given for the living donor, particularly for an anonymous altruistic donation. Such counselling 'can provide living donors with extra emotional support and an opportunity to voice their innermost thoughts in an environment free from judgement'.[26] There is however limited published literature on the role of counselling in organ donation, let alone tissue donation. In the ongoing evaluation of the EU blood, tissues and cells legislation, the lack of mandatory counselling for certain types of potential donors (bone marrow and peripheral blood stem cells) was pointed out at a stakeholders meeting.[27] Various forms of psychosocial assessment are used prior to organ donation, yet there is no definite evidence as to their use to identify 'those individuals who are most at need of additional support or therapeutic interventions pre or postdonation'.[28] In the UK, there is a need for mandatory independent assessment of the potential donor[29] but this does not equate with counselling.

When there is a potential directed donation, from a known living person, the recipient should be involved at the early stages with communication being centred on minimising the psychological conflicts that may arise if the potential donor fails to honour the initial interest in donating or if the proposed donor turns out to be not suitable or if the transplant is rejected by the body. The initial optimism may turn to depression and in some documented cases it has even led to suicidal attempts. These create family relationship problems which may in addition have an adverse effect on the underlying disease progression.

14.2.2 Voluntary Donation

There should be no coercion in obtaining consent, in particular no offer of payment for any donation. It is the norm worldwide, particularly in Europe[30] and the US, for the transplant to be unpaid, and for procurement to be on a 'non-profit basis'.[31]

There is strict observance to avoid the unethical behaviour, which in some countries (e.g. US, reported in 2000), led to some tissue establishments being accused of making excessive profits at the expense of donors, due to unethical practice, including 'non-consented procurement, inadequate testing, inaccurate or false donor files, irresponsible allocations and illegal trafficking of human cells, tissues and cellular and tissue-based products' (see endnote 25). Article 21 of the Bioethics Convention establishes the principle that 'the human body and its parts shall not, as such, give rise to financial gain' (see endnote 14). This is reiterated in the Additional Protocol, which also excludes 'comparable advantage'.[32] Directive 2004/23/EC requires member states to work towards promoting voluntary and unpaid donations of tissues and cells while it allows donors to be compensated for time off work, for travel and for health surveillance (see endnote 31) as does Article 21 of the Additional Protocol (see endnote 32). The Directive requires member states to report, to the Commission, on these practices, every three years (see endnote 31), and Article 12.2 prohibits promotion, including advertising, for donors with the intention of obtaining financial gain (see endnote 31). It is however

acceptable for tissue establishments to charge 'a justifiable fee for legitimate medical or related technical services' (see endnote 32) for processing fees.

14.2.3 Property Rights

Article 1 of the Universal Declaration of Human Rights[33] awards dignity to human beings and Article 8 of the European Convention of Human Rights protects the moral and physical integrity of a human person.[34] It might be appropriate to accept the view that when individuals talk about owning their body or body parts, their main interest is with 'control' and 'rights',[35] in particular in relation to autonomy, rather than what is generally considered as owning property which can be the object of commercial enterprise.

However, a legal solution of who owns the body and body parts is necessary in the context of transplantation. The legal consensus is that the 'there can generally be no property rights in a human body, living or dead' and the 'rights of individual persons in connection with their own bodies are not legally those of 'property ownership''.[36]

There are some indications that this legal position is changing. In the Washington University v Catalona[37] case, regarding ownership of research samples, the Court of Appeals stated that individuals who 'contribute their biological materials voluntarily' do not 'retain an ownership interest'.[38] If property rights could be lost, then there may be a possibility of specifically retaining the rights at consent (see endnote 36). The Court of Appeal, UK, in its decision on the Yearworth case in 2009, regarding destruction of stored sperm, indicated that the sperm was capable of being the property of the men who had produced it; the court made a case for review of the law regarding 'issue of ownership of parts or products of a living human body'.[39]

In certain countries, there is legislation that allows whoever is in possession of the dead body to give consent for the harvesting of both organs and tissues from the deceased. This is not the relative but the management of the clinic, nursing home and hospital. These laws rarely have punishments for failure to donate.

14.2.3.1 Commercialisation

So, though the human body is not considered as property or the object of commerce, in line with Article 21 of the Bioethics Convention, this does not necessarily apply to tissues and cells.[40] Body parts, 'once separate from the body' may acquire property rights 'if, as a result of the application of skill they have changed the attributes of the material',[41] and then can be marketed and traded.

Tissues and their products may be used to develop new therapeutic products or be incorporated in medical devices. Medicines,[42] medical devices[43] and advanced therapy products[44] are strongly regulated by European Directives and Regulations that have been transposed into national legislation but there is also a plethora of guidelines by the European Medicines Agency (EMA).[45] One should note that

Directive 95/34/EC[46] prohibits the use of human tissues and cells in the production of cosmetics to prevent risk of contamination with an infectious agent.

Directive 98/44/EC[47] provides for patentability of 'an element isolated from the human body or otherwise produced by means of a technical process', so tissues and cells may be patentable. Recital 26 states that the donor 'must have had an opportunity of expressing free and informed consent thereto, in accordance with national law'. However, few European member states have implemented this in their law. A main issue is that at the time of donation, there is unlikely to be knowledge of which future product is likely to be developed or which new technologies would become available to make this possible. Obtaining a patent is not necessarily related to the marketing of an invention and to commercial profits.

In Europe, the European Patent Office, established under the European Patent Convention,[48] grants patents for cell lines and cell derived protein products. These are in effect 'parallel national patents' for the European country chosen by the applicant.

The landmark decision of the US Supreme Court in the case of Moore against the University of California, in relation to patent rights and profits[49] arising from cell lines from his T cells, established that once body parts are donated, there is no right of possession. The judge stated that Moore should have 'retained an ownership interest' to enable him to sue against interference with possession and ownership interests.

14.2.4 Confidentiality

The insistence of maintaining confidentiality is covered by Article 23 of the Additional Protocol (see endnote 32) and Article 14 of Directive 2004/23/EC, which in addition specifically states that 'necessary measures' must be employed 'to ensure that the identity of the recipient(s) is not disclosed to the donor or his family and vice versa' (see endnote 31). This viewpoint is understandable as clinical experience has recognised that some donors may develop feelings of ownership, with unnatural stalking of the donor or his family, amounting to almost persecution of the recipient. On the other hand, recipients might be less than grateful to a donor if they develop post transplant complications.

Some transplant associations do however still undertake to facilitate meetings between donor and recipient, or for exchange of letters, if they both so wish.

The responsibility for maintaining confidentiality lies with the procurement organisations and the tissue establishments, which need to ensure safety of the whole process, quality control, traceability and data privacy.

