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CONTRIBUTION TO SAFETY ASSURANCE IN THE CRYOPRESERVATION OF CELLS AND TISSUES USED FOR CLINICAL TRANSPLANTATION

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FOREWORD

The use of cryopreserved cells and tissues for clinical transplantation has expanded as never before in many branches of modern medicine.Together with the benefit that this method has brought for thousands of people, some mishaps have also appeared. Some of these failures, that resulted even to fatal outcomes were caused by the lack of legal regulation of cell and tissue transplantations as well as by technical and methodological failures. Moreover, dissemination of information overestimating the capabilities of existing cryopreservation technologies in the media and the practice of neglecting the ethical and legal aspects of cell and tissue transplantation dented the confidence of the public in the existing system of cell and tissue harvest and transplantation.This work sumarizes the author's long-term effort to contribute to increasing the safeguarding of cell and tissue cryopreservation at his workplace (The Tissue Bank of the University Hospital, Hradec Králové), as well as contributing to an improvement of the tissue banking and transplantation system of the Czech Republic.

The safety aspects of cell and tissue banking are dealt with in three separate chapters. In Chapter 1, the influence of the cryobiological theory on the current practice of tissue banks is reviewed.

In Chapter 2, the ethical and legal aspects of safety in tissue transplantation are analysed in detail.

In Chapter 3, the technical aspects of mainatining the safety assurance level as required by the current legislation are dealt with and the practical solution of the issue is demonstrated.

As the topics of individual chapters differ substantially, the conclusions and references are attached to each one. The text of this thesis was made on the basis of the following publications, lectures and poster presentations. The full texts of selected publications are included in the Appendix.

Publications (full texts, only):

1. Červinka, M., Měřička, P: Etické zásady při využívání lidských tkání pro vědecké účely. In: Veselská, R., Kuře, J.(eds.) Sborník k interdisciplinární konferenci, 1.vyd. Brno, Institut pro bioetiku, 2001,p.21-27
2. Měřička, P.: Úvod do transplantace tkání. In.: Třeška, V. a kolektiv: Transplantologie pro mediky. Karolinum, Praha 2002, p. 112-122
3. Měřička, P.: Brief introduction to application of low temperatures in biology and medicine. In. Chrz V. (ed.): The Seventh Cryogenics 2002. IIR International Conference, Prague, Proceedings. Refrigeration Science and Technology, International Institute of Refrigeration, Paris, 2002, p. 163-198
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Lectures:

1. Měřička, P., Straková, H., Vávra, L., Bláha, M., Štěpánová, V., Voxová, B.: Quality Assurance in cryopreservation of haematopoietic progenitor cells. European Association of Tissue Banks Annuall Meeting, Bratislava, October 23-26, 2002
2. Měřička, P.: Significance of understanding the mechanisms of freezing injury and protection for practice of cell and tissue banks - Commission C-1 session key-note talk. 21st IIR International Congress of Refrigeration, Washington, D.C. August 17-22, 2003
3. Měřička, P.Bláha, M., Vávra, L., Štěpánová, V.: Our system of cross-contamination prevention during storage of haematopoietic progenitor cells.. 21st IIR International Congress of Refrigeration, Washington, D.C. August 17-22, 2003
4. Měřička, P., Navrátil P., Straková, H., Grofová, M., Hradecký, Z., Štěpánová, V.: Some current issues in harvesting and preparation of allogeneic skin grafts. 9 th Annual Conference of the Czech Burn Society, Prague, September, 15-17, 2003
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7. Měřička, P., Stárková, D., Langer, J.: The Czech Republic to harmonize tissue transplant legislation with the requirements of the EU Directive. The impact of the commissions for tissue harvest and transplantation on the legislative process. 13th International Congress of the European Association of Tissue Banks, Prague, October 23-26 2004
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9. Měřička, P., Hodík, K., Kopecký, P., Straková, H., Horynová, A., Štěpánová, V., Drahošová, M.: Cord blood cryopreservation for transplantation,own experience. 13th International Congress of the European Association of Tissue Banks, Prague, October 23-26 2004
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12. Měřička, P.: Introduction to harvest and preservation of tissues for drug delivery studies. Laboratory course and workshop " Biodegradable polymers" „Transdermal Delivery" Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Department of Pharmaceutical Technology, 7th-18th February 2005

Poster presentations:

1. Měřička, P., Počepcov, I., Navrátil, P., Žvák, I., Trlica, J., Voxová, B., Příspěvek k zajištění bezpečnosti rekonstrukce předního zkříženého vazu kryokonzervovaným alotransplantátem typu kost-šlacha-kost. V. konference Transplantace orgánů a tkání Brno, 14.-15. XI 2002
2. Hodík, K., Kopecký, P., Straková, H., Horynová, A., Štěpánová, V.: Kryokonzervace pupečníkové krve pro transplantační účely - vlastní zkušenosti. XXI. Neonatologické dny, Hradec Králové 15-17.9 2004
3. Měřička, P.: Do the ethical and legal issues of tissue banking and transplantation attract attention of undergraduate medical students? 13th International Congress of the European Association of Tissue Banks, Prague, October 23-26 2004

SUMMARY

Aim of the study: The aim of the study was to analyse and to meet in practice current requirements on the safety and quality assurance in collection, processing and cryopreservation of cells and tissues. The new high requirements were set by recent changes in the national legislation of the Czech Republic (Act No.285/2002) and especially by coming into force of the new legislation of the European Union (Directive 2004/23/EC). It is required, now that a system of safety and quality assurance known from pharmaceutical industry be established in the tissue banks and that the tissue banks be licensed or accredited by a national authority. The study was limited to safety assurance in manufacturing cryopreserved, ie viable cell and tissues grafts that cannot be terminaly sterilized.

Methods: The cryopreservation methodology used in the Tissue Bank of the University Hospital Hradec Králové was elaborated by the author and his co-workers in the 80s and 90s and regularly upgraded. The controlled-rate freezing in presence of a mixture of penetrating and non-penetrating cryoprotectants is followed by storage at liquid nitrogen temperatures or in mechanical freezers operating at temperature of −85°C.The Transplantation Act (Act No.285/2002) regulates cell, tissue and organ transplantations and defines the duties of tissue banks. The safety aspects covered by this act are oriented only to defining the contraindications of the cell, tissue and organ donation and assuring the traceability . For this reason the issue of the possibility of infection transmission by transplanted tissue was divided into 2 major groups of tasks. The first one deals with the donor suitability including the serological testing of the donor and long-term archivation of the donor serum as well as with decontamination of the collected tissue. There have been already many publications dealing with this issue and criteria of the suitability of the donor were settled by the Decree of the Ministry of Health of the Czech Republic No 437/2002 Sb.The references dealing with the second issue, the safety asurance in tissue processing preservation and storage, are sparse. As there was not any specific national regulation of these issues the author used the principles of Good Manufacturing Practice of sterile drugs published by the State Institute of Drug Control in Prague in 1998 to elaborate specifications of cell and tissue grafts, to establish or change existing standard operation procedures for processing and cryopreservation,storage and releasing the grafts for clinical application as well as to select appropriate control methods. A special attention was paid to elaboration special rules for storage at liquid nitrogen temperatures as the cases of cross contamination by hepatitis B virus were described by Tedder in storage of red blood cells in the liquid phase of nitrogen in 1995.

Results: Examples of specifications of collected tissues and manufactured cryopreserved cell and solid tissue grafts are included in the appendix of this thesis. Examples of standard operating procedures for donation ,processing and preservation of cells and tissues collected both in living and deceased donors are included as well. The appendix includes also template forms of labels designed by author and used for traceability assurance . These labels are used exclusively for labeling grafts released for clinical application that are stored separately from grafts that did not undergo the output control. The labels contain a unique graft identification number consisting of the code of the producer, the type of tissue preserved, the tissue split number, the donor identification number and the year of manufacturing.

The system of prevention of secondary contamination is based on using clean- room technology for processing of viable cells and tissues before cryopreservation. Processing of cell concentrates or solid tissue samples is performed in the environment of the grade A with the backgroud B, regularly validated by an authorized company.The system of cross contamination prevention during long-term storage of cells is based on meticulous evaluation of the results of serological testing of the donor and/or patient and decision on the optimal storage conditions according to the results of this evaluation. The cell or tissue samples originating from donors or patients free of any markers of active infection can be stored in the liquid phase of liquid nitrogen, the samples originating from donors or patients with markers of active severe infection, such as hepatitis, are stored in the mechanical freezers exclusively. The rest of samples is stored in the vapour phase of liquid nitrogen. The analysis of stability of physical parameters during storage confirmed that temperature stability is achieved best in storage in the liquid phase of the liquid nitrogen. With regard to the possibility of cross contamination prevention described above this way of long-term storage can be used in more than 50% of stored concentrates in the investigated group of persons. In solid tissue grafts with regular findings of bacterial contamination,such as skin, storage in mechanical freezer is preferred.

Discussion and conclusions: The Transplantation Act that was approved by the Parliament and Senate of the Czech Republic after a long-time lasting disscussions in 2002 implements into the national legislation high standards of protection of the autonomy of the cell, tissue or organ donor set by the The European Convention on Human Rights and Biomedicine. The author contributed to covering both tissue and organ transplantation by a single act by his publications, active participation in committees of experts in the field of cell and tissue banking and transplantation as well as during preparation of drafts of the Act. The Act contains an important safety requirement, traceability of the way from the donor to the host of the transplanted tissue and back together with strict keeping of anonymity of the donor. The labeling system used by author since 1998 meets this requirement. The safety aspects of cell and tissue banking in general are expected to be a subject of the future special national legal norm. Application of methods of prevention of secondary and cross contamination was possible because of acceptance of the author´ s proposal to design and to build a new cell and tissue establishment as a combination of cryogenic and clean-room technology.The proposal was submitted in 1998 and the facility was built in the years 2000-2002 with the financial support of the Ministry of Health of the Czech Republic and University Hospital Hradec Králové. The aim of the study was achieved and the results were published; full texts of publications are attached. The practical result was receiving a licence of the Ministry of Health of Czech Republic to operate a multifunctional cell and tissue establishment in the University Hospital Hradec Králové .

CHAPTER 1

THE IMPORTANCE OF UNDERSTANDING THE MECHANISMS OF FREEZING INJURY AND CRYOPROTECTION IN RUNNING CELL AND TISSUE BANKS

1. INTRODUCTION

Understanding the fundamental processes which cause injury during freezing and thawing is important both for cryosurgeons, who try to destroy cells by the application of cold, and for cryobiologists who try to preserve living cells at low temperatures.

In the latter case, prevention of freezing and thawing injury, and/or understanding the mechanisms of cryoprotection, are of major importance. Cell and tissue cryobanks exist worldwide and are being established with varying purposes. Many banks, including the Tissue Bank of the University Hospital, Hradec Králové, (Klen, 1952, 1982, Měřička, 1999, 2000, 2002a,b) store human cells and tissues suitable for therapeutic tissue transplantation or for experimental purposes (Kreuz et al., 1951, Sell et al., 1962, Joyce, 2000, Strong, 2000). The storage of genetic material from domestic animals has been widely used for breeding- purposes (Smith, 1961). The human gametes, or early embryos, are frequently stored in the banks of centres for assisted reproduction (Gunasena and Critser, 1997). The most advanced tissue banks develop methods for the preservation and storage of cell- and tissue- engineered products (Harringer et al. 1997, Sato et al., 1998). Many banks build collections of animal- and plant-cells or micro-organisms for use in experiments and diagnostic tests. Establishing a network of low-temperature genome-resource banks is very important for maintaining biodiversity on earth (Wildt, 1997,Curry and Watson 1998, Harnal et al., 2002).

Freezing is the solidification of a liquid. We can highlight two cases:

1/ Molecules or atoms are formed in regular structures.

2/ Solidification is not accompanied by the formation of regular structures.

The former process is called crystallisation and is accompanied by the release of the latent heat of solidification. Under normal atmospheric pressure, ice crystals are formed in a hexagonal lattice. Cubical ice is formed in temperatures of below -130°C (Meryman, 1966). Other crystallographic forms of ice occur under increased pressure and are mentioned by de Quervain (1975). The latter process is called amorphisation or vitrification. Vitrification during cooling, however, does not exclude crystallisation during warming. During this process, called devitrification, the latent heat of solidification is released. Reviews on vitrification were published by Simatos and Turc (1975), MacFarlane et al. (1992) and Mehl (1996).

Before the discovery of the cryoprotective effect of glycerol by Polge, Smith and Parkes (1949), it was believed that achieving vitrification using rapid cooling was the correct way of assuring survival of cells and tissues. This way was, however, not successful (Luyet and Gehenio, 1940).

Later, Mazur (1961, 1963, 1966a, 1966b) precisely explained the mechanism of avoidance of intracellular freezing in single-celled organisms that led to a good rate of survival after thawing. Slow cooling, enabling the cells to dehydrate and shrink and thus escape from an intracellular ice formation, has been widely used in the preservation of cell suspensions both in the absence (in single-celled organisms) and presence of cryoprotective agents (e.g. in isolated mammalian cells). What was possible in cell-suspensions was not

successful, however, in complex multicellular systems where the formation of extracellular crystals was not innocuous. Also, the role of intracellular ice formation may be different in organised tissues, as explained recently by Acker and McGann (2000). The situation is even more complicated in organs, where freezing protocols based on the use of conventional cryoprotectants completely failed. This was the reason for intensive research analysing the physical and biological properties of different vitrification- solutions in prominent cryobiology centres around the world (Fahy et al. 1984, Fahy 1987, Rich and Armitage, 1991, Mac Farlane et al., 1992, Boutron, 1993, Mehl, 1993). Now, it is well-proven that vitrification of both intracellular and extracellular liquid is the most-promising approach to freezing multicellular systems such as human tissues, and may open the way to freezing whole vascularised organs (Fahy et al., 1991, 1994, Fahy and Ali 1997, Wusteman et al., 2002). In the following text, we will briefly mention some particular issues:

1. Crystallisation and mechanisms of natural frost resistance.
2. Freezing of electrolyte solutions.
3. Freezing of cell suspensions
4. Freezing of multicellular systems
5. Principles of cryoprotection.

2. Crystallisation and mechanisms of natural frost resistance

The process of crystallisation can be divided into nucleation and crystal growth.

2.1. Nucleation

The freezing of a sample of water starts from a nucleus with an ice-like structure. The water-molecules organise on the surface of the nucleus to form an ice- crystal lattice. There are two types of nucleation: homogeneous, where the nuclei are formed by spontaneous aggregation of water-molecules; and heterogeneous, where the water molecules aggregate on foreign particles dispersed in water. The critical size of the natural aggregates of water molecules is temperature dependent. There are about 45,000 water molecules at -5°C and only 70 molecules at -40°C (Burke and Lindow 1990, Vali, 1995). Homogeneous nucleation is generally expected to take place at temperatures of about -40°C; nevertheless, in larger samples, it may occur at even considerably higher temperatures (-20°C) (Bigg, 1953). Zachariassen and Christiansen (2000) expressed the idea that this type of nucleation may even take place in living organisms. Other authors (Wilson et al., 2003) reject this idea and point out that in living organisms only heterogenous nucleation may occur.

Different inorganic and organic substances can serve as heterogeneous nucleating agents. Above a temperature of -10°C, not very many inorganic substances are effective, e.g. CuS, PbJ_2, AgJ. The concentration of freezing nuclei in natural water is $10^6/cm^3$ (de Quervain, 1975). Surprisingly high threshold-temperatures are found in organic substances such as isoleucin D (-1°C), tryptophan D (-2°C) and cholesterol (-1°C) (de Quervain, 1975).

Biological ice-nucleating agents are mostly proteins or lipoproteins. These natural nucleators are encountered in freeze-tolerant beetles, in the Afro-alphine plant Lobelia telekii and in the extra-cellular fluid of other plants: fir (Abies concolor), Juniperus chinensis, Prunus, winter rye (Secale cereale), Citrus fruit and lichens (Zachariassen and Christiansen, 2000). They are also present on the surface of certain bacteria, e.g.

Pseudomonas syringae. Worland and Block (1999) isolated the ice- nucleating bacteria active above -2°C and living in the gut of freeze-tolerant sub-antarctic beetles. On the other hand, natural antifreeze- proteins found in both freeze-tolerant animals and plants can selectively bind the nucleators and thus inhibit heterogenous nucleation (see below). This phenomenon led to the idea of using this mechanism in enhancing vitrification during the cryopreservation of multicellular systems. Synthetic ice-blockers revealing the same mechanism of binding the nucleators were intensively investigated by a group under Gregory Fahy (Wowk, 2000). It was found that the addition of polyvinyl alcohol in very low concentrations strongly inhibited devitrification in cryoprotectant-solutions of polyethyleneglycol, dimethylsulphoxide and glycerol.

2.2 Crystal growth

It is well known that the crystal size is dependent on the freezing-rate: at low freezing -rates, small numbers of large crystals are formed; at high freezing rates, a big number of small crystals is formed (Luyet and Gehenio, 1940). Luyet (1966) described classic types of ice-crystals formed in aqueous solutions. In slow cooling, regular hexagonal dendrites can be grown. With increasing cooling-velocity, the regularity is lost and irregular dendrites are formed. Plain spherulites and evanescent spherulites are formed in high cooling velocities. At extremely high freezing-rates, amorphisation takes place (Meryman, 1966). There are again natural substances that inhibit or modify nucleation or crystal growth. These agents are called antifreeze-proteins and are encountered in fish (Knight et al., 1984), insects, lower terrestrial vertebrates and littoral (intertidal) animals (Zachariassen and Christiansen, 2000). These proteins make it possible to supercool the body-fluids of these animals or plants to temperatures low enough to avoid lethal freezing. This depression of the non-equilibrium freezing-point is based on a non-coligative mechanism.

The difference between the freezing-and melting-point has been termed "thermal-hysteresis" and, for this reason, antifreeze-proteins are also called thermal-hysteresis proteins. Proteins isolated from terrestrial animals are reported to be more efficient than those isolated from polar-fish species. It is likely that these proteins inhibit the growth of crystals formed on natural nucleators present in the body-fluids of these organisms (see above). It is also proven that antifreeze-glycoproteins protect cellular membranes during lipid-phase transition (Hays et al., 1997).

2.3 Recrystallisation

At high freezing-rates, the crystallisation process is not completed and the resulting structure is unstable. During warming, there is a tendency to form a more stable structure and recrystallisation occurs. In constant subzero temperatures, grain growth, (i.e. enlarging of big crystals and the disappearance of small crystals) takes place (Meryman, 1966, Mazur, 1966b). It is well documented that antifreeze proteins of differing biochemical structure inhibit the recrystallisation process (Doucet et al., 2000). The use of synthetic-ice blocking-agents that supress devitrification is a promising path to the future.

The recrystallisation and/or devitrification phenomena are extremely important for finding the optimal temperature for storage of a particular cell or tissue sample in a cell- or tissue-bank. In any case, temperature fluctuations during storage are to be minimised (see Chapter 3).

2.4 Crystallisation in cell suspensions

The mechanisms of freezing-injury and protection in suspensions of single-celled organisms were described by Mazur (Figure 1) in the 1960s (Mazur, 1961, 1963, 1966a,b, 1970). In cell-suspensions, the crystals are formed in the extracellular water first. The intracellular water supercools, and a pressure- gradient between the higher water-vapour pressure (above the supercooled water) inside the cell and the lower pressure (above the ice) outside the cell is formed. This leads to an outflow of water from the cell and to a crystal formation outside the cell. Dehydration and shrinkage of cells is the result of this process. The ability of the cell to shrink and to escape from intracellular freezing is dependent on the cell size and/or its initial volume and the permeability of the cell membrane to water. This process was mathematically described by Mazur (1963, 1966a,b).Thus, intracellular ice-formation is more likely to take place in large cells with a thick membrane than in small cells with a highly-permeable membrane. This is the reason why it is easier to achieve good cell-recovery by freezing cells of standard initial volume than by freezing a mixture of cells of different initial volumes. Sufficient dehydration and/or shrinkage of cells can occur only at low freezing-rates. At high freezing rates, intracellular ice is formed and the cells do not shrink.

The intracellular ice tends to recrystallise during warming which causes cell-death. The mechanisms of intracellular crystallisation are still being discussed (Pitt, 1992). It starts at considerably lower temperatures than extracellular freezing, e.g. intracellular ice in isolated keratinocytes does not form until a temperature of −7°C is achieved (Zieger et al., 1997). In general, when the external medium is frozen, intracellular ice occurs at between −5°C and −13°C; when the external medium contains no ice, freezing can occur at lower temperatures (Acker and McGann, 2000). The same authors (2000) identified 50 % of intracellular ice formed in cells in suspension at -9.4°C ± 0.3 and -8°C ± 0.3 for individual, attached cells. Mazur (1966b) suggested the idea of seeding the supercooled intracellular water with extracellular ice through the natural cell-membrane pores. Later Toner (1993) described two mechanisms:

1. Volume-catalysed nucleation, where nucleation is induced by foreign particles inside cells.
2. Surface-catalysed nucleation, where nucleation is induced by the activity of the internal surface of cells.

Alternatively, Muldrew and McGann (1990) expressed the idea that intracellular freezing is initiated by the seeding caused by extracellular ice growing into ruptures of the cell-membrane previously damaged by the rapid outflow of water from cells during osmotic stress.

3. FREEZING OF ELECTROLYTE SOLUTIONS

Biological fluids can be regarded as electrolyte solutions. In electrolyte solutions, crystalisation leads to considerable osmotic effects that can be observed on the binary system water/sodium chloride. The freezing-process starts with supercooling (Figure 2), i.e. the metastable state of the liquid below its freezing- and/or melting-point. Some authors (Zachariassen and Christiansen, 2000, Wilson et al., 2003) suggest defining the supercooling point of particular systems. The supercooling point can be considerably lower (e.g. because of presence of thermal hysteresis proteins) than the freezing- and/or melting- point, the

primary crystallisation (i.e. crystallisation of water) starts, the freezing-plateau is formed (Figure 1,1a).
The osmolar concentration of the residual liquid increases rapidly until secondary crystallisation of the solute is started at the eutectic point (-21.8 °C) (Figure 1,1a).

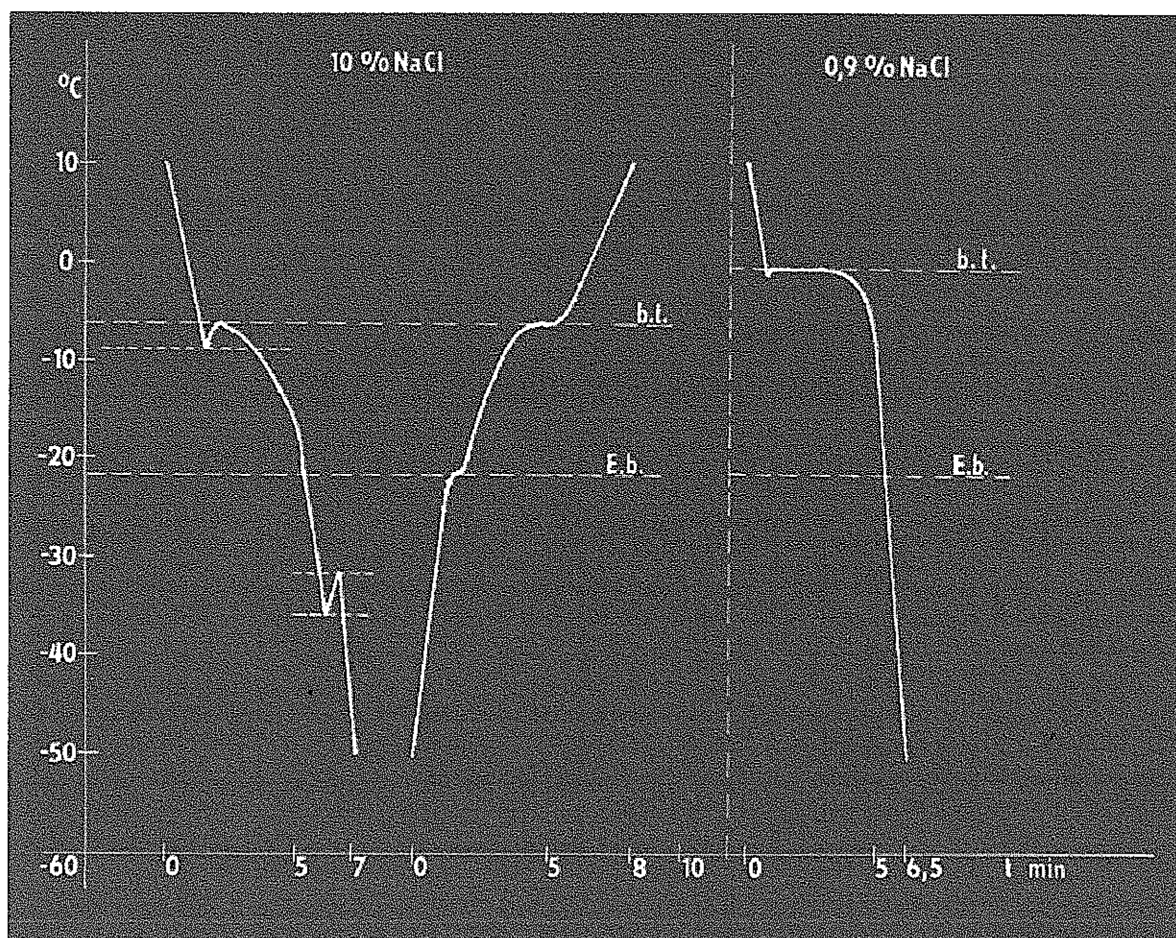

Figure 1: Freezing and thawing curves of a 10% sodium chloride solution (left). Supercooling prior to primary and secondary crystallisation is well detectable during rapid freezing. Eutectic (E.b) and melting points (bt) were detected during rapid warming. In a 0.9% sodium-chloride solution, these points were detectable during slow warming – Figure 1a (Měřička, 1982).

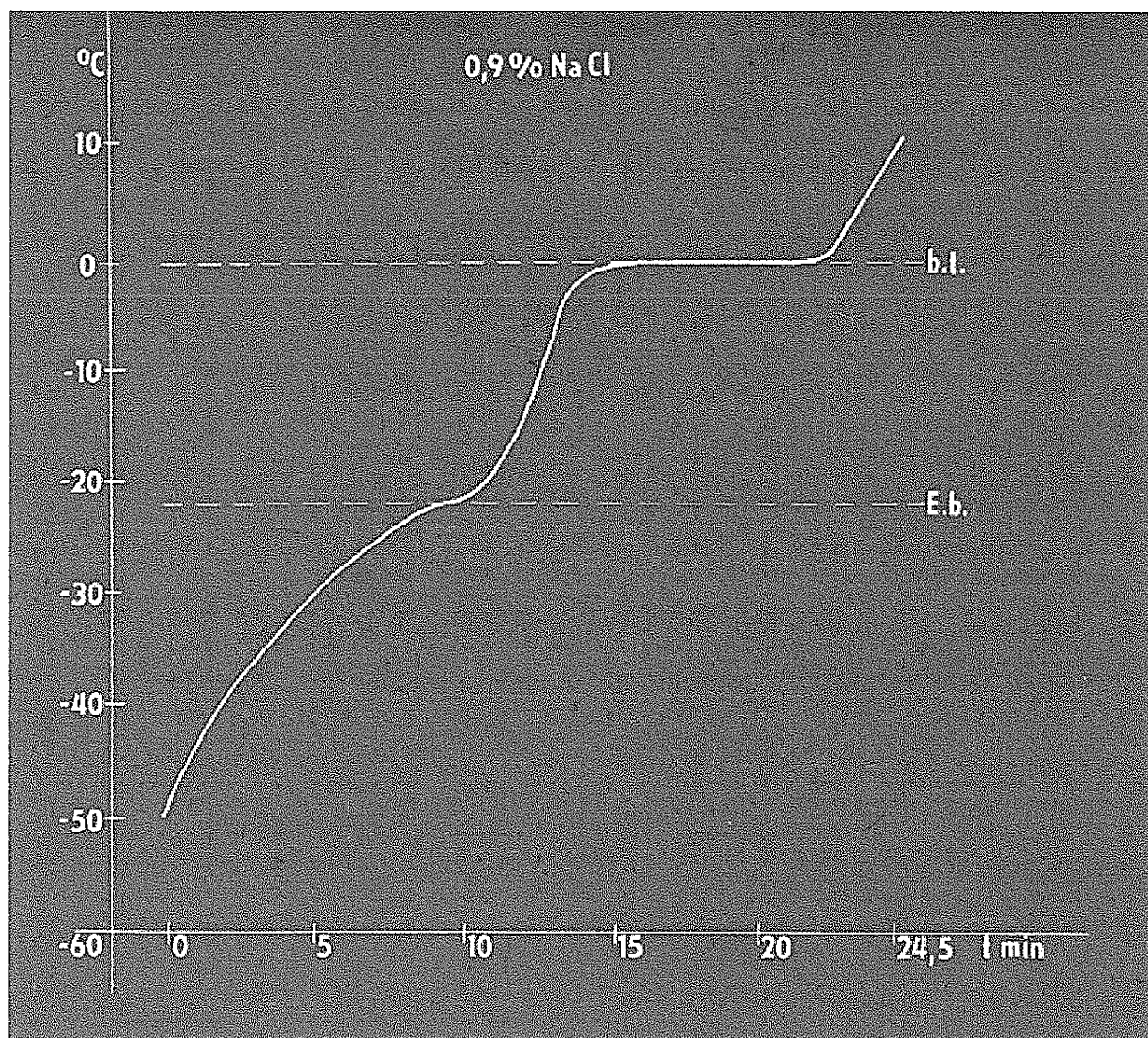

Figure 1 a: Thawing curve of a 0.9 % sodium- chloride solution during slow thawing (Měřička, 1982)

The salt-concentration within the cryoconcentration-zone is dependent only on temperature and independent of the initial salt-concentration, while the volume of the unfrozen fraction at a given subzero temperature is dependent on the initial osmolar concentration of sodium chloride. With decreasing temperature, the concentration of the solute increases rapidly and causes the solution effect – see below.

As supercooling of the eutectic can take place during freezing (Figure 1) and natural body fluids do not represent a simple binary system and/or contain different solutes, it is advisable to determine the temperature of complete solidification in the system. This can be assessed, for example, by electric resistivity measurement as the electric resistivity increases rapidly, when the freezing process is completed, and/or the unfrozen fraction disappears (Meryman,1966).

The high osmolar concentration during freezing is responsible for the solution-effect in freezing cell-suspensions. This effect is caused by an increasing concentration of substrates and enzymes, increasing the rate of biochemical reactions, pH changes and dehydration of membrane-systems (Meryman, 1966). A solution-effect leads to extreme dehydration of cells

dehydration of cells (below a minimal volume), to denaturation of proteins and irreversible disturbances of the cell-membrane permeability. Muldrew and McGann (1990) pointed out that excessive and rapid cell-dehydration may cause ruptures of the cell-membrane.

Besides the intracellular crystallisation encountered in rapid freezing, the solution-effect is the second lethal mechanism that is encountered at slow freezing- rates and can cause cell-death in the absence of intracellular freezing. The terms slow and rapid freezing are valid only for a particular system and are dependent on initial cell-volume, permeability of the plasma-membrane, type of cryoprotectant and its concentration. The freezing rate of -1K/min may be too slow for freezing red blood cells and too rapid in freezing early embryos).

The freezing-regimens used in practice are the result of previous empirical and experimental work by individual cryobiological groups. The empirical approach is now being replaced by computer-simulation of cryobiological processes. Computer programmes may be based on, for example, Kedem Katchalsky equations (Kedem and Katchalsky, 1958), which describe the transport of water and salts across the plasma- membrane, and on data on solute concentrations in freezing ternary systems of NaCl/Glycerol/water and NaCl/DMSO/water published by Pegg (1983, 1986). The methodology approaches to calculating optimal freezing-rates were reviewed by Kleinhaus in 1998 (Kleinhaus, 1998). Besides programmes based on the Kedem Katchalsky equation, a new user-friendly approach is being developed by Katkov (Katkov, 2000).

It is obvious from the above text that in freezing suspensions, the cells can be damaged both by intracellular ice-formation and the solution-effect due to their reliance on the freezing- and thawing- rates. The third mechanism of freezing- injury is the cell-packing effect that occurs in highly concentrated cell- suspensions (Pegg and Diaper, 1983, Mazur and Cole, 1985,). The most likely explanation is that densely-packed cells are damaged by mechanical stresses when the channels containing residual liquid change shape as a result of the recrystallisation of ice- crystals at their boundaries. The cell-cell contact is responsible for this phenomenon (Acker and Mc Gann, 2000).

In practice, small volumes of cell-suspensions are frozen in ampoules of cylindrical shape, and large volumes (tens or hundreds of millilitres) in plastic bags enclosed in metal cassettes or plates to avoid the differences in freezing- and thawing-rates between the periphery and the centre of the sample (Meryman, 1966). The desired freezing-rate is assured by using controlled freezing in programmable freezers (Figure 2,2 a). In many instances, it is possible to use uncontrolled freezing by placing the sample, usually covered by an external insulation, in a mechanical freezer or liquid-nitrogen vapour. Current cryopreservation technologies make possible to achieve freeze/thaw cell- recoveries between 60-90 %. Recoveries of near 100 % are not exceptional.

Figure 2: Controlled-rate freezing of plastic disposable bags with suspensions of blood progenitor-cells in a programmable freezer. The thermocouple is placed in a control bag. The temperature of the freezing-chamber, as well as of the control bag, are recorded (Figure 3a). Control ampoules with cell- suspensions are frozen simultaneously and used for viability control tests.

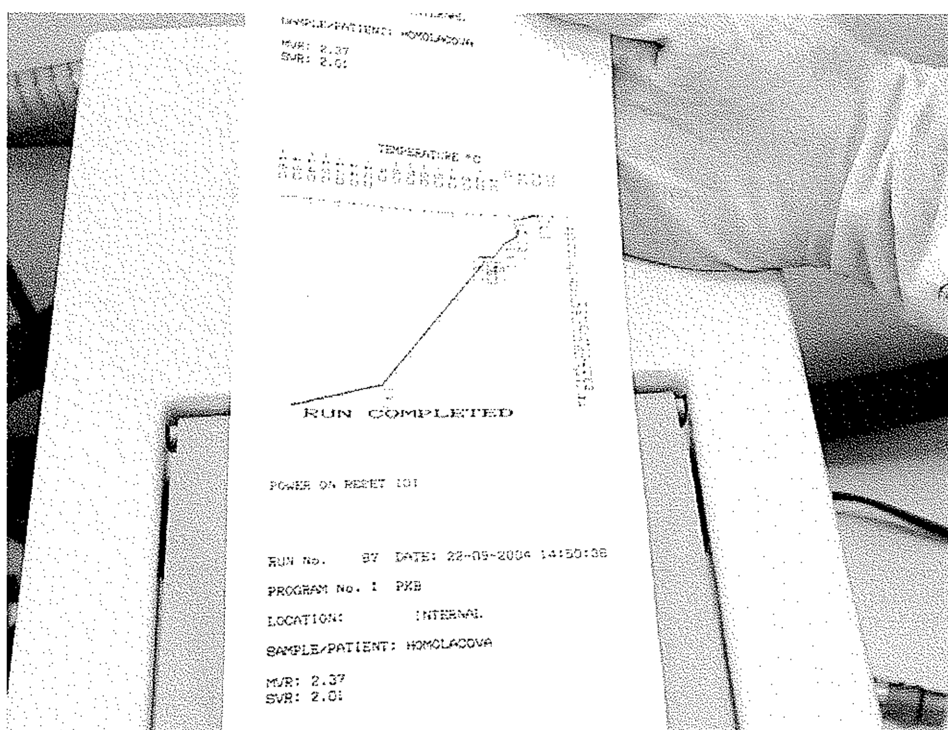

Figure 2 a: Temperature-chart of controlled- rate freezing.

4. FREEZING OF MULTICELLULAR SYSTEMS

In these systems (tissues and organs), damage is also caused by extracellular ice-formation as the ice causes disintegration of tissues. Large crystals can be detected by scanning electron microscopy of soft connective tissues being submitted to slow freezing followed by freeze-drying as shown in figures 3,3a (Měřička et al., 1989).

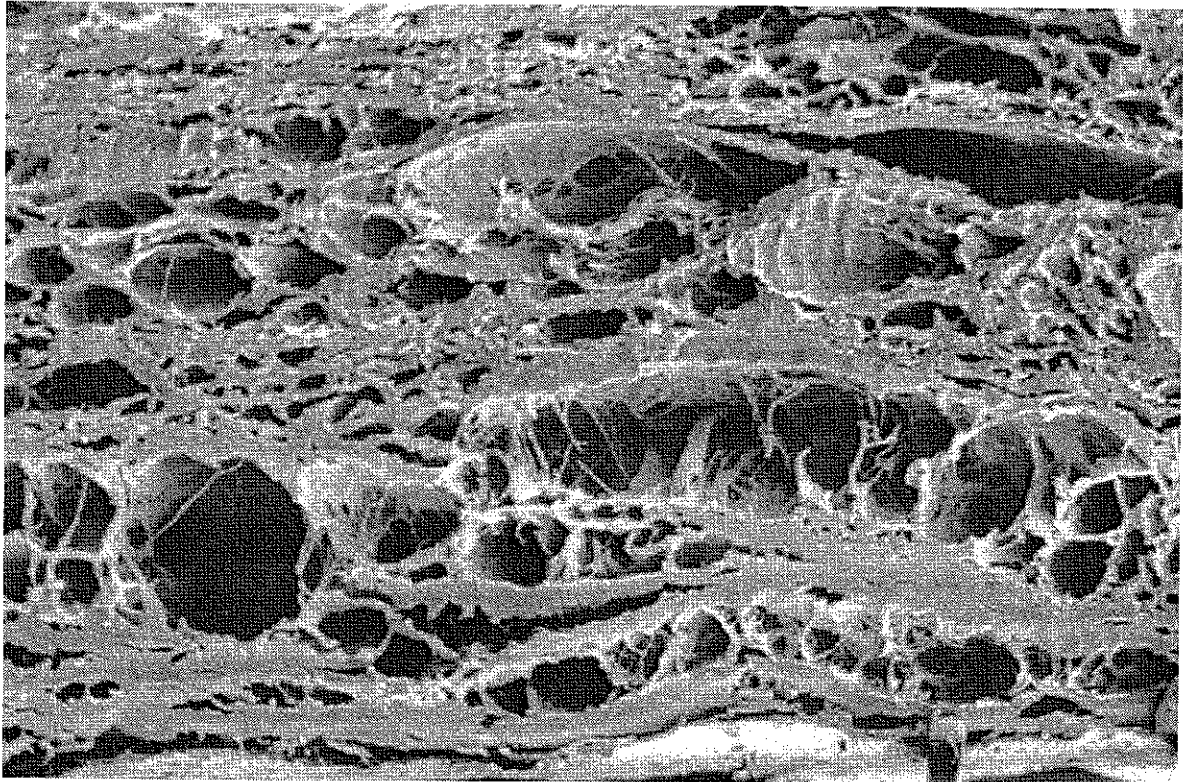

Figure 3: Structure of freeze-dried dura mater graft. Pores of different sizes are formed in the tissue apparently by the sublimation of water from ice-crystals formed during freezing of the tissue to-50°C. The porous structure did not form when the tissue was dehydrated in an ethanol-chlorophorme solution before freezing, as shown in Figure 3a. (Courtesy Prof. J. Špaček).

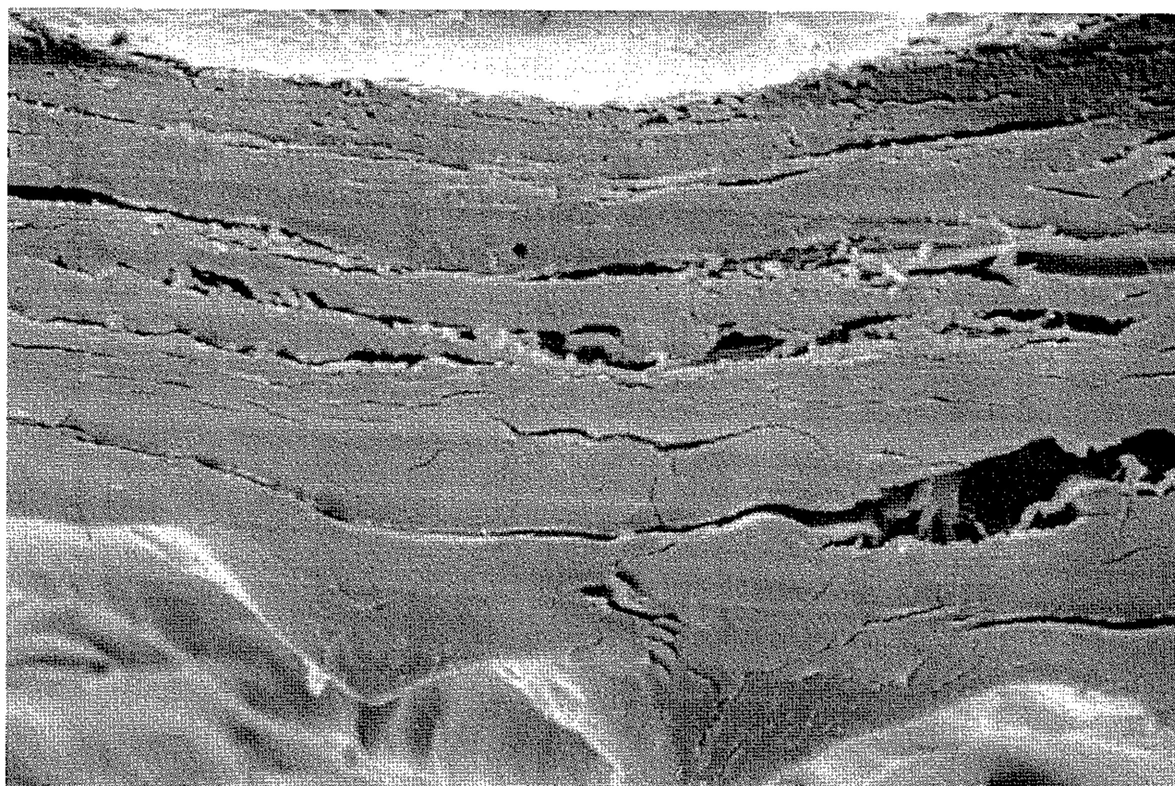

Figure 3 a: Structure of freeze-dried dura mater dehydrated before freezing. (Courtesy Prof. J Špaček).