They must assure the donor that all personal and medical health data, including test results, will be stored securely[50] and will only be disclosed to authorised personnel and bodies. This will ensure confidentiality is maintained without compromising safety of potential recipients or the healthcare workers themselves. Donors are allocated a 'unique identifying code'[51] to enable traceability of donations, with data being kept for 'a minimum of 30 years after clinical use, or the

expiry date'. In addition, a European coding system[52] was introduced to enable traceability across different tissue establishments and in different countries, including reported serious adverse reactions and events.

Safe processing, including storage, of data in European member states must be in line with the General Data Protection Regulation (GDPR)[53] and the national data protection legislation. This would implement safeguards against any data manipulation while allowing genuine procedures to 'resolve data discrepancies' and to prevent 'unauthorised disclosure of information'.[54]

Moreover, healthcare personnel have a legal obligation of professional secrecy and are strictly bound by the relevant professional associations to observe professional ethics, on all matters relating to maintaining patient confidentiality, respecting patients' trust and preventing unauthorised disclosure.

14.2.5 Certification of Death

Naturally for deceased donation, there must be sound protocols in place to enable procurement of tissues after the diagnosis and certification of death. Certification of death on the basis of brain death,[55] or in a few countries brainstem death, in patients on life support systems has enabled the harvesting of organs for transplantation. Most tissues, have always been available following cardiac arrest or cardiac death, even up to 72 hours after death, since it is not as crucial as for organs to maintain viability by reducing ischaemic time. However, increasingly in recent years, with advances in medical technology, there has also been acceptance of organs from non-heart-beating donors, thus also an increase in tissues and cells harvested by this procedure. This process raises multiple ethical issues, related to the dignity of the human person, put at risk by decisions regarding the time span between cardiac arrest and declaration of death and when preparatory procedures should be started and by procedures of no benefit to the dying person. Even more controversial is when to obtain informed consent, with the crucial aspect being which information to deliver and to whom, and who is to give consent. It is extremely doubtful as to the extent of understanding possible when there are few policies available in the public domain. It is also questionable as to whether, if extended cardiac resuscitation were maintained, the individual would have been resuscitated. It is beyond the scope of this chapter to expand further on these controversial issues, which have been grossly debated without a consensus, which explains why there is as yet no global harmonization of procedures across Europe at this stage. However, this makes it imperative to educate society as to the national regulations and/ or legislation concerning death certification.

The medical staff who certify death should be completely separate from donor procurement teams (see endnote 11). 'Donation should not be discussed until the family has accepted the reality of the clinical situation'.[56] Bereavement counselling should be offered to the relatives of the deceased.

14.2.6 Just Access

Patients have a right to the best health care and are increasingly more likely to participate in their own treatment decisions, thus enforcing their autonomy but as a recipient they are generally not involved in decision making as to who their donor is going to be.

Patients rely on the practice of equity by health carers. A just system of allocation, usually devised by the health authorities, is based on medical need - disease severity, possibilities of benefit and time on the waiting list, with no consideration of social status or lifestyle (see endnotes 11, 57). Immunological matching is not generally required with tissues, facilitating allocation, though corneas may need to be matched for age and immunological matching is required for bone marrow cells. The WMA proposes that all policies with regard to transplant procedures should be 'open to public scrutiny' (see endnote 11). Policies should be transparent, so one can appreciate the attempt at obtaining a 'balanced consideration of all relevant ethical principles'.[57] This should definitely apply to bespoke products and tissues.

Access also relies on geographical residence in relation to services, so appropriate health resources should be made available for recipients who live in remote rural areas. Another problem with access is for small states, who may have insufficient donors to provide an adequate donor pool for all their potential recipients, particularly with certain ethnic minorities that have specific immunological differences[58] or if there is an emergency, such as happened in the 2005 London bombings, with requests for skin.[59] Here comes into play the need for solidarity between countries to allow cross-border programmes to maximise the use of all donations and for the state to allocate health resources for such scenarios.

14.2.7 Safety Issues

14.2.7.1 Infrastructure and Procedures

Tissue establishments are responsible for the smooth running of the tissue t service. Administration must be transparent and within the regulations set by the EU Directive (see endnote 31) and all its amendments[60,61,62,63,64] which apply to the collection from donors, the processing of the donated cells and tissues, including rendering them safe from infection, storage and distribution to the recipient.

These institutions generally understand the role ethics plays in transplantation and tissue grafting and it is strongly recommended that they should have a Code of Ethics and a Code of Practice, freely available in the public domain, so as to be transparent in their activities. Ultimately this indicates respect for the donor and recipient, and engenders trust in society, in the services and, in the institution.

The tissue establishments may be sited within a hospital or a clinic or may have dedicated buildings but all must 'be located, designed, constructed, adapted and maintained to suit the intended operations'.[65] They focus mainly on ensuring the safety of laboratory facilities and procedures with emphasis on obtaining and

maintaining laboratory licensing and accreditation through stringent Quality Control in line with established EU regulations and local legislation.

Specific safety issues may arise. The use of ionising irradiation for sterilization must comply with specific regulations.[66] There is also the responsibility to keep tissue in quarantine under separate conditions due to its possible infectious nature, until the donor screening is complete and laboratory results meet the required standards.

Safety issues should be in place to safeguard the personnel, who may be at risk on exposure to infected tissues and cells. National legislation on health and safety at work places requires that such staff is adequately immunised.

There is in addition the aspect of documentation with management of an established data system that ensures full traceability, as already discussed in Sect. 14.2.4. The data system will match the donations to recipients to ensure just allocation in terms of medical scoring of the need of the potential recipient, will keep records of adverse events for both donor and recipient and will report to European authorities to ensure cross-border safety.

14.2.7.2 Healthcare Personnel

Any healthcare personnel, whether working with transplant teams in hospital, directly caring for either the donor and/or the recipient, or those employed by the procurement institution or the tissue establishment, are bound by the same professional standards as any other healthcarer, primarily by national legislation regulating these professions, which tends to be even more stringent than warranted by the Directive.[67] Therefore, the level of qualifications and adequate training of personnel is easy to ascertain from public registers of the professions.

Ethical issues that concern personnel directly working with patients include respect for the patients, obtaining informed consent and adherence to the principles of Patient Charters. Trained staff should be available to provide psychological support and bereavement counselling.

14.3 Societal Issues

14.3.1 Altruism

Pure altruism is only possible for the living donor as they are sacrificing their body integrity for the good of others, whether it is a directed donation towards a relative or close friend or a completely undirected donation. There should be the desire to be of benefit to others without expecting any return. One must not underestimate the sacrifice of losing body integrity, that can result in psychological effects. Certainly one can argue that there could be an element of emotional coercion in reaching a decision to help someone who is close to you. Interestingly it is only in recent times that pure altruistic non directional living donation has been widely accepted. This

obviously reflects the change in culture, which is celebrating individual autonomy, at the expense of condoning self-imposed injury.