Electron-microscopy of frozen skin or cornea reveals damage to intercellular junctions (Zieger, 1997, Acker and McGann, 2000).

Alternatively, the presence of intracellular ice due to rapid freezing may not result in the loss of selective permeability of cells which form the tissue or the cell-monolayer (Acker and McGann, 2000). The tissue of some organs, such as the parathyroid gland or ovary, can be successfully frozen in the form of slices or cubes, Langerhans islets can be frozen in suspension after separation from the exocrine pancreatic tissue.

In vascularized organs, water crystallises in vessels which causes their expansion and rupture. Thus, any resultant haemorrhage can destroy the thawed organ after restoration of the blood circulation (e.g. in kidney transplantation). In organs with cylindrical or spherical shape, differences between freezing-rates occurring at the surface and in the centre of the organ take place during both freezing and thawing. This causes injury by the solution-effect, as well as by volume-expansion at the centre of the organ at the moment of liquid/solid transition. This expansion may lead to organ-rupture as the ice formed at the organ- periphery breaks at the moment of solidification of the liquid in the centre. The introduction and removal of a conventional cryoprotectant, and the resulting osmotic swelling of cells, may lead to compression of the vessels of the organ which causes an ischaemia leading to organ-necrosis after transplantation. For afore-mentioned reasons, the cryopreservation techniques based on classic equilibrium-freezing, and using conventional cryoprotectants, can assure safe preservation and storage of cell-suspensions and tissue-samples which have a flat geometry (e.g. skin). The problem in cryopreservation of organs or complex tissues (such as corneas) obliges a methodological approach based on using cryoprotective solutions, which enhance vitrification (vitrification solutions) (Fahy and Ali, 1997, Brockbank et al., 2000, Meltendorf, 2002) (see below).

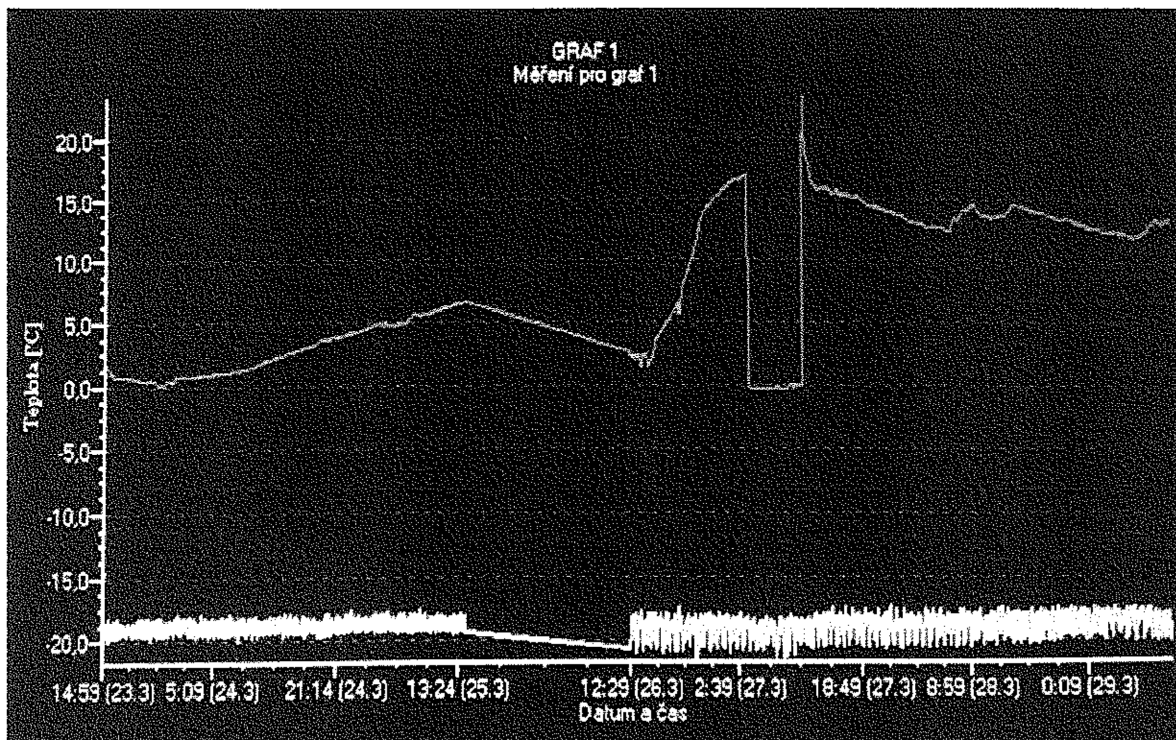

Figure 4:In the current practice, most organs are stored in hypothermic conditions after initial perfusion. Shown is the temperature-chart of a container filled with melting ice (red curve), used for the storage of human kidneys. Temperatures were recorded at 5minute intervals using the "Digiterm"system (Regucon Ltd., Prague)

5. PRINCIPLES OF CRYOPROTECTION

Cryoprotection can be based either on avoidance of intracellular ice-formation and solution-effect, or on extracellular and intracellular vitrification. The former can be achieved in an ideal situation by the simple manipulation of freezing-rates, as described in yeasts by Mazur (1963, 1966a,b). In most cases, such as the freezing of mammalian cells, the addition of cryoprotectants is necessary. The cryoprotectants can be divided into intracellular or penetrating cryoprotectants (e.g. glycerol or dimethylsulphoxide) and extracellular or non-penetrating cryoprotectants (e.g. sucrose, dextran, polyvinylpyrolidon (PVP) or hydroxyethylstarch). The presence of intracellular cryoprotectants modifies the freezing-process both outside and inside cells. They are used in high osmolar concentrations, which leads to considerable freezing-point depression based on the colligative properties of a cryoprotectant. The introduction of cryoprotectant to the cells before freezing can result in considerable osmotic stress (Katkov, 2000).

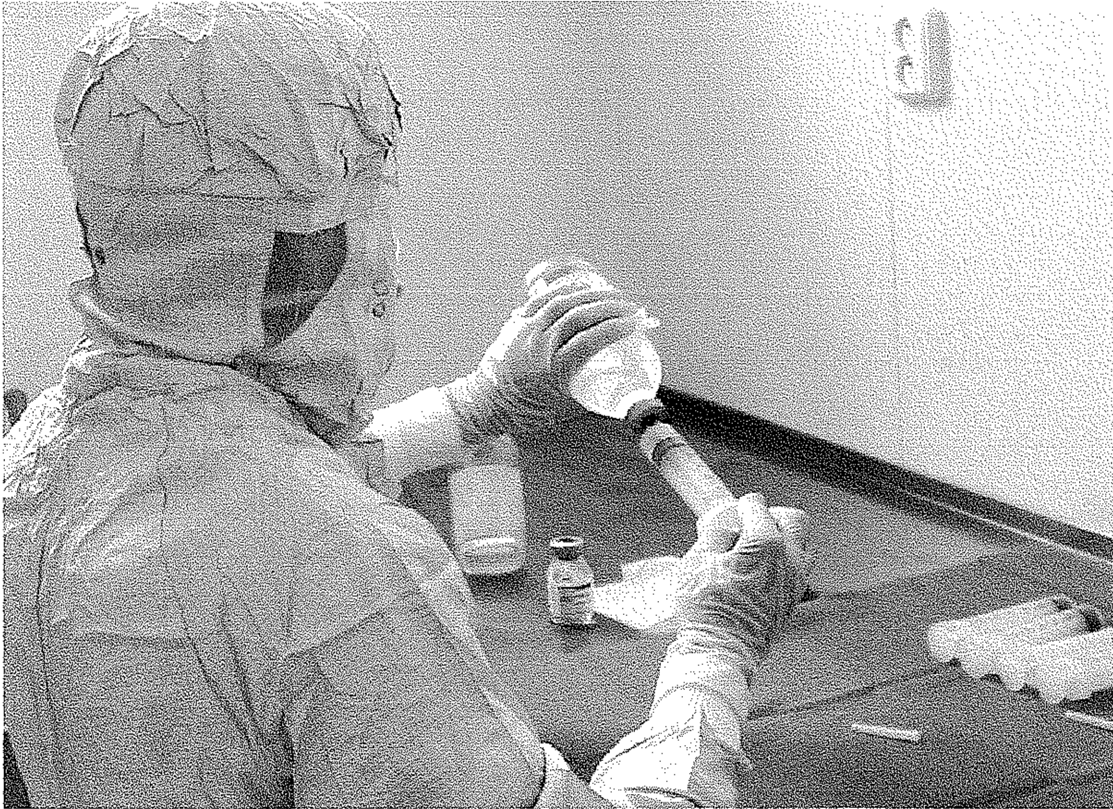

Figure 5: Cryoprotectant-solution for clinical application is mixed under strictly aseptic conditions. 20% Dimethylsulphoxide-solution (Me_2SO) is made from 6% dextran in a physiological solution (40 ml) and 10 ml of Me_2SO of pharmaceutic grade (Figure 6a). Mixing is carried out in a calibrated 50 ml syringe.

Figure 5 a: Mixing a dextran solution with pure Me_2SO.

Figure 6: A concentrate of progenitor-cells collected from peripheral blood (150ml), is transferred from the collection-bag (left) into 3 freezing-bags (each of 50ml). Mixing with an equal volume of pre-cooled cryoprotectant-solution (Figure 5,5a) follows.

Using a cryoprotectant, the unfrozen liquid-fraction below freezing- point is expanded, which prevents excessive and rapid cell-dehydration (Cocks and Brower, 1974). Modification of secondary crystallisation using Me_2SO is also shown on the comparison-chart of thawing curves of a 10% NaCl solution with hydroxyethylstarch and dimethylsulphoxide (Měřička et al., 1983) (Figure 7).

The mechanism of extracellular cryoprotection is not precisely known. Extracellular cryoprotectants are used in low osmolar concentrations, where the depression of the freezing point is only minute. Possible mechanisms are: avoidance of nucleation, inhibition of crystal growth and the binding of water, which prevents extreme cell-dehydration.

In general, the extracellular cryoprotectants are less effective than intracellular ones if used alone. The exception is the successful cryopreservation of red blood-cells with hydroxyethylstarch (Sputtek 1990, 1993). Extracellular cryoprotectants are frequently used, however, in combination with dimethylsulphoxide, in freezing white blood cells, bone-marrow, peripheral blood progenitor-cells and platelets (Figures 5,6).

Another possible mechanism of cryoprotection is vitrification. It can take place even when using classic intracellular cryoprotectants such as glycerol or Me_2SO. Vitrification in these solutions can be further enhanced by using synthetic ice-binders (Wowk et al., 2000)

(see above). Boutron regularly induced vitrification using 1,2 propane-diol or 2,3 butane-diol (Boutron, 1991, 1993). These compounds can be used, with good results, for the freezing of red blood cells. Mehl (1996) pointed out the value of using acetamide, the best glass-former among alpha-alkanamides, since $-NH_2$ groups suppress nucleation better than -OH groups.

It was proven that cocktails of compounds which enhance vitrification, known as vitrification solutions (Fahy, 1991, Mehl, 1996, Wusteman 2002, 2003), can be used with success in the cryopreservation of complex tissues such as the cornea, vessels or skin, and probably also for the cryopreservation of vascularised organs. In 1997, a group led by Fahy successfully demonstrated the immediate function of a rabbit kidney after the introduction and removal of a vitrifiable 7.5 molar solution (Fahy and Ali, 1997). The solution, called VS 4, contained dextrose, Me_2SO, dimethylformamide and 1,2 propane-diol. Vitrification is also used with success in the cryopreservation of plant-cells and -tissues (Steponkus, 1992), as well as for the cryopreservation of embryos (Rall and Fahy, 1985).

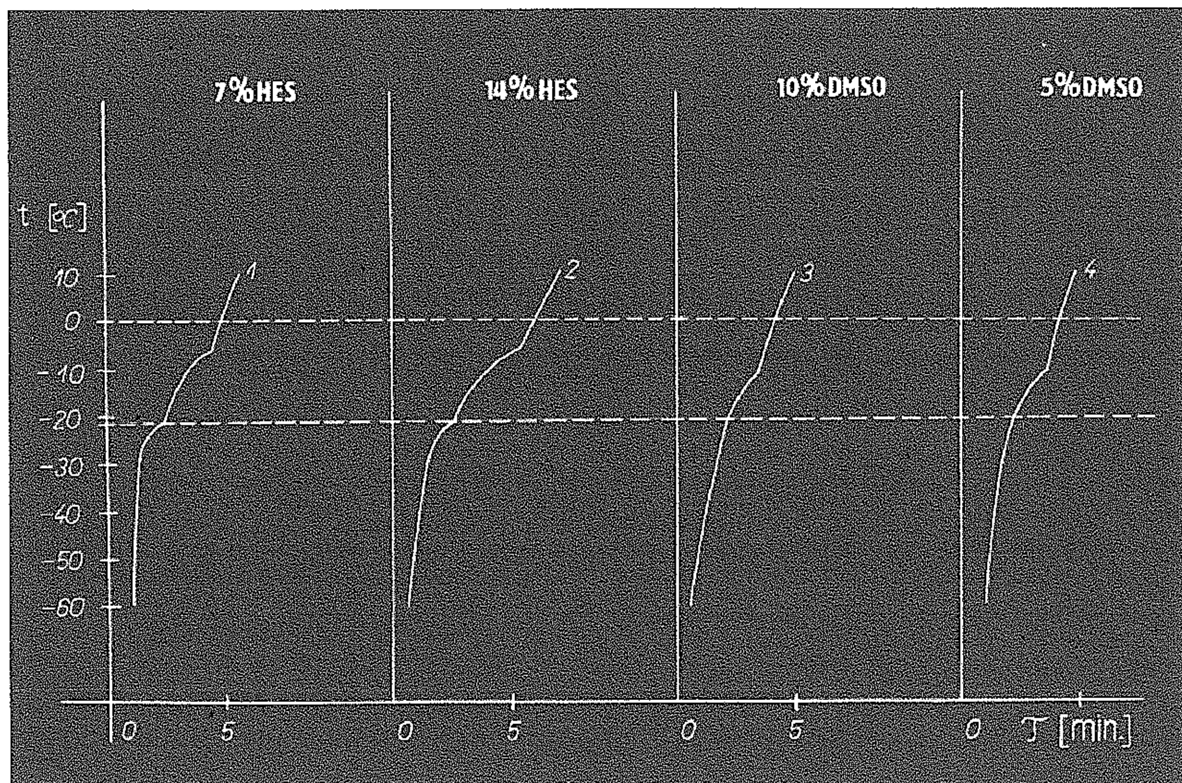

Figure 7: Thawing-curves of a 10% sodium-chloride-solution containing 7% and 14% hydroxyethylstarch (HES) and 5% and 10% Me_2SO. In using Me_2SO, the thawing-plateau of the sodium-chloride eutectic-point is absent (Měřička et al.1984)

5.1 Immediate injury and storage-injury

Cells can be damaged both by freezing- and thawing-processes or by storage under inadequate conditions. The immediate freezing- and thawing-injury can be minimised by the combination of slow freezing and rapid thawing and the application of cryoprotectants.

It is the role of cell- and tissue-banks to limit storage-injury to a minimum so that storage for years, or even decades, can be achieved without significant loss of viability. Storage-injury is avoided if complete solidification of the liquid is achieved, and recrystallisation and devitrification-processes are minimised or avoided. Optimal storage conditions are assured in vapour or liquid phases of nitrogen (Meryman, 1966). At these temperatures, cells can be stored for years. Hunt and Pegg (1996) suggested safeguarding temperature of the vapour-phase of liquid nitrogen below −160°C using a copper heat-shunt.

At the temperature of dry ice (-80°C),which can be achieved in commercially-available mechanical freezers, the recrystallisation processes can take place, and the possibility of the existence of a minute fraction of a liquid, unfrozen phase, can not be excluded. The mechanical freezers can be used for safe storage for months, however (Makino et al., 1991). Storage of viable cells and tissues at higher temperatures cannot be recommended if classic cryoprotectants are used.

5.2 Thawing and removal of the cryoprotectant

While in freezing cell-suspensions the use of a slow cooling-rate is desirable, rapid thawing should be used to minimise cell-injury. A rapid thawing-rate prevents recrystallisation of the ice formed during freezing and minimises the solution-effect. In normal practice, thawing is carried out in a water- bath of 37-40°C. Rapid thawing can be achieved only in small cylindrical containers or containers with a flat geometry such as plastic bags filled with white blood-cell concentrate. Contrarily, some complex tissues, such as vessels, require slow cooling to prevent microfractures. A problem in the removal of the cryoprotectant is encountered when using intracellular cryoprotectants. Extracellular cryoprotectants do not need to be removed. There are two reasons for the removal of the cryoprotectant before further processing or clinical application of the cryopreserved cells or tissues:

1. Cryoprotectant is toxic and can disturb the growth of cells in a tissue-culture. A typical example of this is dimethylsulphoxide.
2. The plasma-membrane permeability for cryoprotectant is much lower than that for water.

Glycerol is a typical example. It drains slowly from the cell after thawing, which can lead to cell-swelling caused by an influx of water into the cell. The swelling may cause cell-death especially if the cells are placed into an isotonic medium rapidly. Excessive influx of water into cells can be prevented by a gradual dilution of the thawed cell-concentrate with isotonic media, as also with dilution media containing substances that bind water outside cells such as sucrose serum or albumin.

6. CONCLUSIONS

Substantial progress has been made in understanding the mechanisms of freezing-injury and -protection during the last 15 years.The classic cryoprotection models, based on avoidance of intracellular crystallisation by slow cooling and using conventional cryoprotectants, are still the most frequently used in cell- and tissue-cryobanks. New approaches based on vitrification are being introduced now, mainly in the freezing of complex tissues. This change may open the way to greater success in freezing vascularised organs. Enhancement of vitrification by synthetic ice-blocking-agents may be a new

contribution to solving this problem. Bringing organ-cryopreservation techniques into current medical practice will need, however, a lot of time and effort. In applying classic freezing-protocols, computer simulation of cryobiological processes contributes to the optimisation of regimens used in the freezing of cells.

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CHAPTER 2

CURRENT NATIONAL TISSUE-TRANSPLANT LEGISLATION IN THE CZECH REPUBLIC AND THE REQUIREMENTS OF THE DIRECTIVE OF THE EUROPEAN PARLIAMENT AND COUNCIL

1. INTRODUCTION

The history of efforts to establish legal regulation of tissue-banking in the former Czechoslovakia has been closely connected to the establishment of the first multi-tissue bank in the University Hospital, Hradec Králové, by Professor Rudolf Klen in 1952 (Klen, 1952, 1954, Plášilová 2001). The first proposal for a Tissue Bank Act was presented at the 1st Conference on Tissue Transplantation held in Hradec Králové, in 1953, by Dr. Dostál, the judge of the Regional Court, Hradec Králové and published one year later (Dostál, 1954). Dostál proposed that specialized institutions be licensed by the Minister of Health of the Czechoslovak Republic to harvest tissues from dead donors for medical and research purposes. It was proposed that donation of tissues for experimental and clinical use be based on an opting-out principle and that any financial compensation paid to the donor be excluded.

Unfortunately, regulation of this ethically delicate issue at the level of a special Act was not accepted, and the legal regulation of tissue-banking was included only in more general decrees of the Ministry of Health dealing with funeral- procedures (Ministry of Health of the Czechoslovak Republic, 1955, Ministry of Health of the Czechoslovak Socialist Republic, 1966). These decrees permitted the harvesting of tissues from dead donors for experimental and clinical purposes in departments of pathology, forensic medicine and tissue banks on the condition that the minimum time-lag between death and tissue-harvest was 2 hours.

With the start of organ-transplantation in the middle of the 1960s, it was permitted, under defined circumstances, to perform an extraordinary harvest before 2 hours after death (Ministry of Health of the Czechoslovak Socialist Republic, 1968). In the 1970's, special decrees defined the conditions of extraordinary harvest in brain-death donors with preserved blood circulation (heart beating donors) (Ministry of Health of the Czech Socialist Republic, 1977). The opting-out principle was preserved in all stated norms both for experimental and clinical use. Only the written refusal of the donor was accepted.

The first review dealing with legal aspects of tissue-banking had been published by Klen in 1957 (Klen, 1957). Eleven years later, the issue of organ-harvest for transplantation was examined by Štěpán (Štěpán, 1968). The legal aspects of tissue- and organ-transplantation were included in a treatise "Law and Modern Medicine" published by a brother of the above-mentioned author (Štěpán, 1989). Hanuš contributed information on the issue by adding a special chapter to a textbook "Selected Chapters of Medical Psychology," published by Charles University in 1994 (Hanuš et al., 1994). This chapter was also separately published (Hanuš and Měřička, 1994). The paper on the ethical and legal aspects of tissue banking was presented by the author of this chapter at the European Conference on Tissue Banking held in Berlin in 1991. A short review, aimed at informing the public of ideas presented at the conference, appeared in the local hospital- and university- journal "Scan" in the same year (Měřička, 1991). In this paper, we reviewed our attitude to tissue-donation and-banking regarding such issues as protection of the autonomy of the donor, exclusion of any financial compensation paid to the donor, reimbursement of expenses to the tissue bank by

health insurance companies on a non-profit basis, etc. We pointed out that transplantation law should protect the donor's right to decide what should be done with his/her body after death.