'The act of donation should be regarded as heroic and honoured as such by representatives of the government and civil society organizations'.[68] This strong statement is present in the Declaration of Istanbul in relation to living organ donation but can be extrapolated to tissues. There is however still an uneasy acceptance of directed deceased donation and it is only considered ethical if there is definite evidence of the will to donate prior to death, such as an Advance Directive; if the latter nominated a specific recipient, the wish would have to be respected.

14.3.2 Education

The benefit of receiving a transplant is obvious to the recipient, but there may also be benefits to society in general, improving the healthy workforce.

Education campaigns geared towards the general public have been used, with varying success rates to increase donations. This has been discussed in the context of 'opt in' versus 'opt out' methods of obtaining consent. There needs to be a strategy to maintain momentum in awareness campaigns, such as continuous education programmes, geared towards different groups of society, including programmes for schools, public dialogue and seminars and media coverage, all aiming to explain and promote transplant procedures. As the field of transplant medicine keeps expanding due to new technology, it becomes even more important to understand the opinions, attitudes and concerns of society in general and also of specific ethnic, religious and cultural groups. These views will help in developing transplant programmes relevant for the community.

14.3.3 Cross-Border Exchange

Acknowledging the scarcity of donations has led society to maximize the effective use of organ donations through cross-border transplant programmes, such as Eurotransplant,[69] a non-profit organisation based in the Netherlands, with collaboration between eight European countries, Austria, Belgium, Croatia, Germany, Hungary, Luxembourg, the Netherlands and Slovenia. They share donor registries and match donor data to potential recipients and analyse follow up data.

The European Registry for Organs, Tissues and Cells (EUROCET),[70] started as a project in 2005 'aimed at setting up a registry on organ, tissue and cell donation and transplantation activity shared by old and new Member States' but has now evolved to become a database. It collects information from European member states on cell and tissue transplant activities, while data for cell activities includes data from non European countries.

14.3.4 Trafficking

In 2003, the World Health Organisation carried out a consultation process on issues of global concern in relation to transplantation, which resulted in a resolution that already recognised the extent of trafficking in human organs and tissues. It urged states 'to take measures to protect 'vulnerable groups from transplant tourism and the sale of tissues and organs, including international trafficking'.[71]

Transplant tourism may unfortunately be a result of ineffective transplant policies in the country, thus pressurising the well to do to find tissues abroad. This places the poor at a disadvantage. On the other hand, trafficking increases the discrimination between persons of different social status but it moreover exploits the poor and may even put their health and life at risk.

Though all countries are against trafficking,[72] abuse of the system is prevalent worldwide though difficult to know the real level - not even for the more common organ trafficking do we have a clear picture, let alone for the less invasive techniques used for cell and tissue procurement. It is difficult to control since vulnerable individuals are always ready to sacrifice body integrity to make some money for their families.

The International Society for Stem Cell Research (ISSCR) urges medical licensing bodies, legal authorities, patient advocacy organizations, physicians, and others to exercise their influence to discourage commercial provision of unproven autologous cell-based interventions outside of clinical trials.[73]

A new form of trafficking is emerging in the digital world we live in now. Australia has reported internet matching, buying and selling of organs, sometimes through specific organisations and community groups that intervene on behalf of recipients.[74] This illicit practice has really grown[75] with a recent publication identifying 417 unique websites advertising stem cell-based therapies, the majority in the US. There is now on the internet direct marketing of stem cell interventions[76] of very little proven value,[77] leading to a new label, that of stem cell tourism. Even though at times the patient does not actually travel abroad.

The EU directive[78] strongly condemns advertisements as these not only include financial gain but discriminate against just access in favour of a well to do person or a specific community group.

There is an established trade of tissues between institutions and across countries. Often donors and recipients are unaware of this, possibly because tissue products are produced years after the donation.

14.4 Regulatory Instruments

14.4.1 Donor Consent

Informed consent is well enshrined in Article 6(1) of the United Nations' Universal Declaration on Bioethics and Human Rights, 2005[79]: 'Any preventive, diagnostic

and therapeutic medical intervention is only to be carried out with the prior, free and informed consent of the person concerned, based on adequate information'. This had already been a well-accepted practice in Europe with Article 5 of the Council of Europe's Convention on human rights and biomedicine (see endnote 14) stating that 'An intervention in the health field may only be carried out after the person concerned has given free and informed consent to it'. The Convention also addresses in Article 19, consent for procurement of both organs and tissues from living donors but only if no donations from 'a deceased person and no other alternative therapeutic method of comparable effectiveness' are available (see endnote 14). The Additional Protocol retains this proviso but specifically allows living directed donation[80] of organs, even beyond close relationships, when national law provides for 'an appropriate independent body, for example an ethics committee, to consider each case'.[81]

Both instruments allow donation of regenerative tissue from living vulnerable persons incapable of consent but only to siblings if there is a potential to save life and if no other compatible donor is available. Cells can be donated to a wider pool of recipients (see endnote 15) beyond family relationships.

Interestingly the Additional Protocol also allows implantation of regenerative tissue and cells not specifically procured for donation 'if the consequences and possible risks have been explained to that person and his/her informed consent, or appropriate authorisation in the case of a person not able to consent, has been obtained'.[82] The Explanatory report considers this article in relation to domino transplants[83] from which tissue may be retrieved but one wonders whether it could also apply to residual tissue left after surgery.

With regard to donation from the deceased, the Protocol insists that no donation should occur if it was known that the deceased had objected to donation but leaves the regulatory framework to the national laws, including specific indication as to what to do if the wishes of the deceased are not clear.[84]

Directive 2004/23/EC (see endnote 31), Article 13, requires consent in line with national laws, provided all the information listed in the Annex is given to the potential donor prior to procurement. This includes an explanation of 'the purpose and nature of the procurement' and the risks, together with the safeguards employed to protect the donor, including data protection and medical confidentiality.

More recently, the WHO Guiding Principles on Human Cell, Tissue and Organ Transplantation, 2010[85] were published to provide an update of the original 1991 guidelines which dealt only with organs.

14.4.2 Transplant Institutions

These are well covered by various EU Directives. Directive 2004/23/EC is concerned with quality and safety for the entire transplant service for human tissues and cells, including 'haematopoietic peripheral blood, umbilical-cord (blood) and bone-marrow stem cells, reproductive cells (eggs, sperm), foetal tissues and cells

and adult and embryonic stem cells'[86] though preamble 12 acknowledges that it is up to the individual states to decide which cells can be used in their country.