The legal situation, before the passing of a special transplantation act, was analysed by Martínek (Martínek, 2002) in a chapter included in the textbook "Transplantology for Medical Students" published by Charles University in Prague (Třeška et al., 2002). Martínek summarized the weaknesses of the legislation existing at that time such as the lack of specification of the duties of medical staff in checking the possible pre-stated dissent of a potential,but than deceased organ- and tissue- donor, lack of definition in the rights of the next-of- kin of the deceased donor, absence of rules for the allocation of collected tissues and organs as well as of criteria for the control of quality, and availability, of transplantation care. Some specific remarks dealing with tissue- transplant legislation were mentioned in a chapter "Introduction to tissue –transplantation" included into this textbook (Měřička, 2002). Dufková published a thorough review of the principle features of the "Transplantation Act of the Federal Republic of Germany" (Dufková, 2000), and Bouška and Klír explained the reasons for considering alternative practical approaches in the Czech Republic, different from those in the FRG. (Bouška and Klír, 2000).

The expected negative effect of the new legislation on the availability of human cells and tissues for experimental use was analysed by Červinka and Měřička (Červinka and Měřička, 2001) in a paper presented at the 4th Interdisciplinary Conference: "Transplantation Medicine – Current Status and Perspectives", organized by the Institute for Bioethics, in Brno, in 2001.

2. ANALYSIS OF THE REGULATORY SITUATION

2.1Contribution of health insurance companies to regulations on tissue-banking activities

The fact that organ- and tissue-transplantations in the Czech Republic were not regulated through a special Act has been strongly criticised both by politicians and the public during the great political changes in the country from 1989. A special Act preserving the opting-out principle in harvesting from deceased donors has been in outline since the establishment of the Czech Republic in 1993.

The basis for real regulation of tissue-banking activities was provided, however, by the Czech General Health Insurance Company, which started to reimburse tissue grafts on a non-profit and tax-free principle in 1993. Grafts produced by individual banks were given identification-codes and the banks were given the identification-codes of manufacturers. The grafts were classified as individually-prepared drugs similar to blood-bank products (General Health Insurance Company, 1993). In 1997, the classification was changed to that of medical devices (General Health Insurance Company, 1997).

Lists of products of individual tissue-banks were published by the insurance company in standard lists of reimbursed drugs and medical devices which were at the disposal of any physician in the country (General Health Insurance Company, 1993, 1998). The lists also included the maximal reimbursement calculated by individual producers (tissue-banks) and approved by the health insurance company. This reimbursement included material costs, salaries and insurance of the staff, costs of using the equipment including its validation, costs of necessary services, tissue bank and hospital overheads. It was possible to alter the reimbursement only if the stated parameters changed. The reimbursement has been always totally independent of actual demand for particular tissue grafts. The approach of the General Health Insurance Company to reimbursement for products of tissue-banks was followed by

other health insurance companies which accepted the maximal reimbursement laid-down by that company.

In 2003, the General Insurance Company increased its standards of safety and quality of work of individual tissue-banks. It stopped, or limited, reimbursement for products of banks that were not able to meet the necessary hygiene-standards, or did not have the necessary equipment at their disposal.

Since September 2004, the insurance company has reimbursed only for grafts of banks licensed by the Ministry of Health (see below).

2.2. Tissue-harvesting and -banking and the drafting of the National Transplantation Act -Contribution of Tissue-Harvest and Transplantion-Committees to the legislative process

Before approval of the transplantation act, the Ministry of Health made two attempts to license an institution for coordinating organ-and tissue-transplantation inside the country, and to cooperate with international organisations. The first institution that received this licence was Czech Transplant, which started work in 1994 (Martínek, 1997). After the dissolution of Czech Transplant by the Ministry of Health in 1997, authorisation to coordinate organ- and tissue-transplants was given back to the Institute for Clinical and Experimental Medicine in Prague which had been responsible for the coordination of organ-transplants for a long time before. Both institutions formed special committees of experts in tissue-banking and transplantation as advisory bodies (Měřička, 1997, 2000).

The first committee was established as the result of the first survey on tissue- banking activities carried out by the author of this paper in cooperation with Czech Transplant, in 1995, and presented to the 4th International Conference of the European Association of Tissue Banks held in Leuven, Belgium, in the same year (Měřička, 1995). In this presentation, it was stated that in the absence of national legislation dealing with the safety issues of tissue banking, the Czech tissue-bankers had tried to implement in their practices the standards of peer international organisations of tissue-bankers, such as the American Red Cross Tissue Service (Eastlund and Campagnari 1992), the European Association of Tissue Banks (EATB) (EATB 1995), the European Association for Musculoskeletal Transplantation (EAMST) and the International Association of Eye Banks (IFEB). The issue of consent was discussed as well. The practice of the tissue-bank in Hradec Králové was, at that time, to accept the dissent of the donor's family expressed in any form including dissent to the autopsy that would have followed the tissue-harvest in any case.

The committee summarized the tissue-banking activities and the demand for tissue-grafts in different medical branches, and issued a report for the year 1996 (Czech Transplant, 1996). It supported proposals to register the tissue-donors and receivers, and to establish a national tissue-bank network. It also supported cooperation among organ- and tissue- harvest teams (Měřička, 1997), and monitored and criticised the export of tissues for commercial purposes.

The second committee was attached to the Department of Organ-Transplantation Coordination of the Transplant Centre at the Institute for Clinical and Experimental Medicine in Prague (Měřička, 2000). It followed the guidelines of the first one, and issued a report covering 1998 (Institute for Clinical and Experimental Medicine, 1999). It proposed accepting the tissue-safety criteria recommended by the European Council and supported the idea of preparing a special national transplantation Act regulating both organ- and tissue- transplants.

The reports prepared by the committees (Czech Transplant,1996,Institute for Clinical and Experimental Medicine, 1999) were used by the Ministry of Health in the drafting of the

Transplantation Act, and the members of the committees served as individual experts to whom the drafts of the Act were sent for comment.

Some data on the number of tissue-harvests and number of prepared grafts in the relevant years are quoted in Tables 1 and 2. The data summarized only grafts delivered by the official tissue-banks and reimbursed by health insurance companies , and did not include data from small hospital-banks attached to the orthopaedic departments of many hospitals which collected, predominantly, femoral heads from living donors.

Table 1: Number of living donors

Collected Tissue	**1996**	**1998**
Bone	159	216
Skin	0	0

Table 2: Number of deceased donors

Collected Tissue	**1996**	**1998**
Bone, fascia, cartilage	173	151
Heart valves	93	66
Skin	5	2
Cornea	913	812

The third committee was formed in Spring, 2004, as an advisory body to the Minister of Health. It consisted of representatives of the government and State control institutions (Ministry of Health, State Institute for Drug Control), representatives of tissue-banks (producers of grafts), representatives of medical societies (users of grafts) and representatives of health-insurance companies who are responsible for the funding of the tissue-banking and transplantation activities. One of the prime duties of the committee was to develop the concept of a national policy in the field of cell- and tissue-transplantation and to prepare a system of collecting data on tissue-banking and transplantation activities. Regular annual collection of these data, according to the requirements of the European Commission, is a duty of this committee as well.

2.3. Relevant international norms influencing the terms of the National Transplantation Act and the impact of the joining the European Union

The drafts of the Transplantation Act, as well as its final text, were strongly influenced by the need to include the terms of the Convention on Biomedicine (European Treaty Series (signed by the Czech Republic in 1998 and ratified in 2001 (European Treaty Series 1997) in the national legislation. For the first time, the Convention defined the legal status of surgical residues. An additional protocol to the Convention (Council of Europe 2002) has not been

ratified yet by the Czech Republic; nevertheless, some of its recommendations have been accepted, e.g. prohibition of financial gain.

The text was also influenced by drafts of the European Council document "Guide on Safety and Quality Assurance for Organs, Tissues and Cells" sent for comment to the EC member states in 2001, and printed in the final version in 2002 (European Council, 2002). This document recommended to the member-states the establishment of national networks of accredited tissue-banks. It also recommended to apply the Spanish model of tissue-donor management (Manyalich et al. 1995). Particular attention was paid to traceability assurance, a principle that had been neglected by some commercial tissue-establishments at that time.

The obligation of the European Union member-states to pay attention to the safety of products of biological origin was already included in the Maastricht Treaty of 7th February 1992. Proposals to protect the European Union States from importing tissues harvested from donors without ensuring donor-suitability were constantly re-iterated by the representatives of Eurotransplant. The idea of preparing a special directive on the issue of the establishment of common standards in the harvesting and processing of tissues, and the assurance of equal access to tissues by all EU member-States, was presented to the Congress of the Transplantation Society in Rome, in 2000. Before issuing the first working-papers of the Directive, the European Commission C – Health and Consumer Protection Directorate - General sent a detailed questionnaire to all member- and candidate-countries (European Commission, 2002) asking them to analyse their national legislation dealing with the safety of cell- tissue- and organ-transplantation. This questionnaire, which should have been returned by May, 2002, helped the candidate-states, including the Czech Republic, to identify any gaps in existing, and also in proposed, legislation. It did not, however, have an immediate and direct influence on the final text of the National Transplantation Act which was already undergoing its final passage through the parliamentary process.

The directive, the draft of which was sent to the member- and candidate-States in 2002, required that each member-State register all tissue-banks, and license or accredit them in compliance with the requirements of the directive (European Parliament and Council, 2004).

Regular publication of reports on the activities of tissue-banks in each country is also one of the principle obligations of member-States prescribed by the directive. They should strongly support non-profit tissue-banks, and were given two years to harmonize their national legislation with the directive.

The directive does not regulate the manufacture of medical devices of human origin. The tissue-donation, donor-screening and testing must be performed, however, in compliance with the directive even in this case. It does not regulate the manufacture of cell-and tissue-engineered products. The pertinent directive dealing with this issue is at the stage of preparation of a working-paper. It neither regulates the harvesting nor preservation of organs.

2.4. Events with negative impact on organ- and tissue-transplantation

There were several events which had a negative impact on public and parliamentary discussion during the drafting of the Transplantation Act. One example which dented public confidence in the existing system of organ-harvesting and transplants, was the so-called "Ostrava case", in which the transplant-surgeons of the local, very active Transplantation Centre were accused of malpractice in organ- harvests and -transplantations. The case was investigated at different levels; nevertheless no evidence was presented against them (Vítko

1999, Vítko 2001, Julínek, 2002). The case led, however, to a considerable decrease in organ-donations in the country (Table 3).

Table 3: Kidney harvesting- activity in the Czech Republic in the years 1997-2003

	1997	1998	1999	2000	2001	2002	2003
Czech Republic	239	198	164	175	172	146	191

A similar effect on the attitude of the public towards tissue-harvests and transplants was produced by further news in the media describing and criticising the persistent export of tissues collected through pathology- and forensic-medicine departments. The history of these exports started before 1989. The exporter was the Institute of Sera and Vaccines in Prague (USOL), a State enterprise. After 1989, private tissue broker-companies continued this practice (Czech Transplant, 1996, Institute of Clinical and Experimental Medicine, 1998, Šteiner and Špaček, 2002, Skočdopole, 2003).

The tissues were probably used for manufacturing various bio-implants in for-profit companies (Šteiner and Špaček, 2002). In the late 1980s and beginning of the 1990s, dura mater was collected for the manufacture of freeze-dried or chemically-treated grafts for dura-mater reconstruction or replacement of other connective tissue-structures. After the risk of transmission of the Creutzfeldt-Jacob disease (CJD) by dura mater was discovered (Pritchard, 1987, Diringer and Braig, 1989), the interest of private companies turned to pericardia (an alternative material for dura-mater replacement) and bone (Institute for Clinical and Experimental Medicine, 1999, Šteiner and Špaček, 2002). Czech TV repeatedly reported on complaints by relatives seeking the purpose of a radical bone- and joint-harvest performed on their deceased next-of-kin at the Dept. of Pathology in Děčín. As a result of this, prominent Czech pathologists (Šteiner and Špaček, 2002) as well as the Board of the Society of Pathologists, Scientific Council of the Czech Ministry of Health (Ševčík and Fakan, 2002) and representatives of the Czech Transplantation Society (Vítko, 2002) criticised the practice of harvesting tissues for commercial purposes. There was a suspicion, however, that these practices continued even after the new Transplantation Act came into force (Skočdopole, 2003). Discussion in the media during preparation of the Transplantation Act was open, often contradictory, and focused on protection of the rights of the donor. This is documented in the recording of our Czech radio-broadcast (in English) of December 2001 (Klement 2001). As a result of these events, and the broad attention paid to them in the media, the Transplantation Act received only a narrow majority in Parliament (Bošková, 2002, Julínek, 2002). Moreover, some changes in the draft submitted by the Ministry of Health consequently included a parliamentary initiative which has made tissue-harvest and preservation-activities much more difficult than expected (see below).

2.5. Principal features of the National Transplantation Act

Act No. 285/2002 On Donation, Collection and Transplantation of Tissues and Organs and changes in other Acts (Transplantation Act) (Ministry of Health, Czech Republic, 2002) regulate both tissue- and organ-transplants. The term "tissue" is also valid for cells.

Gametes, embryonic and foetal-tissues, placenta, hairs, nails and body-metabolism products are excluded from the Act. Harvesting for experimental purposes is excluded as well.

Tissue banks`roles are defined as follows: "The Tissue-Bank is designed to ensure collection, onward processing, testing, preservation, storage and distribution of tissue- grafts for transplantation" and can be established only with the approval of the Ministry of Health as an entity in a hospital which provides specialised or super-specialised medical care. In organizing tissue-collections, tissue- banks are obliged to cooperate with departments of pathology, forensic-medicine and obstetrics as well as with establishments providing tissue-harvests and transplants. They are obliged to use information provided by the National Register of Persons Rejecting Post-mortem Tissue- and Organ-Donation and the National Register of Tissue- and Organ-donors. They are also obliged to keep records on collected and accepted tissues, tests performed, manufactured transplantable-grafts kept in stock and grafts delivered for clinical application. They can also use information from the National Register of Performed Transplants in the evaluation of results of tissue-transplants.

The centre licensed to coordinate organ- and tissue-transplantation must be independent of individual organ-transplantation centres and tissue-banks.

Any tissue- or organ-harvest from a living donor, including the collection of surgical residues, can be made only with the explicit consent of the donor. Presumed consent of a deceased donor is allowed; the dissent can be expressed by registration in the Register of Persons Rejecting Post-mortem Donation or by other means described below. The Act includes strong safeguards for minors and protected adults. Harvesting from these persons is either excluded (prisoners) or can be made only with the explicit consent of the donor's parents or legal guardians.

The Act orders the creation of the following registers:

National Register of Tissue and Organ Donors (not yet established)
National Register of Tissue and Organ Transplantations (not yet established)
National Register of Persons Waiting for an Organ Transplantation (this waiting-list existed before approval of the Act). The Act does not create a register of persons waiting for tissue-transplants.
National Register of Persons Rejecting Post-mortem Organ or Tissue Donation (in operation since 2004, Ministry of Health, 2004).

It is possible to register a general refusal of organ- or tissue-donation or refusal of donation of a particular organ or tissue. The necessary forms are available on the internet. The register accepts mailed forms with a signature verified by a solicitor. It is also possible to register during hospitalisation. The organ and tissue-harvest teams are obliged to check whether the deceased person is registered before starting an organ or tissue-harvest.

Entry in this register is not, however, the only way to express refusal of post-mortem tissue-donation. The donor can leave a written refusal or express an oral refusal in the presence of a witness. For this reason, it is highly advisable to check the donor's attitude by contacting the donor's family.

Safety aspects of tissue-donation were included in Decree No. 437/2002 on the "Health-Suitability of Tissue- and Organ-Donos for Transplantation Purposes" (Ministry of Health of the Czech Republic, 2002). The decree defines criteria for donor-screening, the spectrum of serological and other laboratory tests to be performed, and instructions for performing repeated serological testing of living donors after the 6-month quarantine. It also defines the general contra-indications of organ- and tissue-collection and retains the rule that an autopsy must be performed on each deceased donor. The possible issue of a special decree on safety assurance in banking-procedures was discussed during the drafting of the Transplantation Act. Unfortunately, this proposal was not accepted at that time since the safety aspects of tissue- banking were not considered as urgent as protection of the rights of

the donor. This gap remains to be filled during the harmonization period, i.e. within the next two years. The safety-aspects of cell- and tissue-banking were included, however, in the requirements laid down for licensed tissue-banks by the Ministry of Health in 2004 (see below).

Simultaneous with the passing of the Transplantation Act, several changes in other Acts were made:

1. The Penal Act: This Act includes a new criminal-action clause dealing with the unauthorized handling of tissues and organs.
2. The Public Health Insurance Act: The insurance companies are obliged to pay all expenses connected with tissue- and organ-harvesting, preservation and transplantation. Reimbursement is settled on a non-profit and tax-free basis.
3. The Offences Act: Advertising with the aim of stimulating demand of tissues and payment of incentives to stimulate donation are regarded as offences. Disclosure of identity of the donor and the host is also regarded as an offence..
4. Change in the "Public Health Care Act": Includes special regulations for manufacturing bio-implants.

This change in the Public Health Care Act was made as the result of a parliamentary initiative without reference to the representatives of tissue-bankers. The aim of this initiative was to restrict the opportunity for tissue-trafficking and/or for exporting tissues for commercial purposes. The major change was to abandon the principle of presumed consent and turn to the opting-in principle. The tissue used for manufacturing bio-implants can be harvested now only with the explicit consent of the donor, or of his/her next-of- kin.

Bio-implants were defined for the purpose of the Act as tissue-grafts depleted of living cells. Tissues preserved by simple deep-freezing were not regarded as bio-implants. The parliamentary decision to create a special rule for manufacturing bio-implants came from the assumption that bio-implants can only be produced by foreign companies and outside the territory of the Czech Republic. It did not take into account that many products of Czech tissue-banks included in the list of tissue-grafts for reimbursement by Czech insurance companies also , belonged in this category (Table 4).

Figure 1: Minimally-manipulated deep-frozen, or cryopreserved, tissue-grafts are stored in a standard position in cardboard-boxes which hold grafts sealed in sterile, disposable peel-packs (Figure 1a). The boxes are labelled using standard abbreviations and insurance-company codes (in brackets).

Figure 1 a: Deep-frozen fascia lata graft ready for clinical application. The graft is finally labelled with a standard label including all data necessary for identification of the graft, the donor (in anonymous form) and the manufacturer - Tissue Bank, University Hospital, Hradec Králové (the insurance-company code of the manufacturer is 230).

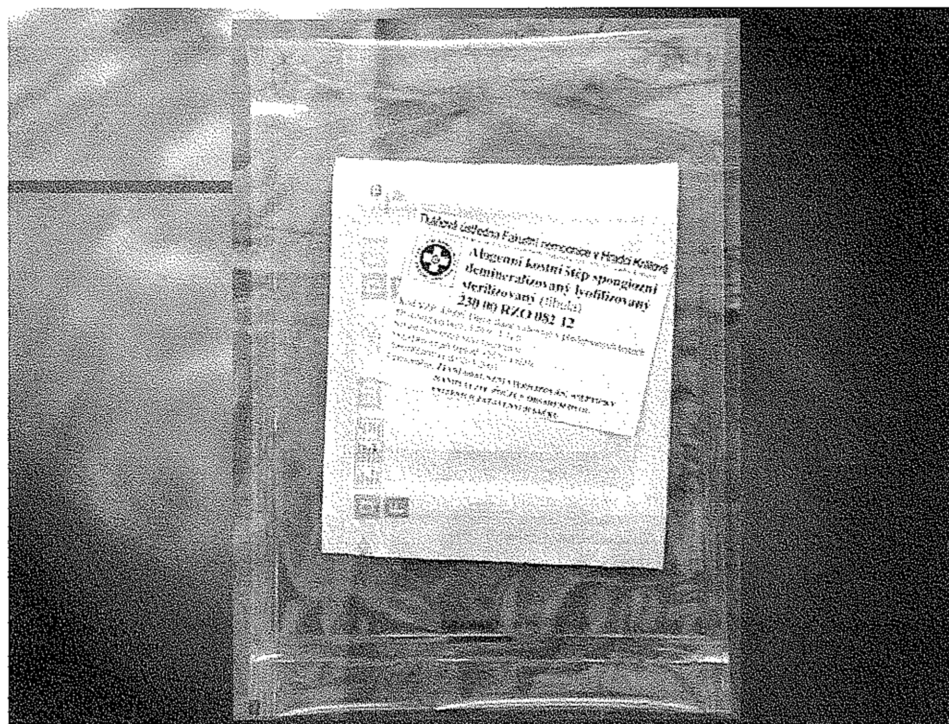


Figure 2: Freeze-dried, demineralised bone-grafts were included in the category of bio-implants. The inner-bag is a standard bag used for gas-sterilisation. The outer-packaging and labelling is identical, as shown above.