Safe practices are identified in Directive 2006/17/EC.[87] There are 'selection criteria' for both living and deceased donors with an emphasis on excluding cases where there is insufficient medical evidence to ensure a safe donation, as well as the required laboratory tests for donors and coding for traceability. This includes documentation on all products coming into contact with the tissues and cells. These procedures are covered by standard operating procedures (SOPs) as are all other laboratory processes. Directive 2006/86/EC[88] covers licencing and certification of tissue establishments and the reporting of serious adverse reactions in both donors and recipients by procurement organisations and organisations responsible for human application of tissues, with reporting to the tissue establishments and notification to the competent authority. Adequate documentation with a set of medical information, must be retained for 'at least 30 years' to ensure traceability. In fact, 'a single European code' (see endnote 63, 88), is to be used to facilitate movement, including cross-border, of tissues and cells.

Cross border movement is regulated and tissues imported from outside the EU have to reach the standards laid out in Directive 2015/566 (see endnote 64).

In the US, there are two main legal instruments covering donation of human biological material.[89] The revised 1968 Uniform Anatomical Gift Act (UAGA) and its 1987, 2006 and 2009 amendments,[90] is universally adopted in all US states. It provides for an 'opt in' donation by a living person, deals with consent and donor rights to donate and to refuse to donate and allows gifting through an Advance Directive. It also provides for a relative to consent to donating the body, or parts of the body, of a deceased relative who had not made a gift in life and had not been against donation. The 1984 National Organ Transplant Act (NOTA)[91] is the federal law responsible for the service, procurement, registries, allocation and matching of donations from the deceased, through the establishment of the Organ Procurement and Transplantation Network (OPTN), managed by the United Network for Organ Sharing (UNOS). Despite the name, it also covers tissues without distinguishing between organs and tissues. Since tissues are so different from organs, there is an argument for a separate tissue-specific law.

The Patient Self-Determination Act, 1991, is the federal law that makes it mandatory for healthcare institutions to notify admitted patients about their rights in healthcare decisions, including the right to prepare an Advance Directive.[92] However, not all Advance Directives include a section on views regarding organ donation.

14.4.3 Data Protection

An essential part of transplant procedures is the collection of medical data with the donation of biological material, the donor data, which helps to ensure the best allocation as well as guarantee safety to the recipient. All processing, testing and storage procedures must be documented for traceability, including information on

all materials that come into contact with the tissues and cells. Donor data of imported products is also maintained by the tissue establishment. The EU directives require such data to be kept for a minimum of thirty years (see endnote 63, 88), whether this is in paper format or an electronic record.

Data is protected through strict adherence with the General Data Protection Regulation (GDPR).[93] 'Data concerning health' meaning 'personal data related to the physical or mental health of a natural person, including the provision of health care services, which reveal information about his or her health' is categorized as one of the 'special categories of personal data'[94], which can only be processed for specific conditions, in this case, healthcare.[95] However, this is overridden if there is explicit consent from the data subject, to process the data for a specified purpose.[96] Even more relevant to transplant procedures is that processing is specifically allowed when: 'processing is necessary for reasons of public interest in the area of public health, such as protecting against serious cross-border threats to health or ensuring high standards of quality and safety of health care and of medicinal products or medical devices'.[97]

Informed consent from the potential donor should cover consent to the retention of donor data and of processing data. Tissues and cells will be pseudonymised[98] but the donor should be aware of who is the Data Protection Officer of the tissue establishment and who has access to the data, such as the data controller and the data processor/s. The facility would have undertaken a Data Protection Impact Assessment, as this is mandatory for 'processing on a large scale of special categories of data'.[99]

The GDPR, Article 15, gives the right of access to health data, including medical records and as outlined in the Annex of the Directive 2004/23/EC, the donor has 'the right to receive the confirmed results of the analytical tests, clearly explained' (see endnote 31). There is also the right to request correction of any 'inaccurate personal data'.[100] A person can withdraw consent to being a tissue and cell donor and request the right to erasure of personal data be employed and the name be removed from the donor register.

Transplant data needs to be shared between the hospitals and clinics and institutions in the programme, on a local and international level. The transfer of data to third countries, a common event in the search for the best transplant tissue for the recipient, is protected by the provisions of the GDPR, Chapter V. The Commission decides who has 'an adequate level of protection', otherwise data transfer can only occur if there are appropriate safeguards in place.

The Council of Europe addresses the need to 'develop and maintain harmonised national living donor registries'.[101] There should be 'interoperability with currently existing transplant databases, including linkage with corresponding transplant recipient registries, to facilitate data exchange'.[102]

14.4.4 Training

Countries have a responsibility to ensure that the transplant services offered are up to date with current medical and technical knowledge and that personnel providing these services have 'appropriate qualifications' and are receiving basic training and further 'timely and relevant'[103] training. European Directives lay down the mandatory requirements for training of the procurement teams[104] and for staff at tissue establishments[105] since this ensures quality of the grafts and safety of donors, recipients and staff. Professional competence also ensures that licences and accreditation of facilities are renewed. The Commission[106] provides guidelines regarding regular inspections of tissue establishments and the required training and qualifications of the inspectors. There are various associations and academic bodies that provide training courses, both at introductory level and advanced courses for continuing professional development. These can be hands on, or on line, courses and may be aimed specifically at different groups of professionals.

In 2017, the European Commission's Directorate-General for Health and Food Safety embarked on a public consultation to assess the current transplantation legislation and its effectiveness and whether it was still 'fit for purpose'. Although most agreed legislation had been beneficial, it was felt that 'it is not up-to-date with scientific, technological, epidemiological or societal developments and that process of updating is not flexible or quick enough to adapt to them.' Stakeholders would prefer technical legislation to be changed to guidance, quoting the Council of Europe's guidelines' as a 'suitable reference for up-to-date technical standards'.[107] This Guide[108] states categorically that it 'includes recommendations considered to be minimum standards that align with the principles set out in the various relevant European Union (EU) directives' but offers much more, as it sets out relevant and current scientific information that is necessary to formulate transplant policies.

14.5 Special Considerations for Cellular Therapies

Some of the most challenging aspects of cellular therapies concern informed consent and traceability. A number of complex issues exist, including;

- the implications for the donor in the event of identification of known or novel infectious or genetic disease markers, either at the time of donation or at any time in the future;
- the implications for potential previous recipients of the development of post-donation disease in the donor, which may have an infectious or genetic basis;
- the extent to which the development of clinical problems in recipients may have implications for the donor and/or his/her family; and
- the nature of informed consent from a donor's perspective, and whether or to what extent they should be able to waive feedback.

Cells can be either minimally manipulated (e.g. haematopoietic stem cells or cord blood cells), somatic cellular therapies (e.g. corneal limbal stem cells or mesenchymal stromal cells) or stem cell lines (e.g. induced pluripotent cells and human embryonic stem cells). Somatic cells are collected from the donor, isolated and cultured for varying periods of time prior to transplantation. They can be given to one recipient or potentially to a large number of recipients. Stem cell lines have been considered together, as many of their characteristics are shared: such cell lines will proliferate indefinitely in culture and can differentiate into most, if not all, of the cell types present in an adult. They therefore present the possibility of indefinite scalability and of a single donor contributing multiple cell or tissue products to multiple recipients over an extended period.

The matrix below is meant to illustrate where each product sits in relation to the others with respect to two key areas:

- the complexity of the product, and
- the potential risks of the product.

Generally speaking, as the complexity of each product increases, the number of recipients who might benefit from the product also increases. This relationship is a correlation rather than a causal one and is a broad generalisation. It can be assumed that in the current state of technology, simple (or relatively simple) products are usually given to one or very few patients. As the biological culture systems become more complex, the number of patients that could potentially be exposed increases significantly and it is envisaged that the number of patients that could be exposed to a product derived from a single biological system may reach thousands, or possibly even hundreds of thousands. Clearly, there are exceptions to this and some simple products can be given to large numbers of people.

Further, in an attempt to compare the issues involved within the different products, three key issues have been chosen:

- the *regulations* that govern each product (shown in green)
- the *ethical and consenting issues* surrounding each product (shown in red)
- *traceability* issues (shown in yellow) (Fig. 14.1).

The size of each coloured sphere is intended to reflect the complexity of each key issue considered. These are shown in the matrix above. Cellular therapies have been grouped in boxes and those that are in the same box are generally considered to have approximately equivalent levels of risk/complexity.

It is important to note that the chart above is an oversimplification since there is a considerable degree of overlap between the various products/categories. However, it helps to tease out the various complex issues involved.

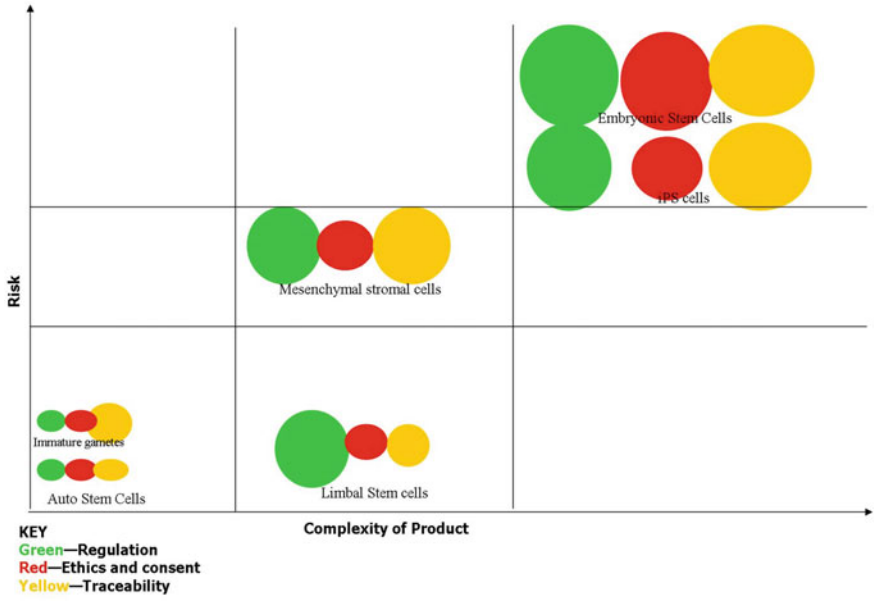

Fig. 14.1 Consent & traceability matrix Source: Donation of Starting Material for Cell-Based Advanced Therapies: a SaBTO (the Advisory Committee on the Safety of Blood, Tissues and Organs) Review (June 2014). https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/326823/Cellular_Therapy.pdf

14.5.1 Capacity to Consent

It is essential that the donor has capacity to consent. When the donor is a child, the principle is that those minors who have the ability to consent should be asked to do so following the Gillick principle.

14.5.2 Limits of Certainty of Consent

As an emerging technology that is developing rapidly, uncertainty is inherent in many aspects of cellular therapies, including the exact therapeutic advantages that may follow; there will be limits to the extent to which the risks and benefits can be identified and quantified. When people are being given information on which to make a decision, whether it is as a donor of cells or as a recipient of treatment with a cellular therapy, these limits of certainty should be communicated to them, as part of the consent process; and it is important to manage the expectations of those coming forward to donate.

14.5.3 Scope of Consent

The scope of the consent needs to be reasonably explicit, although a balance needs to be struck. A blanket, generic consent may mean that the person giving consent does not know or understand the implications of what they are consenting to, and yet if the initial consent form is too detailed, it may be too restrictive and exclude the possibility of future (non-controversial) use. This illustrates the importance of consent being thought of as a process, rather than an event. It is also important for people to understand the limitations of traceability and the circumstances in which they may or may not be alerted to an issue arising in relation to their traceable donation. It is essential for example to contact a donor if incidental findings have implications for themselves, their families or public health. Donors may choose whether or not they wish to be informed of findings of uncertain import. As supply lines lengthen, and as cellular therapies become more complex, involve more intermediaries and have potentially global reach, the practical ability to trace back donations becomes significantly more difficult.

14.5.4 Duration of Consent

Consent can be given for a limited period of time, or can be enduring. It is especially important to be explicit about duration when dealing with advanced cellular therapies because periods between donation and subsequent therapeutic use can be extremely long: in some cases, several decades. In most cases of cellular therapies, it would not be appropriate for consent to be time-limited - but again, the donor needs to be aware of the consequences of this. Given the lengthy and potentially complex existence of some donated material, it is particularly important to be clear about the ability to withdraw consent, and the stages at which this can be done / the point at which it becomes impossible. It is therefore of key importance to ensure that initial consent is right. It is possible and desirable to take enduring consent, initially, provided sufficient thought has been given to potential future uses of the starting material. This is much more preferable to trying to re-consent.

14.5.5 Commercialisation

Whilst research in cellular therapies is currently spread across academia, the public sector and private enterprises, it is likely that translational work in the future will be led by commercial enterprises. Indeed, commercial involvement may be the only way, in most cases, that developments will take place and the potential benefits of such therapies be realised. Therefore, in obtaining consent from donors in respect of any type of starting material, any commercial involvement needs to be explained upfront and be explicitly covered. If commercial involvement is possible or likely, donors may need to be given an option to consent to their cells and tissues being used for non-commercial purposes but excluded for use by commercial organisations, though in some instances this might mean the donation would not be taken.