Table 4: Classification of codes given by the insurance company to the allogeneic tissue-grafts manufactured in the Tissue Bank, University Hospital Hradec Králové (1997-2002)

	Transplants Number of codes	**Bioimplants** Number of codes	**Total** Number of codes
Bone grafts	4	5	9
Soft connective tissue-grafts	4	3	7
Biological skin-covers	2	2	4
Ocular tissues	2	2	4
Total	**12**	**12**	**24**

The change in the Public Health Care Act caused a confusing situation in which there were two parallel Acts regulating tissue-harvesting and banking; one based on opting-in, the other on opting-out. Unfortunately, even more information that appeared in the press immediately after approval of the Act was either misleading or interpreted incorrectly, e.g. that tissues, in general, can be collected only with the explicit consent of the donor (Bošková, 2002). It is probable that this anomaly will disappear during the process of harmonization of national legislation that comes with the EU Directive.

Tissues collected for experimental or education purposes can be harvested only with the explicit consent of the donor or of the next-of-kin

2.7. Practical consequences of the EU Directive and the process of licensing tissue-banks in the Czech Republic

Directive 2004/23/EC of the European Parliament and Council "On Setting Standards of Quality and Safety for Donation, Procurement, Testing, Processing, Preservation, Storage and Distribution of Human Tissues and Cells" (European Parliament and Council, 2004) came into force on March 31, 2004. In the Czech Republic, it came into force on the date of accession, i.e. 1st May 2004. It led to the biggest changes in the practice of tissue-banks within the last 10 years. The Directive has a unifying power, which is different from recommendations of the European Council. All member-states are obliged to harmonize their national legislation with the Directive within 2 years. The Directive regulates both autologous and allogeneic cells and tissues, including gametes, foetal and embryonic ones (different from the Czech Transplantation Act). The Directive does not include organs (different from recommendations of the EC). The Directive emphasises the need of establishing the safety and quality assurance programmes in processing cells and tissues of human origin.

The differences between the National Transplantation Act, the EC guide and the EU Directive are summarized in Table 5.

Table 5:

	National Transplantation Act	EC Guide to Safety	EU Directive
Organs	Included	Included	Excluded
Autologous cells and tissues	Included	Excluded	Included
Allogeneic cells and tissues	Included	Included	Included
Gametes	Excluded	Excluded	Included
Foetal and embryonic cells	Excluded	Excluded	Included

The Ministry of Health started the process of licensing tissue-banks in Spring, 2004. All hospitals that applied to license existing tissue-banks, or new tissue-banks, were obliged to submit answers to detailed questions, published on the internet-pages of the Ministry, concerning:

lists of tissues collected and processed, and their specifications,
description of premises of the tissue- bank, system (methods) of sanitization, methods of cross- contamination prevention, system of environmental control,
description of the technical equipment of the tissue bank, results of the validation of the equipment,
organization of the bank, qualification of the staff, the names of persons authorized to release grafts for clinical application,
description of the documentation,
system of monitoring adverse reactions,
the tissue bank quality-management policy.

The applications were evaluated by independent experts. The list of the licensed banks was published on the internet-pages of the Ministry of Health. The licensed banks were divided into the following categories:

1. multi-function banks; fully-licensed banks for the harvesting of cells and tissues from living and dead donors, preservation by different methods including cryopreservation and freeze-drying, use of tissue-culture and cell- and tissue-engineering methods,
2. cell tissue-banks; licensed to collect and process living cells for transplantation and cellular-therapy,
3. eye-tissue banks; licensed to preserve corneas and other ocular cells and tissues,
4. specialized tissue-banks, e.g. heart-valve bank,
5. bone-tissue banks.

In each licence, it was precisely defined whether a bank was approved to collect tissues only from living donors, as with some bone-tissue banks, or whether it was licensed to also collect tissues from dead donors. Banks that had a licence to collect tissues from dead donors were given authorisation to search in the National Register of Persons Rejecting Post-mortem Tissue Donation, which also started to operate on the 1st September, 2004 (Decree No. 434/2004: Details of the range and content of data obligatorily stored in the National Register of Persons Rejecting Post-mortem Tissue and Organ Donation, Ministry of Health of the Czech Republic, 2004).

3.METHODS OF MEETING THE REQUIREMENTS OF THE CZECH AND EUROPEAN LEGISLATION-OWN APPROACH

The author's approach to ways of matching the new quality- and safety-criteria prescribed by national legislation and the EU Directive was based on the similarity of the cell- and tissue- banking methods and manufacturing of sterile drugs.The general requirements of pharmaceutical control-institutions described in the rules of the Good Manufacturing Practice (GMP) and issued by the State Institute for Drug Control (State Institute for Drug Control, 1998) were used as a methodological guide.

As a result of this similarity, it was necessary to change a lot of the standard operating-procedures used before; to describe specifications of the collected material and final products; to establish a system of traceability- reliability and cross-contamination prevention, and to specify the storage-conditions. One of the necessary measures was the introduction of clean-room technology and environmental control to ensure an aseptic way of processing cells and tissue- products that did not tolerate terminal sterilisation.

In this paper, which is an expanded version of the paper presented to the International Conference of the European Association of Tissue Banks held in Prague in October, 2004, (Měřička et al., 2004), examples of specifications of cryopreserved cell- and tissue-grafts, standard operating-procedures for cell- and tissue-donation, banking and bacteriological control of grafts and the traceability reliability system are shown.

Chapter 3 describes the system of ensuring requisite storage-conditions, a system of cross-contamination prevention and the results of monitoring the clean rooms used for the processing of cells and tissues before cryopreservation.

4. RESULTS

4.1 Specifications for the collected material and final products

Specifications for the collected tissues as well as for the final products were written using the general criteria for specifications published in the Bulletin of the State Institute for Drug Control (SÚKL, 1998). Specific requirements of the Transplantation Act and of the decrees of the Ministry of Health were taken also into account. Examples of specifications for collected cell-suspensions (bone marrow) and solid tissues (skin, bone-tendon-bone allografts) are presented in appendix 1a.

Examples of specifications for cryopreserved cell-grafts (unseparated bone-marrow) and solid-tissue-grafts (dermoepidermal and bone-tendon-bone grafts) are demonstrated in appendix 1b.

4.2 Standard operating procedures for cell and tissue donation and collection

Standard operating procedures(SOP´s) for cell- and tissue-donation and -collection from a living and deceased donors including examples of informed consent, are attached. The major change is to include information on the possibility of tissue-harvesting to the family of the deceased donor – Appendix 2. Examples of informed consent for donation of cells or tissues for experimental purposes are also shown.

4.3 Standard operating procedures for cell- and tissue-cryopreservation

The general SOPs used for the cryopreservation of cells and tissues ciover two categories – one for grafts that are to be implanted (bone–tendon-bone allograft),and one for cryopreserved biological skin-covers. The SOPs were written using the requirements of GMP (Appendix 3).

4.4 Standard operating procedures for bacteriological control of tissue- grafts

Control is carried out by the Department of Clinical Microbiology of the University Hospital. The standard operating procedures for culturing bacteriological swabs as well for sterility-testing are included in Appendix 4.

4.5. Traceability-reliability in cryopreserved cell-and tissue-grafts

Traceability is guaranteed by using specific labels for collected,as well as for released, final products (Měřička et al., 2002). The labels include:
identification of the producer using the identification code of the insurance company and of the Ministry of Health,
anonymous identification of the donor using his/her identification number;in autologous grafts the name and birth code of the donor/receiver is quoted,
identification of the date of collection or year of manufacture,
identification of the tissue using the tissue identification number.

Examples of labels used for traceability-reliability in cryopreserved products are shown in Appendix 5. After using the grafts, the labels are to be included in the patient's documentation.

4.6 Results of the licensing process

On the 1st September, 2004, 15 institutions received a licence to establish tissue-banks. Only two institutions received a licence to establish multi-tissue banks. The tissue-bank of the University Hospital, Hradec Králové, received a multi-function licence, i.e. the broadest type of licence. It was given the registration code MTB 006. The data summarizing the results of the licensing-process, including reimbursement for grafts by the insurance companies, are shown in Table 6. Only 7 banks have been able to submit all the necessary data required by the insurance company for acceptance of reimbursement for their products since the 1st January,2005.

Table 6: Tissue-banks added to the list of producers

Year	Monobanks	Multifunction banks	Total
1993	4	2	6
2004	6	3	9
2005	5	2	7

5. DISCUSSION

The effort to quickly integrate the requirements of the EU Directive into the practice of Czech tissue-banks led to an improvement in the level of safety and quality assurance in tissue-transplants in the country. The major achievement was the establishment of a national network of licensed tissue-banks of different types. This network also includes small banks in surgical departments that do not distribute their products outside their hospitals, and which had been exempt from any supervision in the past. All tissue-banks received the status of "final producer," responsible for the whole technological chain starting with tissue-collection and ending with the distribution of the final product. This status also includes responsibility for the selection and interpretation of all mandatory contro-tests. This legislative change happened very quickly and allowed very little time for adaptation. For this reason, even some tissue-banks with a long-term tradition disappeared from the list of those approved to distribute their products (Table 6). Two monobanks and one multi-function bank were removed from the list, and of the new establishments that received a licence last year, only one monobank was included in the list. This situation confirmed the validity of the long-term approach towards quality- assurance which aims at the gradual implementation of the rules of Good Manufacturing Practice (Měřička et al., 2002).

The decision to regulate the quality and safety of tissue-banking to an European norm was not a surprise. This was a reaction to the failure of a system of voluntary accreditation within organisations of tissue-banks as practiced in the U.S.A. at the beginning of the 1990s. This system was not able to eliminate non-accredited tissue-banks or the use of tissues imported from countries where collection was performed without proper supervision.(Warwick and Kearney 2002) This situation was strongly criticised by peer American tissue-bankers and representatives of the FDA. This practice, together with the practice of pooling tissues collected from several donors in one processing batch, led to an increased danger of infection-transmission (von Versen, 1995). In 1993, the FDA decided to issue special rules assuring the basic level of safety and quality in the process of tissue-donation and -harvesting (Food and Drug Administration 1993, Sandler,1994, Fragale, 1997). Later, specific instructions for the application of GMP rules into tissue-banking, called Good Tissue Practice, were prepared by the FDA (Department of Health and Human Services, 2001).

In the European Union, there was a strong lobby in the middle of the 1990s to regulate tissue-banking through a Medical Device Directive (Roscam Abbing, 1995, Dupont, 1997). This approach was not accepted, however, as it would have been extremely difficult to guarantee compliance with the Convention on Biomedicine and its Additional Protocol. Nevertheless, it probably influenced the Czech General Health Insurance Company into a decision to include tissue-grafts on the list of medical devices (General Health Insurance Company, 1997).

As in the U.S.A., specific rules for tissue-banking were also issued in Europe, firstly by the British Department of Health (Department of Health, 2001).

In the Czech Republic, the General Health Insurance Company was, for a long time, the only institution having a controlling role. Its policies were aimed, however, mainly at financial control. Safety and quality criteria were considered only during the last two years. The process of tissue-bank licensing, in 2004, was initiated and controlled by the Ministry of Health, with only temporary 2-year licences being issued. After the expiration of this period, it is expected that licences,or accreditation, will be granted by a specialised, independent institution.

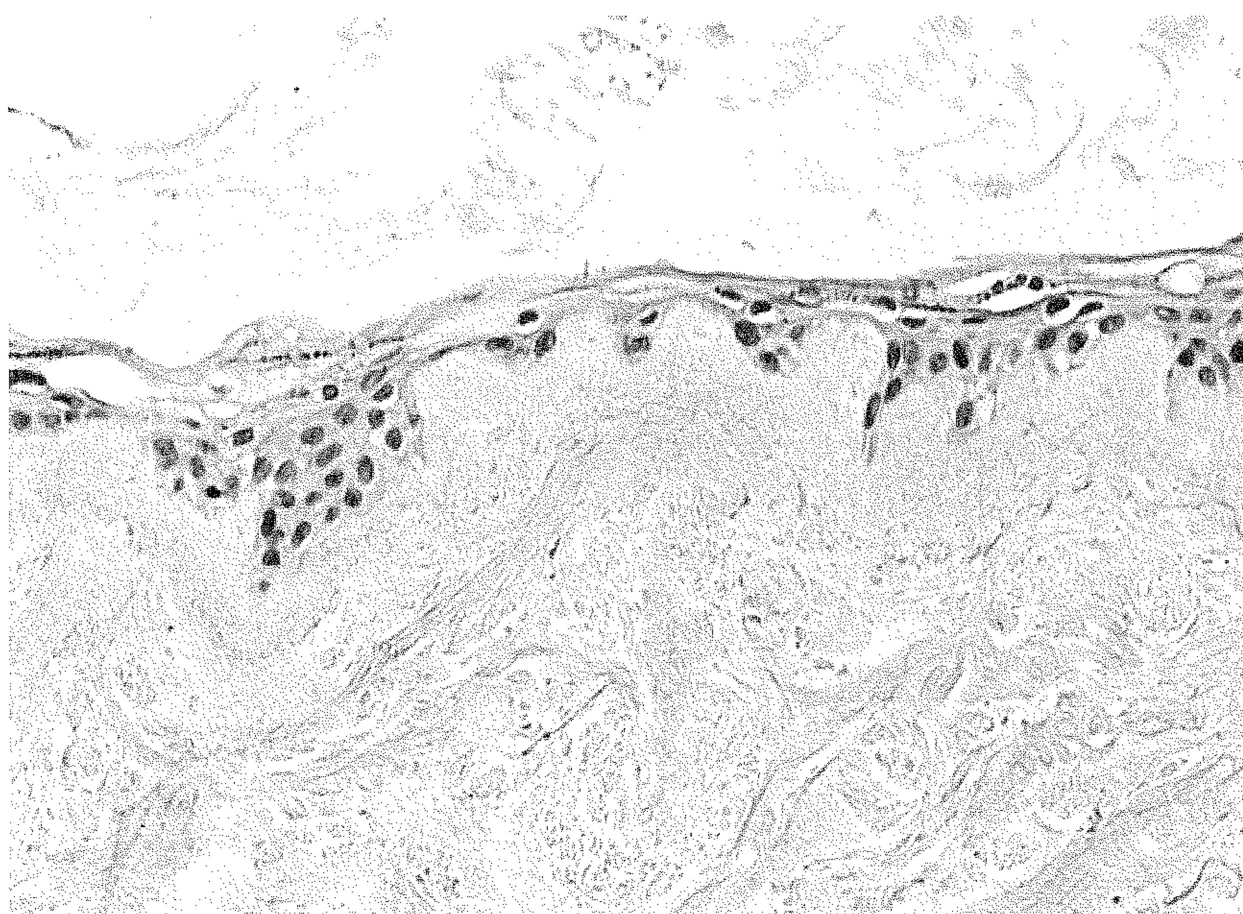
For this reason, it was very important to modify the safety- and quality-management in the University Hospital Tissue-Bank in compliance with the GMP rules recommended by the State Institute for Drug Control (State Institute for Drug Control, 1998).

The detailing of specifications for collected tissues and end-roducts became a new feature of its practice, which had been not included in previous operational manuals. Defining the properties of grafts, including the control-methods used in the specifications, makes it possible to inform clients on the properties of the grafts, or to make corrective actions in case of any deviation. It is also possible to measure the probability of meeting the requirements of the specifications through a validation process.

The major change in the SOPs for tissue-donation and -collection was the inclusion of information on possible tissue-harvesting from a deceased donor to the donor's family. In addition to the ethical value of such a decision, contact with the family may help in completing the medical history of the deceased donor, especially in cases where verbal contact with the donor was impossible.

Collection of cells and tissues for experimental purposes was limited to living donors. In the illustration below an experiment carried out in 19987 is shown. The experiment ,which enjoed financial support from the Internal Grant Agency of the Ministry of Health (Straková, 1995, Měřička et al., 2000),reveals the keratinocytes taken from one deceased donor grown on the de-epidermised dermis of another deceased donor. Nowadays, this experiment could be repeated only with explicit consent as defined in the Public Health Care Act.

Figure 3: Human epidermal keratinocytes taken from a deceased donor forming a new epidermis after being seeded to a de-epidermised, acellular dermis prepared from a split skin-graft taken from another deceased donor (Courtesy Ass. Prof. Šubrtová). Financial support of IGA MZČR, Grant No 3696-3 (Měřička et al, 2000).



In addition, the author stopped collection of cadaveric split-skin for experimental purposes. This skin had been used over a long time in drug- penetration studies carried out in the Faculty of Pharmacy, Hradec Králové (Doležal et al., 1993, Vávrová et al., 2002). Human skin was replaced with pigskin taken from the ear by a standard procedure developed for pigskin-grafts being manufactured for clinical use (Měřička et al., 2002).

The SOPs for the processing and control of cell- and tissue-grafts were submitted for audit to an authorized company in 1999. The control-procedures were strictly separated from the manufacturing itself and were carried out by individual departments of the University Hospital, or by licensed independent institutions. Bacteriological monitoring of cryopreserved cell- and tissue-grafts is performed by the Department of Microbiology. Swabs taken from processed tissues are cultured in aerobic and anaerobic conditions (Měřička et al., 2002). The methodology of the Paul Ehrlich Institute (FRG) is applied to the sterility tests (see Appendix 4).

The traceability-assurance system is an efficient tool for the control of infection-transmission in cell- and tissue-transplantation, and was traditionally included at all levels in voluntary tissue-banking organizations. In the tissue-bank of the University Hospital, Hradec Králové, it has been applied since its opening in the 1950s. The new approach to traceability –accuracy, using specific labels containing anonymous data on the donor together with the identification-codes of the producer, was introduced by the author in 1999, and the results were published in 2002 (Měřička et al., 2002). The traceability-system will be implemented nationally after the establishment of registers for tissue-donors and -receivers as ordered by the Transplantation Act.

Implementation of the new norms will, without doubt, increase the safety and quality of the work of tissue-banks. On the other hand, availability of tissue-grafts for clinical application may be jeopardized. In table 1, we demonstrated the influence of the dissemination of negative information on the work of organ-transplantation teams. It is the lack of current transparent rules for international co-operation that may lead to the publication of negative information now. It is expected that rules on this issue will be approved during the harmonization process. Regular annual reports on the activities of tissue-banks, required by the EU Directive and issued by the Ministry of Health or Centre for Transplantation Coordination, should be made publicly accessible. They represent an efficient tool for the strengthening of public confidence in the existing system of cell- and tissue-collection,-banking and -transplantation.

Alternative ways of achieving greater availability of transplantable tissues are to increase the use of tissues collected from living donors and/or to use xenogeneic tissues, where possible.

6. CONCLUSIONS

The existing national legislation regulating tissue-harvesting and transplantation in the Czech Republic is not in contravention of the Directive of the European Parliament and Council.

The gaps in existing legislation are to be eliminated with the help of a newly-formed committee of experts.

Publicly-accessible, regular, annual reports on the activities of tissue-banks are regarded as an efficient tool in strengthening the confidence of the public in the transparency of the system of tissue-collection,-banking and -transplantation.

The procedures for tissue-bank licensing led to the establishment of a national tissue-bank network, and rapidly increased standards of quality and safety of tissue-transplants in the country.

Availability of tissue-grafts may be jeopardised by the implementation of the requirements of the national Transplantation Act. The increasing use of tissues collected from living donors, or the use of xenogeneic tissues, where possible, may help to resolve this issue.

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CHAPTER 3

THE CELL AND TISSUE BANK AS A COMBINATION OF CRYOGENIC AND CLEAN-ROOM TECHNOLOGY

1. INTRODUCTION

The history of establishing large cryobanks is bound together with the large-scale use of cryopreservation techniques in the breeding sciences as well as with the establishment of national and international cell-culture collections. In medicine, it is connected closely to the application of cryopreservation in haematology, transfusion-medicine and burns- and disaster-medicine (Smith, 1961, Meryman, 1966, Dobrý,1971, Sumida,1974, Puškar and Belous, 1975, Kobylka 1989, Konigová, 1990, Moserová and Houšková, 1989, Smit Sibinga et al., 1990, Areman et al.,1990, Měřička et. al., 1990,1992,1993, 1995, Griščenko and Belous 1994, Klein 1997, Klein and Měřička, 2000, Měřička et al., 2002, Měřička and Hošek, 2002). The project of creating a cell-culture collection as a part of the Tissue Bank of the University Hospital, Hradec Králové, already existed in the early 1970s (Heger et al., 1971), and led to the introduction of the cryopreservation of living cells (Hroch and Srb, 1971, Srb and Hroch, 1971). The project aimed at creating a bone-marrow bank in the University Hospital, Hradec Králové, as a part of the tissue-bank was introduced by Prof. Klen at a meeting of experts from the International Atomic Energy Agency in Moscow, in 1968; and at a symposium on tissue-banking in Washington, D.C. in 1975 (Klen, 1968,1976). A review of availabibity of technological support necessary for establishing a bone-marrow bank in the former Czechoslovakia was published by the author of this paper in 1983 (Měřička, 1983). The programme of clinical application of cryopreserved blood progenitor-cells in the University Hospital, originated in its Department of Intensive Haematological Care, 2nd Dept. of Medicine, in the years 1988-1989 (Bláha et al., 1990). It used metal containers manufactured by the Research Institute of Food and Refrigeration Technology, Hradec Králové (now Ateko, Ltd. Hradec Králové) (Měřička et al., 1990,1991), controlled-rate freezing, and a mechanical freezer (-80°C) for storage. Storage in liquid nitrogen was introduced in 1991 (Měřička et al., 1995,1999).