14.6 The Future

We are at an exciting stage as we move into future medical practice and new techniques. Advanced therapies, such as the use of gene therapy and tissue engineering, are already on the market, regulated by Regulation (EC) No 1394/2007 (see endnote 44) and Directive 2009/120/EC[109] and EMA guidelines. Xenotransplantation offers potential for increasing availability of organs, tissues and cells. While xenogeneic cell based therapy is already available and regulated by EMA guidelines,[110] we are still awaiting the outcome of clinical trials of tissue xenotransplants in human recipients. One of the main ethical concerns has been the risk of transmission of zoonosis but new techniques for genetic engineering of pigs have helped to delete porcine viruses and suppress immune responses.

Detailed review of these is beyond the scope of this chapter.

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Human Embryonic Stem Cell Banking for Clinical Applications—20 Years from Their Isolation

15

Paul A. De Sousa

Abstract

Twenty years on from the first reported isolation of pluripotent human embryonic stem cells (hESC) from in vitro fertilised preimplantation embryos, clinical evaluation of derivative cell products is ongoing. Contrary to expectations that pluripotency induction by genetic reprogramming would replace commitment to hESC source material basic and translational research and application has continued. Here we update on current clinical, translational and scientific progress and issues in human embryonic stem cell banking. These reflect transformation of an academically lead research into quality assured good manufacturing practice grade cell banking underpinning active clinical investigations.

keywords

Human embryonic stem cells · Induced pluripotent cells · Advanced cell therapy · Epigenetics

15.1 Introduction

The production and banking of human embryonic stem cells for clinical application is a subject upon which much has been written by us [1–4] and others [5–7]. With the advent of cell reprogramming methodologies, the spotlight has shifted in recent

P. A. De Sousa (✉)
Centre for Clinical Brain Sciences, College of Medicine and Veterinary Medicine, University of Edinburgh, 49 Little France Crescent, Edinburgh EH16 4SB, Scotland
e-mail: paul.desousa@ed.ac.uk

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years to induced pluripotent stem cell banking. However, as the original source of human pluripotent stem cells first reported twenty years ago [8], embryonic stem cells derived from surplus to infertility treatment preimplantation conceptuses procured by informed consent of donors undergoing assisted conception, continue to be regarded as the gold standard against which induced pluripotency is defined. This is reflected by a greater knowledge base arising from 10 years of research preceding the first reports of pluripotency induction [9, 10], not to mention the 20 years of mouse embryo stem cell research before that [11, 12]. By way of estimation, a best match search of biomedical citations on Pubmed using the terms "embryo stem cells" versus "induced pluripotent stem cells" yields almost 3 fold more citations (www.ncbi.nlm.nih.gov/pubmed; at time of writing 41,569 versus 15,000, respectively). The key conclusion arising from this collective research is that whilst there is an undeniable resemblance and relation between the two resources, embryo and induced pluripotent stem cells are distinct entities with overlapping and distinctive properties. These reflect their tissue of origin, method of creation and subsequent cultivation and storage, all of which have potential to induce genetic and epigenetic perturbations altering cell growth and lineage potency [13, 14]. Thus, advances in the state of the art of banking of both entities has relevance to each other but still requires adaptation or verification of utility and efficacy. The aim of this chapter is to update on progress of practical and scientific issues associated with human pluripotent stem cell banking, with a specific emphasis on human embryonic stem cells. Beginning with a comment of the relative merit and ethics of induced versus embryonic stem cells as source material for advanced cell therapies, we update on the state of clinical evaluation of derivative cell therapies and the development of consensus standards for cell banking in recent years, with particular attention to ethics and data standardisation. Lastly, recent scientific developments impacting on quality control and safety are discussed.

15.2 Human Embryonic Versus Induced Pluripotent Stem Cells as Clinical Source Material

Although both embryo and induced pluripotent stem cell resources have potential to be applied as source material for autologous therapy, the predominant focus of the field to date has concerned their utility as "one to many" allogeneic cell therapy resources. Without manipulation, embryo and adult tissue derived pluripotent stem cells and derivative cells will present cell surface histocompatibility antigen repertoires possessed by the donor of origin, whose expression varies in accordance with derivative cell type. These will normally be heterozygous for Type I and II Multiple Histocompatibility Complex (MHC) alleles. Sourcing adult donors which are homozygous for major MHC alleles has been touted as a route to creating finite collections of hiPSC to facilitate immunological matching of derivative cell products with prospective recipients [15–19]. However, the creation of uniparental haploid or diploid parthenogenetic (maternal genome only) or androgenetic

(paternal genome only) embryo derived hESC, constitutes an alternative avenue to achieving the same end from embryo sourced tissue [20–24]. Single donor genome specific somatic cell nuclear transfer embryos also provide a route to creating adult donor-specific MHC antigen constitution [25, 26]. In principle all methods of pluripotency induction and construction of genomic and non-genomic (ie. cytoplasmic/mitochondrial) components carry with them potential for epigenetic and genetic perturbations that may impact on MHC antigenicity as well as resulting cell function and potency [27–30] These arguably would be the most pronounced in uniparental embryos lacking maternally or paternally imprinted gene expression [23, 31], thus favouring hiPSC as the preferred resource for MHC haplotype representation.

Practical advantages aside, the creation of adult tissue of origin induced pluripotent stem cells is commonly described as more ethical than embryos, based on strongly held but variable religion-based convictions and perspectives on the commencement of human life [32–34]. On this note, it is worth remembering that adult tissue procurement only shifts the focus of ethical concern from the use of embryos for purposes other than reproduction to safeguarding the rights and freedoms of adult tissue donors. This includes privacy arising from the use of cell line associated genetic information generated in the course of characterisation and commercial exploitation [32–36]. Further, the creation of human entities with embryo-like features, such as is the case for hiPSC and derivative organoids, necessitates a rethink of ethical distinctions and boundaries which previously were defined by the natural origins of tissue [32].

15.3 Human Embryonic Stem Cell-Based Therapies

15.3.1 Currently Under Clinical Evaluation

Momentum for hESC based Advanced Cell Therapy (ACT) has built up in recent years after a false start in 2009 owing to subsequent commercial abandonment of a phase I safety evaluation of hESC derived oligodendroglial cell therapy for spinal cord injury in 5 recipients. Long term follow-up for this study communicated in 2016 reported no adverse responses and reduced deterioration of patients' spinal injury [37]. At time of writing (June–August 2018) searches of the US based international clinical trials registry (www.clinicaltrials.gov) for recruiting or completed hESC based interventions retrieved 15 trials. Eleven of these were for Phase 1/2 evaluation of retinal pigmented epithelial cell products for retinal diseases. Published follow up assessments for some of these have reported no adverse effects and gains in visual acuity [38–41]. The remaining recruiting or completed studies have been for neurodegenerative diseases (Amyotrophic Lateral Sclerosis, ALS, and Parkinsons), and ischaemic heart disease. A clinical evaluation of safety, tolerability and efficacy of an hESC based cell product for Diabetes is currently listed as active but not recruiting. Meanwhile, other competing academic and

commercially led programmes for these and other conditions, such as Huntingtons Disease, and skin wound healing, approach clinical evaluation [42, 43]. Additionally, commitment to preclinical development of next generation 3-dimensional tissue constructs with/without interfacing biomaterials and extracellular vesicle products remains strong [44–51]. These all substantiate further progression and evolution of hESC based ACT for decades to come.