Before starting the cryopreservation of large volumes of cell-suspensions for clinical application solving specific problems was necessary, such as finding a plastic material resistant to liquid- nitrogen temperatures suitable for manufacturing freezing-bags and for the storage of cells; finding optimal freezing-rates which assured standard freeze/thaw recovery-rates. In freezing haematopoietic progenitor-cells, it was also necessary to resolve the issue of the toxicity of the cryoprotectants that were to be infused intravenously together with the thawed suspensions, and to find optimal stimulation-protocols (Bláha et al., 2001). The majority of the above-mentioned issues seemed to have been resolved in the work of the haematopietic cell- banks in the early 1990s (Areman et al.,1990, Warkentin et al.,1993, Měřička et al.,1995,). The issue of the quality of plastic, disposable freezing bags returned, however, after the publication of Tedder's paper describing cross-contamination by the hepatitis B virus in red blood-cell concentrates which had been stored in bags immersed in liquid nitrogen (Tedder et al., 1995, Hawkins et al. 1996).

Increasing requirements for the safety and quality of cryopreserved tissues and cells used for therapeutic purposes, as well as the rapid development of methods of cell- and tissue-engineering in the last decade (Lanza et al. 1997, Polak et al.2002), led to the necessity of allying cryobanks closely with laboratories able to process the cells and tissues prior to cryopreservation. After thawing (e.g. using cell expansion method), the process was to be carried out under conditions which meet the criteria required by current legislation (see chapter 2). As living cells and tissues do not withstand terminal sterilisation, it was obvious, even in the middle of the 1990s, that the process should be performed under those conditions used for manufacturing sterile pharmaceutical products, i.e. using clean-room technology. Evaluation of these trends led to a proposal for the creation of a new cell- and tissue-establishment, based on a combination of these technologies, at the University Hospital..

After critical evaluation of this proposal by the University Hospital Administration, the bank was designed in 1998/1999 by Atelier H-1 Hájek, Hradec Králové, and the Chironax Project, Prague, using the guide-lines for the design of pharmaceutical facilities laid down by International Society for Pharmaceutical Engineering (ISPE Headquaters, ISPE European Branch Office, 1999) and the recommendations of the State Institute for Drug Control, Prague (Věstník SÚKL, 1998). The whole project for the bank was reviewed by G.M. PROJECT, Opava, a company with international experience in planning and building facilities for the pharmaceutical industry and registered with the European Commission (Reg. No. CZE-19216). After receiving a positive go-ahead from the State Institute for Drug Control, the project was given financial support by the Ministry of Health, and the bank was built between 2000-2002 by Stako (Hradec Králové). The heating, ventilation and air-conditioning systems (HVAC) for the clean-room facility was manufactured by Pulsklima, Liberec. In this paper, the two-year experience in operating the new cell- and tissue-bank, built with a combination of cryogenic and clean-room technology is reviewed.. The aim of the paper is to determine and analyse the most critical parameters bearing the safety of the working staff and of the product.

2. DESCRIPTION OF THE BANK

The bank consists of a cryogenic facility used for the controlled-rate freezing of cells and tissues and storage at either temperatures of –85°C (mechanical freezers) or at liquid-nitrogen temperatures, and a clean-room facility. The outer-view of the bank is shown in figure 1. The bank is supplied with liquid nitrogen from the outer-vessel which is connected by an insulated tubing to the cryostorage-facility as well as to a laboratory for the controlled-rate freezing (Figure 1).

Figure 1: The outer view of the Tissue Bank, University Hospital, Hradec Králové. The outer tank can contain, maximally, 4,000 kg of liquid nitrogen. Critical parameters, i.e. the liquid-nitrogen weight and the pressure inside the container, are continuously monitored and recorded. The container is connected by an insulated tubing to the cryostorage rooms and cryopreservation laboratory (front right). The clean-room facility, with the critical processing-areas, is situated in a building seen at front – left. The high building behind the bank is the Department of Gynaecology and Obstetrics.

Figure 2: Internal view of the bank. The large containers for the storage of haematopoietic progenitor-cells are filled automatically from a back-up, low-pressure liquid-nitrogen container (volume 230 l). A container of the same size is used as a back-up, safety-cooling system for two mechanical freezers operating at a temperature of –85°C (right).

Liquid nitrogen in the outer tank (Figure 1), as well as in the back-up liquid-nitrogen vessels placed inside the bank (Figure 2), is stored at low pressure to minimize liquid-nitrogen loss through evaporation. The tubing connects the outer tank to the cryobank itself (Figure 3).

Figure 3: The insulated tubing connects the outer liquid-nitrogen container to the cryobank. The metal construction around the tubing lowers the possibility of mechanical damage.

The tubing is filled with liquid nitrogen only at times of transfer of the liquid into the vessels placed inside the bank. The critical pressure for opening the tubing relief-valves is adjusted to 0.3 MPa. This operation is carried out once or twice a week, and lasts approximately one hour. For the rest of the time, the tubing is left without pressure in order to avoid any leakage of liquid nitrogen in the case of mechanical damage to the tubing. Automatic filling of the large biological containers with liquid nitrogen is effected from the back-up, low-pressure containers placed inside the bank (Figures. 2,4). The same type of low pressure container is used for supplying the programmable freezer (Figure 4).

Figure 4: The programmable freezer is supplied with the liquid nitrogen from a low-pressure, 230 l container. The back-up vessel is connected to the external vessel by the tubing.

The following issues are dealt with in this paper:

A. Cryogenic facility:

1. evaluation of the oxygen-deficiency hazard.
2. setting the normal operational- and alarm-limits in the cooling equipment used for storage of cells and tissues by operational qualification.
3. system of cross-contamination prevention; this issue is dealt with separately in the published papers (Měřička et al., 2002, Bláha et al., 2003, Měřička et al., 2003). Full texts of these papers are included in the appendix.

B. Clean- room facility:

1. application of general guide-lines for aseptic pharmaceutical production in the tissue-bank.
2. results of the microbiological validation of clean-rooms at the beginning of operational use and after one year of operation.

3. METHODS AND RESULTS

3.1 Cryogenic facility

3.1.1 Evaluation of the oxygen-defficiency danger

The level of atmospheric oxygen, a critical factor in safety of the staff, was analysed in the paper "The Importance of Oxygen-level Monitoring in the Cryostorage Facilities" presented at the conference "Cryogenics 2004" in Prague, and published in the Conference Proceedings (Měřička et al., 2004). Full text of the paper is uincluded in the appendix.

3.1.2 Setting of normal operational- and alarm-limits for the storage of biological material

The critical factor for maintaining the quality of the product (cell or tissue graft) is the temperature inside the cooling-equipment used in the storage of these biological materials. This temperature is continuously monitored and recorded, and is required to remain exactly within the process- limits.

When continuous monitoring or recording is necessary, the ISPE Guide (ISPE 1999) recommends adjustment as follows:

1. An alert to register that the temperature has deviated from the normal operating- range.
2. An alarm to indicate that the temperature has deviated from the process-limits.

The standard way of setting the normal operational range and process-limits is to observe the operational qualification of the equipment set by an authorised company (Figure 5).

3.1.2.1 Methods of operational qualification of the cooling equipment

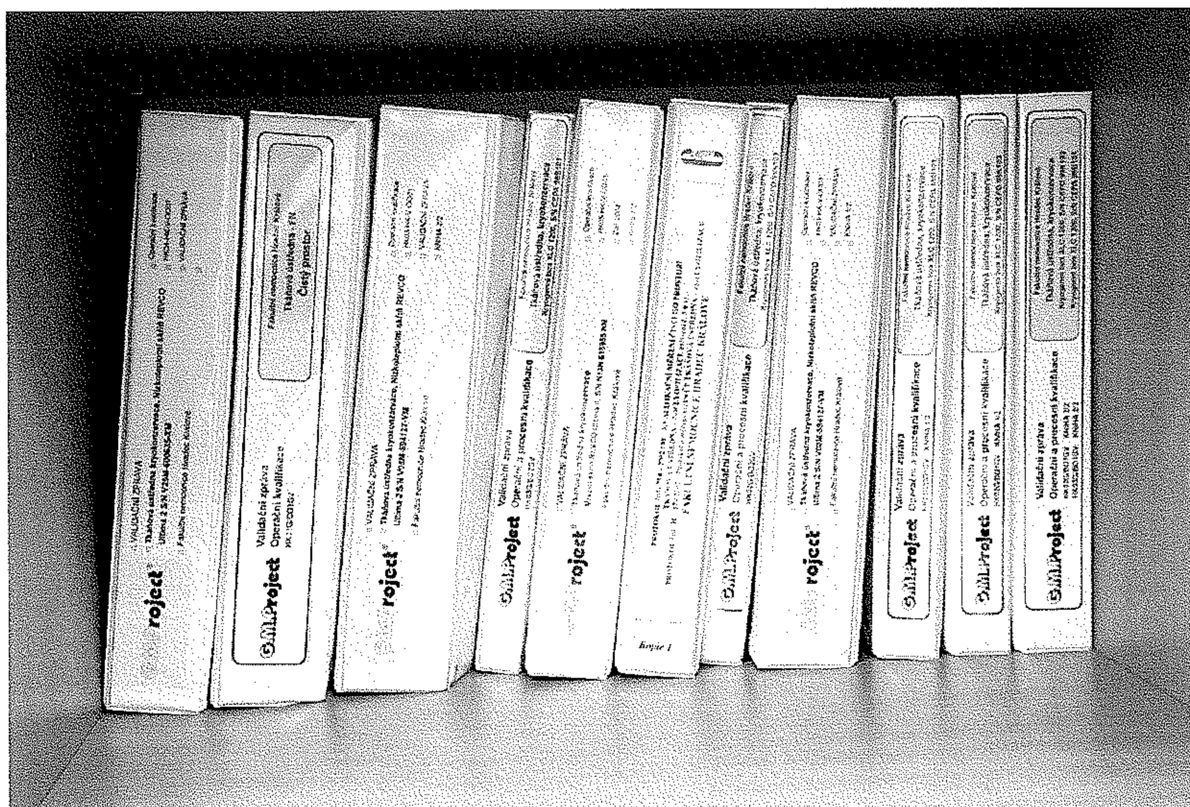

Figure 5: Validation-reports are stored in the department for future reference

The operational qualification of the two large liquid-nitrogen containers, the two mechanical freezers (Figures 2,5) and the two pharmaceutical refrigerators was set by the company G.M. PROJECT Opava, registered with the European Commission. The validation-team consisted of representatives of the user (tissue-bank staff, the hospital administration) and a representative of the authorised company. Validation was performed during normal operation of tested equipment as well as during simulation of alarm situations using standardized power-off and door-opening tests.The acceptability-criteria were established by the representatives of the tissue-bank on the basis of the following assumptions:

A: Liquid nitrogen vapour-phase storage:

1. The normal operational temperature-range is affected by temperature-fluctuations during regular, automatic filling of the containers (Figure 6) .
2. The upper process-limit is achieved at the temperature of cubic hexagonal transformation of ice (-130°C); the lower process-limit, by the liquid-nitrogen temperature.

B: Liquid-nitrogen liquid-phase storage:

1. The normal operational temperature-range is affected through temperature-fluctuations during automatic filling of the container (Figure 7).
2. The upper process- limits are achieved at a temperature of –150°C; the lower process-limit at a temperature of –199°C, as adjusted by the manufacturer.

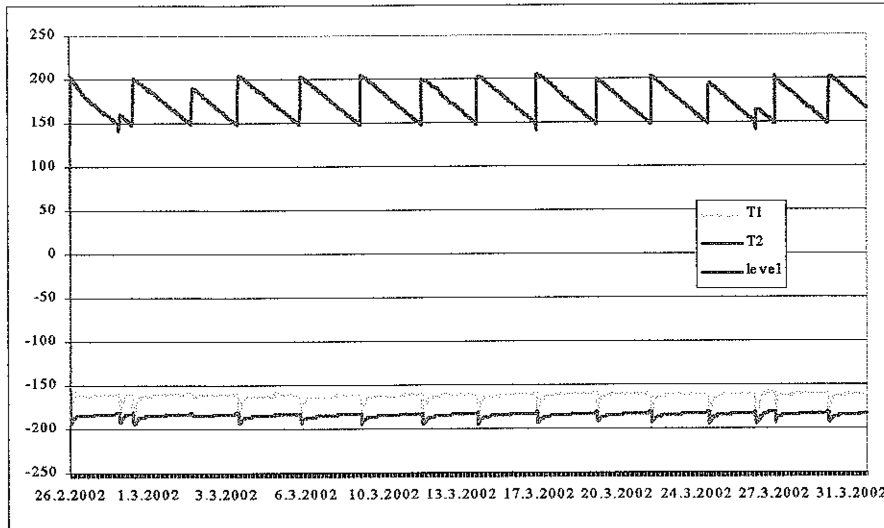

Figure 6: Adjusting the liquid-itrogen levels in a container used for storage in the vapour-phase. Temperature-drops occur during automatic filling from the back-up vessel.

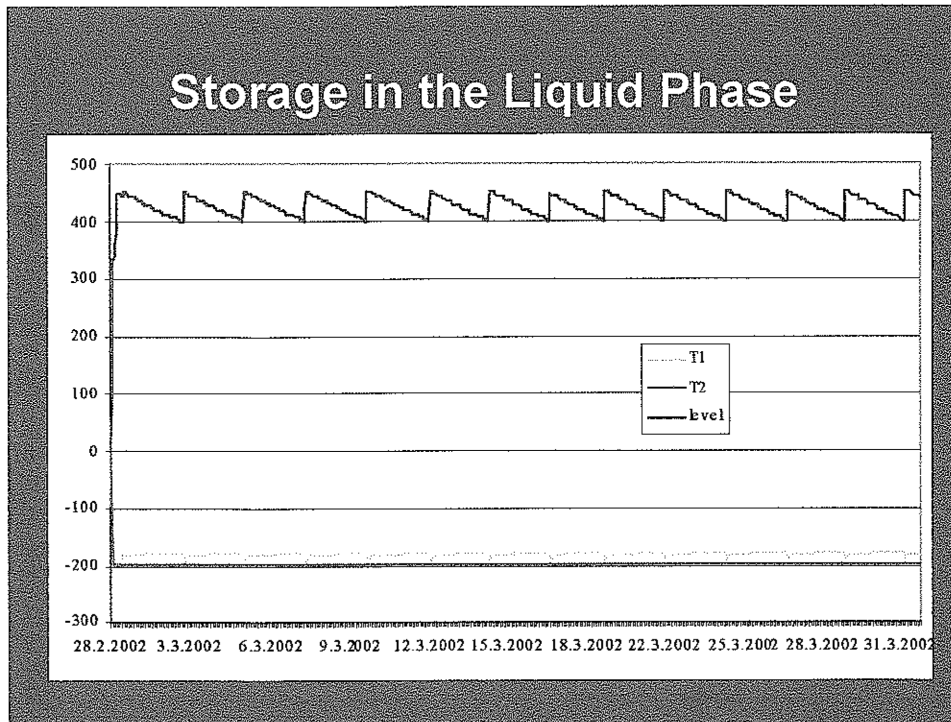

Figure 7: Adjusting liquid-nitrogen levels in the container used for storage in the liquid-phase. Temperature-drops occur during automatic filling of the container.

Automatic filling with liquid nitrogen is regulated by adjusting the upper and lower level of liquid nitrogen inside the tank. The upper/lower-level difference in both cases is adjusted to 50 mm; the frequency of filling is clear from Figures 6 and 7.

C: Mechanical freezing (-85°C):

1. the normal operational-range is accompanied by temperature-fluctuations during mechanical cooling (Figure 8).
2. the upper process-limit is reached at the temperature of –70°C, at which point the back-up, liquid- nitrogen cooling system starts to operate.
3. the lower process-limit is reached at a temperature of –90°C, which indicates a breakdown in the regulation of the cooling- system.

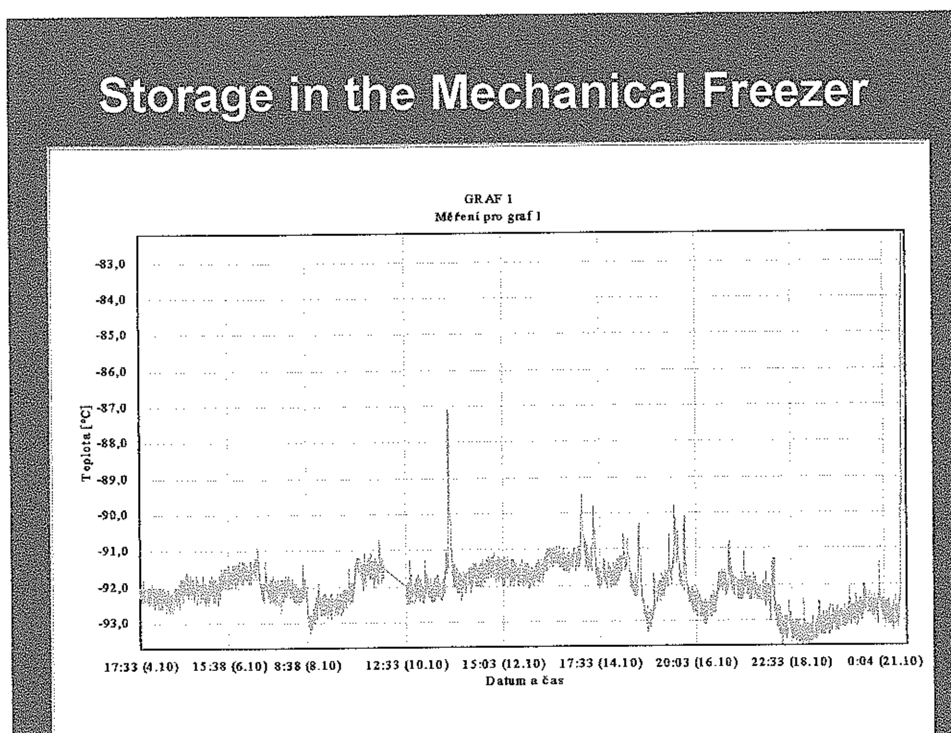

Figure 8: Graph of temperature-fluctuation during mechanical cooling. The regular temperature-fluctuations are caused by switching on/off the compressor; the temperature-peaks are caused by opening the door of the low-temperature cabinet.

D: Mechanical refrigeration (+5°C):

1. normal operational range is characterised by temperature-fluctuations during mechanical cooling.
2. upper process- limit is + 8°C, as recommended by the Ministry of Health.
3. lower process limit is 0°C.

3.1.2.2 Tested cooling-equipment and methods of temperature measurement

A summary of types of checked cooling-equipment, displayed values and performances, and a list of alarm-functions is shown in Tables 1–3. The acceptability-criteria for calibrated probes are summarised in Table 4.

Table 1: Types and number of items of cooling-equipment tested

	Volume (l)	No. of items	Manufacturer	Year of testing
Cryogenic box	1,200	2	Chart USA	2002
Upright mechanical freezer	486	2	REVCO USA	2004
Upright pharmaceutical refrigerator	280	2	Electrolux	2004

Table 2: Displayed values and functions

	Adjusted T/LN_2 level	Warm control set point	Cold control set point	Actual T	Power ON/OFF	Function of back- up system	LN_2 usage /day
Cryogenic boxes	yes	yes	yes	yes	yes	yes	yes
Freezer –85°C	yes	yes	yes	yes	yes	yes	no
Refrigerator +5°C	yes	yes	yes	yes	yes	no back-up	no back-up

Table 3: Alarm functions

	Warm T alarm	Cold T alarm	High/low LN_2-levelalarm	Power-failure alarm	Back-up supply alarm
Cryogenic boxes	yes	yes	yes	yes	yes
Freezers −85°C	yes	yes	no	yes	yes
Refrigerator +5°C	yes	yes	no	yes	no back-up

Table 4: Conditions for calibration of probes and acceptability-riteria

	Cryogenic boxes	Mechanical freezer −85°C	Pharmaceutical refrigerator +5°C
Precalibration temperatures	-197 °C 0 °C,-25°C	-197°C 0°C,-30°C	-20°C +20°C,10°C
Postcalibration temperatures	-25°C	-30°C	-10°C
Acceptable deviation	2°C	0.3°C	0.3°C
Acceptable stability	0.2°C for 2 min	0.2°C for 2 min	0.2°C for 2 min

Storage at liquid nitrogen temperatures - used liquid nitrogen containers:

Type of container: Cryogenic-box XLC 1200 with an internal inventory-system for the storage of biological material in racks with bags, or boxes with ampoules. The inventory-system and racks are made of stainless steel. The box is used for the storage of bags with human blood progenitor-cells enclosed in metal (aluminium) cassettes ST 100 (Consarctic, Schöllkrippen, FRG) on two levels. The internal diameter of the box is 780 mm; the depth 850 mm (Figure 9). The distance from the top of the inventory -system to the upper-lid of the box is 300 mm; the distance of the top of cassettes stored on the upper layer from the upper-lid is 350 mm.
Figure 9: Internal view of a cryogenic-box. The space is divided into 16 individual compartments by an inventory-system. The cassettes with bags containing cryopreserved suspension are stored in racks placed on two levels.

Temperature-measurement inside the liquid-nitrogen containers was performed by an Intelligent RTD Temperature Probe Modul M 2801 (Kaye Instr. Inc. USA) using multiple probes placed at different positions inside the container. The data were processed by a portable validator modul X 1310 (Kaye Instr. Inc., USA).The temperature was recorded in 1-2 minute intervals. Printed records were included in the validation reports .

3.1.2.3 Results of the operational qualification of the cooling equipment

A .Storage in liquid-nitrogen containers – vapour-phase

The lower limit of the liquid-nitrogen level was adjusted to 150 mm; the upper limit, to 200 mm: the low-level alarm-limit to 99 mm; the upper-level alarm-limit to 250mm. The position of the probes, and the temperatures, at the start and end of the validation-process are summarised in Table 5:

Table 5: Temperature-conditions in the vapour-phase of liquid-nitrogen

Probe-distance from the LN_2 level (mm)	Temperature at start (°C)	Temperature at end (°C)
355	-188.1	-174.1
297	-182.1	-169.3
10	-188.7	-195.1
50	-184.2	-189.7

The data on temperature-differences during a 7-day recording of temperatures on two levels (at 30-minute intervals) in each container, as well as temperature-differences encountered in bags, during the operational qualification are summarised in the Table 6:

Table 6: Temperature conditions in the vapour-and liquid- phase of liquid-nitrogen and inside the stored bags containing 100 ml of 10% dimethylsuphoxide solution

	Maximum temperature (°C)	Minimum temperature (°C)	Maximal difference (°C)
Vapour- phase storage upper-level	-170.2	-187.3	17.1
Vapour- phase storage Lower-level	-189.6	-193.8	4.2
Maximal difference vapour-phase storage	-	-	23.6
Liquid- phase storage upper-level	-182.6	-194.6	12.0
Liquid phase storage lower-level	-197.4	-197.7	0.3
Maximal difference, liquid-phase storage	-	-	15.1
Temperature in bags – vapour-phase storage	-171.6	-185.1	13.5
Temperature in bags – liquid- phase storage	-197.4	-197.5	0.1

B.Storage in liquid-nitrogen containers – liquid-phase storage

The lower limit of the liquid-nitrogen level was adjusted to 350 mm; the upper limit, to 400 mm. The low-level alarm- limit was adjusted to 300 mm; the upper-level alarm-limit, to 500 mm. The position of the probes from the upper-lid of the container and the temperatures at the start and end of the validation process are summarised in Table 7:

Table 7: Temperature-conditions in the liquid-phase of liquid nitrogen

Probe-position below the LN_2 level (mm)	T min (°C)	T max (°C)	T mean (°C)	Maximal difference (°C)
30	-196.3	-195.2	-195.6	1,1
330	-196.5	-195.3	-195.7	1,2

C. Storage in mechanical freezers – used equipment

Type of freezer: Upright, air-cooled, mechanical freezers without forced air-circulation, and divided into 5 equal compartments by stainless- steel shelves. Each compartment is sealed by plastic doors.

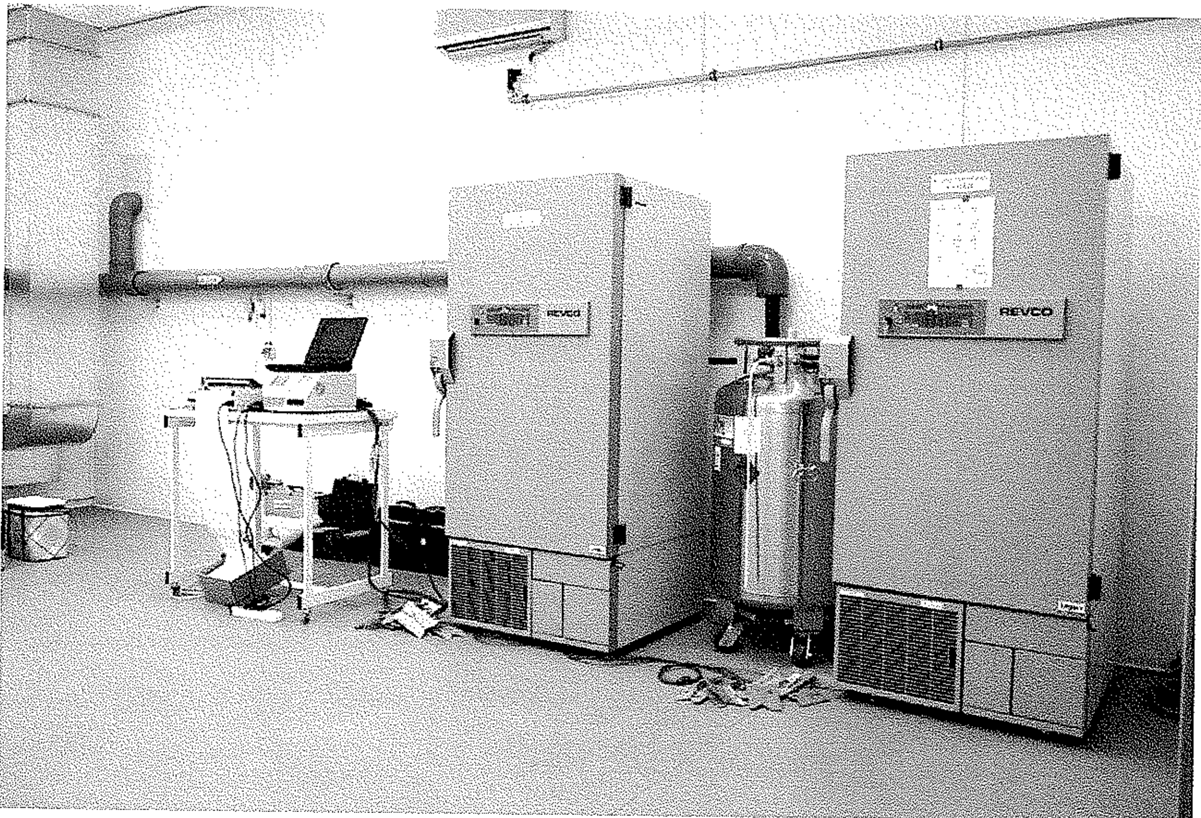

Figure 10: Operational qualification of the two mechanical freezers with a liquid-nitrogen, back-up cooling-system. Temperatures in different areas of the equipment are recorded by multiple probes and registered in a validator.
The results of the operational qualification are quoted in Tables 8 and 9:

Table 8: Temperature-conditions in the unloaded and loaded mechanical freezers - mean values.Temperature was recorded at 1to 2 minute intervals

	Adjusted T (°C)	Duration (hours)	Number of values	Distribution of values	Mean T (°C)	SD (°C)
Freezer 1-unloaded	-85	26	37 548	normal	-83.45	2.29
Freezer 1 - loaded	-85	25	33 132	normal	-83.85	2.22
Freezer 2 unloaded	-85	24	36144	normal	-83.55	1.67
Freezer 2 loaded	-85	24	103692	normal	-84.04	1.97

Table 9: Temperature-conditions in the unloaded and loaded mechanical freezers – temperature-range, differences of mean temperatures and maximum temperature-differences

	Range (°C)	Deviation from adjusted T (°C)	Max. difference of mean T (°C)	Maximum T difference (°C)
Freezer 1, unloaded	-76.3 to –86.9	1.55	6.3	10.6
Freezer 1, loaded	-74.6 to –87.8	1.15	7.1	13.2
Freezer 2, unloaded	-78.0 to –87.0	1.45	4.2	9.0
Freezer 2, loaded	-77.8 to -88.2	0.96	6.6	10.4

D. Storage at temperatures above zero- used equipment

Storage at these temperatures is used in the bank only for short-time storage of material before cryopreservation (the refrigerator placed in the cryolab – Figure 4), or for storage of sterile solutions, or material in the materials corridor, before processing.

Type of refrigerator: Upright, mechanical, pharmaceutical refrigerator with forced air-circulation and transparent door; divided into 5 compartments by glass-shelves. The fan is situated at the bottom of the cabinet.

Results of operational qualification:

Table 10: Temperature-conditions in the unloaded and loaded pharmaceutical refrigerators - mean values. The temperature was recorded at 1 to 2 minute intervals

	Adjusted T (°C)	Duration (hours)	Number of values	Distribution of values	Mean T (°C)	SD (°C)
Refrigerator 1, unloaded	+5.0	12	16995	normal	+5.1	1.54
Refrigerator 1, loaded	+5.0	24	20985	normal	+5.1	1.62
Refrigerator 2, unloaded	+5.2	12	8676	normal	+5.1	1.71
Refrigerator 2, loaded	+5.2	24	19530	normal	+5.7	1.68

Table 11: Temperature-conditions in the unloaded and loaded mechanical refrigerator – temperature-range, differences of mean temperatures and maximum temperature-differences

	Range (°C)	Deviation from the adjusted temperature	Maximum T difference (°C)
Refrigerator1, unloaded	+0.5 to+8.4	0.1	7.9
Refrigerator 1, loaded	+0.4 to +8.58	0.1	8.18
Refrigerator , unloaded	0.5 to +8.3	-0.1	7.8
Refrigerator 2, loaded	+0.5 to +9.0	-0.1	8.5

3.1.2.4 Testing of alarm situations

The results of tested, standard alarm-situations – opening-of- the-door test or power-off test in all types of cooling equipment are summarised in Tables 12 and 13.In these instances, the temperature was recorded at 10s intervals

Table 12: Power-off test

Type of cooling equipment	Duration of the power breakdown (min.)	T 1 °C	T 2 °C	Temperature difference (°C)
Liquid-nitrogen container	not done			
Mechanical freezer 1	61	-85	-72	+13
Mechanical freezer 2	61	-84	-74	+10
Refrigerator 1	60	+5.2	+12.4	+7.2
Refrigerator 2	60	+5.4	+12.4	+7.0

Table 13: Opening-the-door test

Type of cooling equipment	Duration of opening (min.)	T 1 °C	T 2 °C	Temperature difference (°C)
Liquid-nitrogen vapour- phase	4	-172.9	-165.1	+7.8
Liquid-nitrogen liquid- phase	4	-195.7	-195.6	+0.1
Freezer 1	62	-84	-66	+22
Freezer 2	not done			
Refrigerator 1	1.5	5.6	5.8	+0.2
Refrigerator 2	1.5	5.8	6.2	+0.4

Function of the back-up cooling-system

Function of the back-up cooling-system was verified by a long-term power-off test. The temperature rose above the process-limit (alarm temperature –70°C) after 90 minutes. Spraying liquid nitrogen into the cabinet enabled maintenance of the temperature at between –58.5 and –75°C for 45 hours until the battery was empty. In the second freezer, the battery was empty after only 8 hours.

3.1.2.5 The final setting of the process limits –new acceptability criteria

The expected operational-range and process-limits are presented in Table 14; the revised limits, in Table 15. The revised operational-range was determined as the difference between maximal and minimal temperatures within the observed period, but expanded by the uncertainity of the temperature- measurement determined during calibration (Table 14).

Table 14: Anticipated operational- and process- limits

	Operational range (°C)	Process-limits (°C)
Liquid-nitrogen vapour-phase	–183 to - 161	–195 to -130
Liquid-nitrogen liquid-phase	–195 to -177	–199 to –150°C
Mechanical freezer	–86 to –80°C	–90 to –70°C
Mechanical refrigerator	+ 3.2 to +7.2	+2.2 to +8.2

Table 15: Revised operation- and process- limits

	Operational range	Process- limits
Liquid nitrogen-vapour-phase	-195.8 to –167.3	-195 to –130
Liquid-nitrogen liquid-phase	-197.7 to –186.7	-199 to –150
Freezer –85°C	-88.5 to –74.3	-90 to –70
Refrigerator +5°C	+0.2 to + 9.2	0.1 to 9.5

3.1.2.6 Current monitoring of and registration of critical parameters

A list of continuously-monitored critical parameters, and data on the relevant supervisory systems, is summarised in Table 16.

Table 16: List of continuously-monitored critical parameters

Type of parameter	Parameter	Recording-interval (min)	Supervisory system	Duration of memory	Record
Environmental	Air oxygen-level (%)	5	Comet	3 months	Electronic
	Relative humidity (%)	5	Comet	3 months	Electronic
	Temperature (°C)	5	Comet	3 months	Electronic
Cooling-equipment	Freezers	5	Digiterm	3 days	Electronic
	Cryogenic boxes	30	Tec 2000	7 days	Electronic
Outside liquid-nitrogen supply	Pressure (MPa)	5	Comet	3 months	Electronic
	Weight (t)	5	Comet	3 months	Electronic

3.2 The Clean-Room Facility

3.2.1 The general features of the clean-room design

The design of the clean-room facility is based on the classic, nested organization of the manufacturing zones arround the critical processing-area, as described in the ISPE Guide (ISPE Headquaters, 1999). The critical processing-area is designated Grade A (class 100) with a background of Grade B (class 10 000). Maintaining the designated grade is contingent on the use of high-efficiency particulate-filters (HEPA), unidirectional air-flow and the differential pressure-stepped cascade away from the critical area.

3.2.2 Application of general guide-lines to aseptic pharmaceutical production in a tissue-bank

The ISPE guide recommends monitoring the following parameters considered critical in clean-production areas (ISPE, 1999):

1. temperature
2. percentage of relative humidity
3. differential pressure
4. particle count
5. airborne microbiological levels
6. air-velocity for Class 100 areas (Grade A).

The method of monitoring temperature- and relative humidity-levels is described above (Table 16). The differential-pressure is continuously displayed by manometers placed at entrances to the processing rooms (Figure 11). Values outside process-limits are indicated by acoustic- and light-alarms. The pressure-differential between rooms with different grades is documented once a-day.

Figure 11: Differential-pressure manometers are placed at entrances to personal corridors. Here, a clean-room operator, wearing a suit used for Grade C areas, enters the corridor into a Grade B area. Each entry is registered.

Parameters 4-6 are measured once a-year during operational qualification and /or requalification carried out by an authorised company (Labox, Prague, Ltd, G.M. PROJECT, Opava).

The results were evaluated using criteria established by the State Institute for Drug Control, Prague (SÚKL 1997) (Table 17) and EU rules: (The Rules Governing Medicinal

Products in the European Community, Volume IV: Good Manufacturing Practice for Medicinal Products, 1992, Annexes 1, Manufacture of Sterile Medicinal Products). Regular operational qualification (2001, 2002, 2004) showed that the criteria established in European and national recommendations were met.

Table 17: The maximum number of particles at rest

Grade	Maximum number of particles/m^3	Maximum number of particles /m^3
Particle size (µm)	0.5 µm	5 µm
A	3,500	0
B	3,500	0
C	350,000	2,000
D	3.500,000	20,000

Other important rules for clean-rooms include:

1. Special clothing for the staff – anti-emission overalls.
2. Periodical training of personnel.
3. Cleaning and sanitising of rooms using certified materials (non-emitting particles).
4. A special requirement in processing cells and tissues is the use of non-powdered, sterile gloves;non-emitting, disposable towels and other materials.

Clothing for the staff

The anti-emission overalls (Cleantex, Prostějov) differ for the grade B (Figure 12) and grade C (Figures 4,11) areas. The clothes are washed separately from common surgical garments. The garments for use in the grade C room are not sterilised; the clothes used for the grade B room are steam-sterilised and kept in transparent packaging material. Both types of clothes are tailored and labelled individually for all staff-members (Figures 4,11,12,13).

Cleaning and sanitization

Cleaning and sanitization are carried out according to local SOPs and instructions (Straková et al. 2005). The working-surfaces in the critical production-area are cleaned with sterile water for injection only, and disinfected with ST – tissue-towels (Bode,Chemie,Hamburg,FRG) after each cell- or tissue-processing run.

Figure 12: The clean-room operator working in the grade B area. She wears a sterilised anti-emission overall, tailored individually, with labelled sections. The operator is cleaning the surfaces of stainless-steel tables with special towels soaked in a disinfectant.

Figure 13: A clean-room operator cleaning the floor of the Grade B area. Sterilised towels are used for this procedure. The cleaning-instruments and towels are made from non-particle-emitting materials.

Sanitization of grade B rooms is carried out using filtered water,which meets the pharmacopoeial standards for Aqua purificata (ČL,Ph.B 97,1997). Sterilised towels made from non-particle-emitting material are used for cleaning and sanitization (Figure 13).

Training of the staff

Staff- training was carried out by an authorized company (G.M. PROJECT, Opava) before the start of clean-room operations. All staff-members received printed instructions (G.M. PROJECT, 2003). Repeated training is provided once-year by a pharmacist specialised in pharmaceutical technology and the manufacture of drugs of biological origin.

Daily operation of the rooms

The clean-rooms can be operated maximally twice a-day. In the morning, the critical production-area is usually used for the manufacture of solid tissue-grafts; in the afternoon, after cleaning and sanitization, the room is used for the processing of haematopoietic progenitor-cells before cryopreservation. The operating-regimen is supervised by a qualified pharmacist. A normal procedure lasts between 1.5 and 2.5 hours and requires the presence of two persons inside the grade B area, and one person in the material corridor. The subsequent cleaning and sanitization lasts from 1 to 1.5 hours, and is done by one clean-room operator (Figures 12,13).

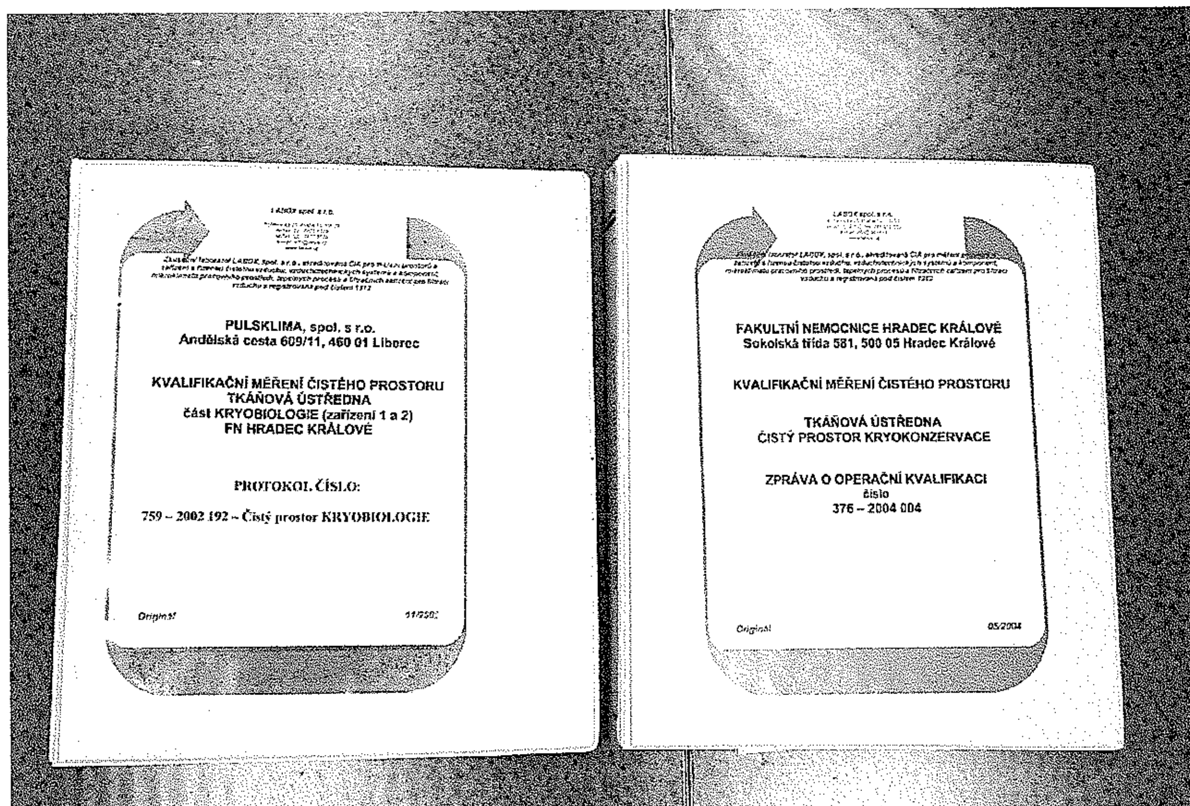

Figure 18: Reports on an operational qualification of the clean rooms (left) and requalification (right).

3.3 Methods and results

In this paper, detailed mention is made only of the results of the microbiological validation performed by ITEST Plus, Ltd., Hradec Králové, the company accredited by the State Institute for Drug Control, Prague. Validation was carried out firstly before starting work, and then again after 1 year of operating the critical processing-area. Validation was performed at rest in both cases.

The measurement was performed using the Biotest HYCON RCS plus Air Sampler, FRG device, calibrated by Merck Service Centre, CR.

For each measurement,a volume of 500 l of air was sampled (rate of 50 l of air/min) for10 minutes. During air-sampling,the device was placed on the floor or on a table in an upright position. At time of measurement, 2 to 4 persons were present in the clean-room areas. Strips of the type TC were used to trap bacteria and fungi. The strips were cultured for 2 days at temperatures of between 30 and 35°C, and for 3 additional days at temperatures of between 20–25°C. The CFU were counted, the microbial strains identified, and the count per 1 m^3 was determined.

The results of the count of number of CFU/m^3 are shown in Table 17. A list of strains found is given in Table 18.