15.3.2 Clinically Qualified Source Materials

The first clinical evaluation of hESC-derived oligodendroglial cells for spinal cord injury utilized one of the original research grade hESC lines sourced from an Israeli donor [8], subsequently manufactured and tested to comply with US Food and Drug Administration Centre for Biologics Evaluation and Research (CBER) standards. Since then there have been reports of the creation of clinical grade hESC lines compliant with US FDA CBER [52, 53] and European Union Tissues and Cells Directives [2, 54–72], and many lines self-declared by developers and/or the centres at which cell lines have been deposited as being clinical grade (eg. UK https://www.nibsc.org; US https://www.wicell.org; Korea https://kscr.nih.go.kr). An important distinction between US and European standards is guidance to advanced cell therapy developers from the former on the transitioning and qualification and testing of research grade cell resources to a quality assured Good Manufacturing Practice (GMP) standard. This differs from European Directives and Regulations emphasising implementation of quality standards for procurement, processing and banking from the outset [2]. Confidence in retrospective qualification of a cell resource as currently guided by CBER, is constrained by the availability of information to do so. This is particularly important for screening of adventitious pathogens for which validated tests with known thresholds of sensitivity are lacking.

15.4 Consensus Standards for Cell Banking

Over the last decade, the International Stem Cell Banking Initiative (ISCBI) funded by the International Stem Cell Forum (www.stem-cell-forum.net) has yielded consensus guidance for quality assured human embryo and induced pluripotent stem cell banking and supply [73, 74] for clinical [5] and non-clinical [75] applications. Co-ordinated by the UK Stem Cell Bank, the overarching aims of the ISCBI have been to "*create a global network of existing stem cell banks, support the development of new banks in member countries, share knowledge about ethical and regulatory issues and assist in the development of international standards for banking, characterisation and testing, thereby creating a solid ethical framework for international stem cell banking and research*." Updates from workshops address hurdles and progress of individual participating members [76] and consensus

achievements. The last workshop in 2016 [76] highlighted advances in ethics and data standardisation summarised here with progress since.

15.4.1 Ethics

ISCBI efforts to foster common approaches for development and evaluation of ethical issues associated with informed consent and procurement of tissue are balanced by respect for moral diversity and sovereignty as reflected by individual national regulatory frameworks. These have focused on analysing policy convergence and identifying commonalities in the adoption of ethical safeguards to respect donor autonomy and privacy interests and to ensure the ethical integrity of research as well. Useful guidance for best practice in establishing ethical safeguards which have been developed for bio-repositories is available from the International Society for Biological and Environmental Resources (https://www.isber.org/page/ISBERTools). Additionally, in 2015 the International Society for Stem Cell Research (ISSCR) updated its guidelines for global standards for stem cell research and clinical translation originally drafted in 2006 and updated in 2008 (see Commentary, Daley et al. 2015). In Europe, the new General Data Protection Regulation (GDPR; https://ec.europa.eu/commission/priorities/justice-and-fundamental-rights/data-protection/2018-reform-eu-data-protection-rules_en) which took effect in May 2018, has increased the regulatory burden on biorepositories to safeguard privacy particularly with respect to donor identifiability resulting from genetic sequence analysis. While this has been seen as a particular concern for human induced pluripotent stem cell banking and research, this is no less relevant to human embryonic stem cell banking. Key recommendations from bio-ethicists for human induced pluripotent stem cell banking include [77]:

- Explicit consent for derivation of pluripotent stem cells and processing of personal and genetic data that will be used to characterise the cell lines.
- Explicit consent for stem cell lines and associated data to be made available to the wider scientific community, including commercial companies.
- A requirement for institutions to have a long-term plan for maintaining connections between pseudonymized data and sample donors. This connection must be durable and enable access from subsequent data controllers, even if the samples/data are produced by a fixed-term project or consortium.
- Awareness of data controllers and processors of increased administrative responsibility associated with GDPR accountability and transparency provisions.
- Implementation of a privacy by design approach to all operations.
- Compliance with the principle of data minimisation wherein collected data is related to purpose for which donated tissue is processed. Thus, usage for which consent is sought should be broadly conceived, and mechanisms should be in place to recontact donors to update their data or provide additional information in the event intended usage is outside the scope of consent.

- Restriction on transfer of data on EU citizens, including pseudonymised data to data processors in non-EU countries unless adequate protection for data security in place. This should be ensured by formal Material Transfer Agreements stipulating standard data protection clauses.

15.4.2 Data Standardisation

A critical facet of cell banking is unambiguous cell line authentication to preclude loss of associations between data and cells. The risks of such have increased over the last decade with the rapidity with which human induced pluripotent stem cell lines have been generated, exchanged and used by individual research labs. Although a nomenclature for hPSC has previously been proposed [78] its widespread acceptance has required the establishment of a central registry and agreements on a minimal set of measures to identify and authenticate cell lines with which a unique identifier (UI) can be established in addition to data access policies that comply with the data protection provisions stipulated by the donors informed consent. This has been recently achieved [79], with an automated tool for establishment of a standard nomenclature for referencing and authentication of pluripotent stem cells accessible online as a manual data entry form available via the global registry hPSCreg [80] (https://hpscreg.eu/user/cell_line_name/create). The UI which this creates modifies and integrates the previously proposed nomenclature for hPSC [78] with the nomenclature system of the International Cell Line Authentication Committee (ICLAC; https://iclac.org/resources/cell-line-names/) so that codification of cell source, cell line, and cell type is complemented by codification of relatedness to its origin and genetically modified derivatives. The first exemplified implementation of this standard has been in the context of the European Bank of Induced Stem Cells (EBiSC) providing centralised and standardised access to hiPSC lines generated by independent laboratories across Europe [81].

Concurrent global development of hPSC banking and registries has also defined a necessity to establish minimum information standards to consistently structure data and make it reproducibly available to users, other banks and the public. The first minimum information guidelines for standardising data entries was organised by international consortia to integrate microarray data from various platforms, followed by the same for biomedical or biological Investigation projects and cellular assays [82, 83]. As of 2015 at least 20 human pluripotent stem cell information resources have been established around the world amongst whom collaboration has yielded a proposal of guidelines for Minimum Information About A Cellular Assay for Regenerative Medicine (MIACARM) covering both omics assays applied to cells being banked and all other practical information underpinning their subsequent usage [84]. Further international development and implementation of these

will benefit from engagement with World Health Organisation Regulatory Standards for Biologicals (https://www.who.int/biologicals/en/) and the Organisation for Economic Co-operation and Development (https://www.oecd.org).

15.5 Basic Scientific Developments Impacting on Quality Control and Safety

15.5.1 Genetic and Epigenetic Instability Affecting Lineage and Oncogenic Potential

The immortality of human pluripotent stem cells in principle conferring the unique practical advantage of infinite expansion capacity has, since their discovery, motivated study and concern for the genetic and epigenetic stability of cells during expansion and differentiation, and in the case of induced pluripotent stem cells, following cell reprogramming. Concern was substantiated by evidence in early studies of both minor (ie. microsatellite, copy number) and major (chromosomal rearrangement and aneuploidy) genomic alterations, and aberrant methylation of DNA (imprinted genes, gene and non-gene associated CpG islands, and retroviral elements). In some cases, these were associated with growth advantage (eg. chromosome 20 amplicon), alteration of gene expression and differentiation or oncogenic potential (eg. gain of chromosome 12 and 17q) [85–94]. Latterly, whole genome studies have reinforced concern reporting variation in genetic structure/integrity across research [94] and clinical grade cell lines [95]; associated with cell culture media conditions [96, 97], length of culture [98], and handling practices (eg. single cell passaging [99]; high density culture [100]; and exposure to trace levels of mitomycin C, historically used to inactivate supportive feeder cell populations [101]). It is easy to overemphasize the significance of these findings and to lose sight of the fact that they are based on study of cell populations under diverse and often sub-optimal culture environments attempting to maintain a cellular identity which either does not exist in vivo, or if it does, does so only transiently. Under more optimised culture conditions DNA microsatellite instability, chromosomal mosaicism and rearrangements, aneuploidies occur as low-grade events [102, 103] [104]. Further, whilst loss of imprinting can be observed [105] undoubtedly impacting on subsequent differentiation potential, conserved epigenetic signatures associated with pluripotency are maintain across cell populations irrespective of cell line provenance and culture conditions [105, 106]. Collectively these studies underpin a necessity to monitor genetic and epigenetic stability of undifferentiated and differentiating cells but also to define rational and informed quality control specifications that respond proportionally to observed changes on the basis of proven or evidence substantiated risks to lineage potency or oncogenic potential.

DNA replicative stress and chromosome condensation defects downstream of alterations in the expression of actin cytoskeleton genes and their regulatory

transcription factor (ie. Serum Response Factor, SRF) have been cited as a driver of genomic instability manifesting as chromosomal aneuploidies impacting on oncogenic potential [104]. The actin cytoskeleton is highly important for chromosome condensation and segregation during mitosis [107] and unlike somatic cells, hPSC have inherent deficiencies in cell cycle checkpoints which normally govern these events (ie., intra-S [108, 109]; G2/M [110]). SRF was first identified in studies investigating the response of fibroblasts to serum addition. These findings raise the question of whether chronic or periodic depletion of bFGF (which results in decreased levels of SRF in hPSC [104]) could augment the incidence of chromosomal aneuploidy in culture.

Recently whole exome sequencing of 140 hESC lines, 26 of which were produced for clinical use) and 117 hiPSC lines disclosed numerous mutations in the G2/M checkpoint Tumour Suppressor protein TP53. Five unrelated hESC lines had six inactivating mutations of the type normally seen in cancers. Another nine mutations predicted to cause errors in the DNA binding domain of p53 were found in hiPSC. The proportions of TP53-mutated cells increased with the number of passages in culture and during some steps in differentiation [111]. Dominant negative single copy deletions that affect TP53 function have also been reported by others in hESC [96] and hiPSC [112, 113] with the effect of markedly increasing cell proliferation and survival [114]. TP53 is required for mesendoderm differentiation and transient silencing or inactivation of TP53 increases the efficiency of producing iPSC [115–118]. Interestingly, silencing of TP53 also promotes CRISPR-CAS9 gene editing/engineering of hPSC [119]. An implication is that hPSC lines which may be particularly amenable to genetic engineering may harbour mutations affecting TP53 or associated DNA repair mechanisms resulting in greater oncogenic and/or diminished mesendoderm lineage potential. Collectively these studies substantiate diligent quality control screening and serial assessment of TP53 and associated pathway mutations and gene expression throughout the spectrum of stem cell isolation, expansion and differentiation, following any genetic engineering and before transplantation of derivative products.

15.6 Summary

Consistent with expected multi-decade timelines for translation of basic science into real world practice, the current state of play of human embryonic stem cell banking for clinical applications reflects a broad spectrum of progressive activity. This ranges from basic science impacting on quality control assessment of lineage and oncogenic potential, through consensus standards governing translation to clinical practice, and phase I/2 clinical evaluation of therapeutic products generated from qualified source materials for multiple indications (summarised in Fig. 15.1). The next twenty years will hopefully see the realisation of aspirations to apply this resource in medical practice. Along the way the outcomes of basic and translational research will serve to augment safety and efficacy, as well as innovate as yet

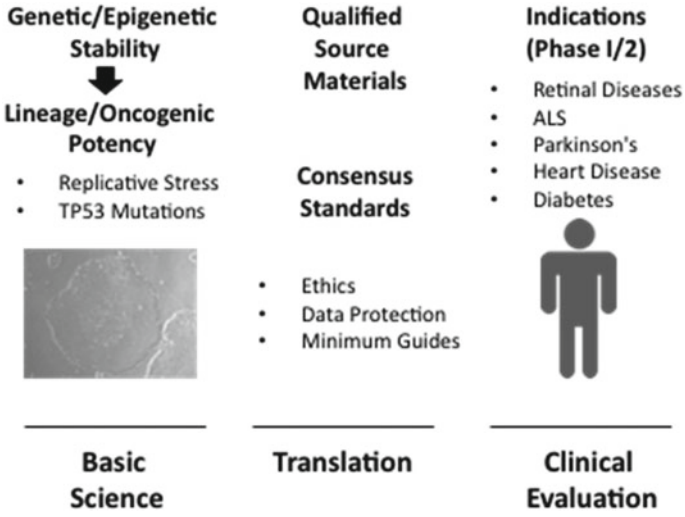

Fig. 15.1 Current clinical progress and translational and scientific issues in human embryonic stem cell banking for clinical applications, twenty years on from their isolation

conceived integration with other biotechnological achievements. For those who have personally and professionally committed to the development of this resource over this time, the prospect of one day being a beneficiary could also one day be a reward.

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