Table 17: Results of the microbiological validation

Grade	A	B
Acceptability-criteria CFU /m^3	1	10
First validation 2003 CFU/m^3	0	4
Second validation 2004 CFU/m^3	0	2

Table 18: Microbiological strains found during validation

	Strains
First validation 2003	Micrococcus sp.
	Bacillus sp.
Second validation 2004	Bacillus sp.

4. DISCUSSION

An evaluation of a two-year experience of operating the new tissue-bank contributed to identification of the critical parameters for both cryogenic and clean-room facilities of the bank, and to an assessment of the normal operational- and process-limits of selected parameters. In the cryogenic facility, the temperature inside the liquid-nitrogen containers, mechanical freezers and refrigerators, and the atmospheric oxygen-level, were analysed in detail. Other parameters, such as the pressure inside the liquid-nitrogen tanks and the liquid-nitrogen evaporation-rate remain for future detailed analysis. Some of the above-mentioned parameters, such as the temperature inside the cooling-equipment, are important for assuring the quality of the product; the other ones, such as the level of atmospheric-oxygen, are essential for the safety of the staff. Monitoring of the liquid- nitrogen pressure and the weight of the stored liquid nitrogen is necessary for calculating the liquid-nitrogen losses due to evaporation. This parameter is important for the economy of the bank.

Evaluation of the athmospheric oxygen-level showed that the lower limit of the normal operational-range lies below the critical level approved by national legislation for female personnel. For this reason, women were excluded from performing some procedures in the bank (Měřička et al., 2004).

Evaluation of the temperature inside the cryogenic-boxes, freezers and refrigerators was made within a 3-year period by the same authorized company. The results are well comparable since the measuring and processing of data was carried out using the same equipment. The accuracy of the measurement improved with time; whereas in 2002, probes with an uncertainity of 2 °C were used, in 2004, the uncertainity dropped to 0.3 °C (Table 4).

The results of the temperature-measurement inside the mechanical freezers (-85°C) showed that the temperature-range inside the cabinet was wider than expected (Table 8, 9). The expectation was based on knowledge of temperature-fluctuations measured from one probe position (Figure 8, Table 14). The differences are wide regardless of the fact that control of the temperature was very finely adjusted (to 1°C). Table 15 shows the corrected, normal operational-range. The process-limits remained unchanged (Table 15). Thus, the difference between the normal operational-range and process-limits became very narrow. A similar situation is encountered in the evaluation of temperatures inside the pharmaceutical refrigerators (Table 10, 11). In this case, meeting the criterion for safe storage at temperatures of +3 to +8°C, as recommended by the Czech Ministry of Health, was not possible and the lower operational-limit remained very close to the freezing-point temperature. This result is not surprising as a recent European study determined that a substantial proportion of refrigerators produced in EU countries did not meet the criteria established by national legislation (Ben Amara et al., 2003).

While a methodology of qualification of mechanical freezers is well-established in authorized companies, qualification of cryogenic equipment represented a new field even for well-known institutions. When using a standard measurement-duration of 24 hours, it is not possible to register more than one filling-cycle, which is insufficient for the assessment of normal, operational temperature-range (Table 5,7). For this reason the data obtained during the qualification process were supplemented by data from routine monitoring of the temperature inside the cryogenic-oxes (Table 6). During the planned requalification of the equipment, it will be necessary to make some modifications to the methodology, and to pay more attention to the analysis of temperature-changes during the automatic filling-process when the highest temperature-differences are expected to be encountered.

The vapour-phase storage operational temperature-range is more suitable than expected (Table 14, 15); however, not so good as described by Hunt and Pegg while using a copper heat- shunt compatible with the inventory-system (Hunt and Pegg, 1996).

Tests of extraordinary situations (Tables 12 and 13) showed that the proces-limits were exceeded in 2 cases: during a power-off test of refrigerators (duration 60 minutes,the temperature rose to +12°C; and, in a door-opening test on a freezer, the temperature reached –66°C after 60 minutes of remaining open.

During simulation of a long-term power-breakdown or breakdown of the mechanical cooling-system (compresssor-breakdown, leakage of the coolant), the back-up cooling system was able to maintatin the temperature above the process limits by spraying liquid nitrogen into the low-temperature cabinet, and at still relatively safe level. In both cases, the spray control was stopped by discharge of the battery (after 8 or 45 hours), and not by exhausting the whole liquid-nitrogen back-up supply. This fact emphasises the need for including an emergency back-up power-supply which kicks-in shortly after a power-breakdown occurs. This requirement is met in the University Hospital Bank. In any case, a regular, personal check of all systems cannot be excluded, especially during week-ends.

The results of the assessment of the critical parameter-range inside the cooling-equipment proved without, any doubt, the advantages of storage by immersion in liquid nitrogen with stable temperature, minimal temperature-fluctuations, and minimal influence from unforseen events (Table 12, 13). The advantages of storage by immersion in liquid nitrogen had already been stressed by A. Smith (Smith, 1961) and H.T. Meryman (Meryman, 1966). Also, efforts to achieve airproofing while constructing metal containers (Měřička et al., 1991) and to choose plastic bags with high reliability (Měřička et al., 1993) came from the assumption that the material should be stored by immersion in liquid nitrogen. The current approach to the safe storage of biological material requires, however, avoiding

and/or minimizing the possibility of cross-contamination that may lead to the transmission of severe infection, as described by Tedder (Tedder, 1995). Tedder´s paper dealt with cryopreserved red blood-cells; however, the issue can be even more urgent in the cryopreservation of blood progenitor-cells, since in autologous setting, the cells are collected from non-healthy persons (Měřička et al., 2002, 2003, Bláha et al., 2003) and then re-infused into patients conditioned by aapplication of immunosuppressive drugs or total body-irradiation. An analysis of group of patients and donors included in this study showed that the most likely source of cross-contamination stems from an active infection present in the donor or patient (Měřička et al., 2003, Bláha et al., 2003), while the possibility of transmission of bacterial infection is negligible. Surprisingly low bacteria- contamination rates were found, even in the group of cord- blood collections (Měřička et al., 2005).

The quality of disposable-bags is a factor that critically influences the likelihood of cross-contamination. The quality of bags was regarded as standard for many years (Warkentin 1993, Měřička et al., 2002, 2003). In 2002, however, Khuu described, a high cracking-rate in some batches of bags manufactured in 2000 and 2001 and used for the storage of peripheral blood progenitor- cells (Khuu et al., 2002). Cracking occurred in nearly 7% of thawed bags. In our group of patients the cases of cracking bags occurred very rarely; no bags were used, however from any batches with the shown high cracking-rate.

For the above-mentioned reasons, storage in the vapour-phase of liquid nitrogen is now preferred. Analysis of the obsereved groups of patients and donors showed that in approximately one third of cases, positivity in markers of active infection occur, and collected and cryopreserved material should not be stored in the liquid. In positive cases of severe infection, such as hepatitis,the bags are not stored at liquid-nitrogen temperatures at all, but in mechanical freezers (Měřička et al., 2002, 2003, Bláha et al., 2003).

Deep-frozen or cryopreserved solid-tissue grafts were described repeatedly to be more likely to transmit viral- or bacterial- disease than transfusion medicine products (Veen 1994, Eastlund and Strong, 2003, Martinez et al., 2003). A higher rate of bacterial contamination in solid-tissue grafts than in cryopreserved progenitor-cell concentrates was confirmed also in our experience; not only in skin grafts, where contamination by normal skin flora is acceptable (Měřička et al., 2002) but also in bone-tendon-bone grafts harvested during combined organ /tissue collection from brain-death donors (Měřička et al., 2004). This is one of reasons why storage in mechanical freezers for solid tissue grafts is preferred as a means of prevention of cross-contamination by bacteria. Further investigations aimed at standardisation of temperature- conditions in the liquid-nitrogen vapour-phase at the lowest possible temperature- level are, however, fully justified since long–term storage (for years or decades) is required in such instances as cord-blood banking for clinical transplantation (Barker et al 2003) and prophylactic cryopreservation of progenitor-cells in patients (e.g. in chronic myeloid leukemia treated by Glivec) (Voglová et al., 2005) or in healthy persons with a high risk of damage of haematopoiesis (Klen, 1968, Měřička,1983).

The operation qualification of the clean processing-areas before starting operations was through a check of the correct performance of the HVAC system only. Requalification after 1 year examined also adherence to the standard regimen for clean-room areas, i.e. correct procedures by of personnel, efficiency of cleaning- and sanitization- practices as well as the proper use of disposable non-particle-emitting materials. The microbiological validation also tested the efficiency of the preventive system in the processing of contaminated, biological material. The operation qualification was carried out, however, only at rest. The EU Directive requires the testing of grade A areas in operation, using the criteria listed in table 19. Methods for checking these the critical processing- areas during operation are to be introduced in the nearest future, and will include the use of particle- counters as well as standardised, microbiological tests.

Table 19: Maximum numbers of particles during operation

Grade	Maximum number of particles/m^3	Maximum number of particles/m^3
Particle- size (μm)	0.5 μm	5μm
A	3,500	0
B	35,000	2,000
C	3500,000	20,000
D	not defined	not defined

The reported experience was, without any doubt, the first attempt at operating a bank based on a combination of cryogenic and clean-room technology in Central Europe. At the EATB meeting in Prague, in October 2004, a paper was presented from Austria describing the use of a clean room in a tissue-bank. It was limited, however, to the processing of bone for preservation by freeze-drying (Beranek and Winkler, 2004).

It is probable that similar banks will be built in the near future to support haematology-transplantation centres and for the purpose of cell- and tissue-engineering. This type of tissue-establishment, called cell-factory, is starting to operate in several locations in Europe (Giordano et al., 2004). In the University Hospital, Hradec Královė, the availability of a clean-room cell-processing laboratory makes it possible to introduce the different types of cell-manipulations necessary in the application of cell therapy (e.g. the use of stem- cells in the treatment of a myocardial infarction) (Pudil et al., 2005).

5. CONCLUSIONS

The two year experience of operating a cell and tissue bank based on a combination of cryogenic and clean-room technology proved that it was possible to achieve internationally-accepted standards of aseptic work.

In the cryogenic- facility,an analysis of critical parameters showed:

1. The level of atmospheric oxygen can be lowered to below the acceptable national norm for female staff. The potentially dangerous level of 18% is not reached in normal ventilation conditions.
2. The availability of portable and personal oxygen-monitors is critical for distinguishing between the occurance of an oxygen monitor breakdown and an actual drop in the atmospheric oxygen-level.

3. Detailed analysis of temperature-conditions inside different types of the cooling-equipment led to the accurate gauging of a normal, operational temperature- range.
4. From the purely physical point of view, storage by immersion in liquid nitrogen is still the most reliable way. The possibility of cross-contamination led to a preference for vapour- phase storage, however.
5. The most likely source of cross-contamination in the observed group of haematology patients or donors is the positivity of active virus infection in a patient or donor. Own system of cross contamination prevention is based on meticulous selection of the storage method . This storage method depends on the results of the serology tests. This approach ensures the advantageous availability of liquid- nitrogen storage for the majority of cryopreserved cell- concentrates.
6. Recent reports describing high cracking rates in plastic disposable bags used for storage of haematopoietic progenitor cells were not confirmed in the practice of our centre.

In the clean–room facility, it was found that:

1. the results of repeated validation vindicated meeting prescribed acceptability-criteria before the start of operation and after one-year´s operation of the bank.
2. all measurements were performed at rest, however and are to be re-inforced by the checking of critical areas during operation in the nearest future.

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UNIVERZITA KARLOVA V PRAZE
LÉKAŘSKÁ FAKULTA V HRADCI KRÁLOVÉ

MUDr. PAVEL MĚŘIČKA

CONTRIBUTION TO SAFETY IN CRYOPRESERVATION OF CELLS AND TISSUES FOR CLINICAL TRANSPLANTATION

DISERTAČNÍ PRÁCE

PŘÍLOHY

DOKTORSKÝ STUDIJNÍ PROGRAM: LÉKAŘSKÁ BIOLOGIE

HRADEC KRÁLOVÉ

2005

ŠKOLITELÉ: prof. MUDr. RNDr. MIROSLAV ČERVINKA, CSc.
ÚSTAV LÉKAŘSKÉ BIOLOGIE A GENETIKY UNIVERZITA KARLOVA V PRAZE, LÉKAŘSKÁ FAKULTA V HRADCI KRÁLOVÉ

prof. MUDr. MILAN BLÁHA, CSc.
II. INTERNÍ KLINIKA FAKULTNÍ NEMOCNICE HRADEC KRÁLOVÉ

SEZNAM PŘÍLOH

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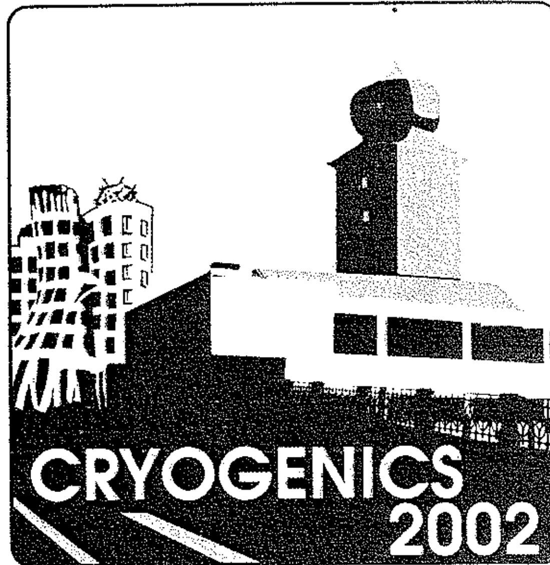

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BRIEF INTRODUCTION TO APPLICATION OF LOW TEMPERATURES IN BIOLOGY AND MEDICINE

Pavel Měřička
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ABSTRACT

In this survey we deal only with cases in those the tissue undergoes freezing. Freezing is used in biology and medicine for three major purposes. 1. To destroy viable cells or tissues by formation of intracellular ice crystals during rapid cooling in cryosurgery. 2. To preserve viable cells and tissues or biological material not containing cells for diagnostic, therapeutic or experimental purposes. 3. To preserve morphological shape of cells in histology. For a long time cryopreservation has been applied to maintain collections of microorganism and cell culture cells in national and international levels. Use of cryopreserved sperm for assisted reproduction in breeding service has been expanding since the fifties and later the same methods were applied in man. The current global problem to be solved is establishing of genetic resource banks (GRB) of endangered species. The IIR is one of the international organisations ready to contribute to manage this issue.

INTRODUCTION

In this survey we will deal only with cases in those the solidification of the liquid in tissue occurs and not with many applications of cold in biology and medicine, where cooling is applied locally for therapeutic purposes, e.g. in balneology, surgery, etc. Low subzero temperatures are used in biology and medicine for three major purposes: 1. To destroy viable cells or tissues by formation of intracellular ice crystals during rapid cooling. This method is used in cryosurgery as an alternative to surgical removal of the tissue affected with pathological changes. The cryosurgical application of cold will be explained in the following papers. 2. To preserve viable cells and tissues or biological material not containing cells for diagnostic, therapeutic or experimental purposes. 3.To preserve original morphological shape of cells in histology. In this case formation of small intracellular crystals inside cells and/or preserving of their original volume is desirable and enables the histologist to use this method as alternative of chemical fixation. The cell does not survive freezing, its morphology is however well preserved as well as its chemical composition, what is scarcely achievable with using classic chemical fixatives. This result is very important if use of histochemical or immunohistochemical methods is necessary to make diagnosis in a patient or to perform experimental investigations. In this paper cryopreservation of cells and tissues will be dealt with in detail. If preservation of viable cells is required, formation of intracellular ice crystals must be prevented by finding optimal freezing rates Mazur (1966), application of special chemical compounds, so-called cryoprotectants or combination of both approaches. Glycerol and dimethylsulphoxide (Me_2SO) are examples of cryoprotectants penetrating into cells, that prevent formation of highly concentrated solutions of electrolyte during freezing. This so-called "solution effect" may be as detrimental for cells and tissues as intracellular ice formation. Dextran or hydroxyethylstarch are macromolecular compounds serving as examples of extracellular cryoprotectants that prevent the solution effect by binding a significant amount of extracellular water. Adjusting of desired cooling rate of straws, vials or bags placed into a freezing chamber of the programmable freezer is achieved by

spraying with liquid nitrogen. An alternative approach is based on finding an optimal geometry of a sample that is placed to a freezer with stable subzero temperature. Shelf life of frozen materials is dependent on the fact, how the temperature used for storage eliminates the residual liquid fraction inside and outside cells and/or prevents recrystalization phenomena during storage. As vitrification often occurs in freezing complete mixtures of cryoprotectants and colloids that are formed after introduction of a cryoprotectant to the cell suspension or solid tissue, devitrification during storage should be prevented as well. It is commonly accepted that safe storage of viable cells and tissues can be achieved if temperatures below –80°C are used. If storage for years is required, it is better, however, to use vapour or liquid phase of nitrogen. Hunt and Pegg (1996) described the system maintaining the temperature in the vapour phase of a cryostorage tank below -160°C, even if the tank was opened during manipulation with stored samples. Temperature fluctuations inside the vapour phase of liquid nitrogen should be minimised and the same requirement is valid also if the material is stored in mechanical freezers.

METHODS AND RESULTS

The methods used for preservation of biological material not containing viable cells as well as material containing viable cells are demonstrated on example of the Tissue Bank of University Hospital Hradec Králové, established in 1952 (Klen, 1952).

1. PRESERVATION OF MATERIAL NOT CONTAINING LIVING CELLS

Storage of sera or plasma collected in patients included in research projects or clinical trials of new drugs is a very common example of long-term storage of material not containing living cells. Peripheral blood is collected into a vial not containing anticoagulant (serum) or containing anticoagulant (plasma). The collection undergoes centrifugation and the supernatant is divided into cryotubes of different size (the most common volume being 2 ml). The cryotubes are put into the cardboard boxes and placed into the freezer with the temperature -80°C (REVCO, U.S.A.). If very sensitive components of the plasma are to be analysed cooling with liquid nitrogen with subsequent storage in liquid nitrogen is used.

2. PRESERVATION OF VIABLE CELLS AND TISSUES

Preservation of viable cells includes in our practice preservation of sperm used in assisted reproduction in man, blood progenitor cells (bone marrow, peripheral stem cells, cord blood) used in autologous and allogeneic transplantation in haematology and oncology, suspensions of human epidermal keratinocytes* used for reconstruction of skin after excessive skin loss (Straková et al., 1995, Klein et al., 1997), suspension of human chondrocytes used for reconstruction of articular cartilage* and tissue culture cells used for experimental pupnoses. In cryopreservation of sperm we use 10% glycerol as a cryoprotectant, in cryopreservation of blood progenitor cells combination of 10% Me_2SO with 6% dextran and human serum albumin is used (Měřička et al., 1999). In cryopreservation of keratinocytes and chondrocytes we use 10% Me_2SO in combination with fetal calf serum. Sperm, suspensions of keratinocytes and chondrocytes are frozen in vials, blood progenitor cells in plastic bags closed in metal cassettes. Controlled rate freezing in a programmable freezer (Planer-Biomed, England) is performed and storage in liquid nitrogen is preferred. In preservation of blood progenitor cells storage in mechanical freezer at –80°C (REVCO, U.S.A.) was found fully sufficient if the time between collection and clinical application of the graft did not exceed 1 year. In preservation of solid tissues it is always necessary to know if the presence of viable cells is necessary for the function of the graft in the host's body. Examples of tissues not requiring presence of viable cells are bone, fascia lata and sclera that are used in different indications in traumatology, orthopaedic

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surgery, neurosurgery, stomatosurgery and ophthalmology. For these tissues we use similar conditions as for material not containing living cells, i.e. after rapid freezing of tissue sealed into disposable plastic bags storage at temperature -80°C follows. If preservation of viable cells is necessary, prevention of freezing injury by application of cryoprotectants must be made. Allogeneic and xenogeneic skin used especially in burn treatment, vessels and articular cartilage are examples of such tissues. In xenogeneic skin we use 10% glycerol combined with 6% dextran, in allogeneic skin we use 10% Me_2SO combined with 6% dextran (Měřička et al., 2002). The same combination is used for vessel cryopreservation. In our practice cryopreservation with Me_2SO is also applied for storage of ligamentum patellae used for reconstruction of the crucial ligament of the knee and amnion used for treatment of cornea ulcerations. Skin, amnia and ligaments are stored at –80°C, in vessels we prefer vapour phase of liquid nitrogen.

3. STORAGE CONDITIONS

Storage conditions are submitted to continuous monitoring of temperatures inside and outside mechanical freezers and cryostorage tanks. The author has 2-year experience with monitoring of temperature in mechanical freezers with the liquid nitrogen back-up cooling system using the system Digiterm with the programmes Netcom and Read 95, Regucon Prague, Ltd, that is adjusted to record data in 5 minute intervals. The temperature inside the freezers is not influenced by changes of ambient temperature in an unairconditioned room. For monitoring temperature inside liquid nitrogen storage tanks XLC 1200 (Chart, U.S.A., Planer Biomed, U.K.) the system Tec2000 with records in 30-minute intervals is used. The tanks are filled automatically from the Cryocyl 230 LP (Chart, U.S.A., Planer Biomed, U.K.) container, connected with the tubing with an external liquid nitrogen container (Aga Cryo, AB Sweden) containing 6,000 litres of liquid nitrogen.

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* The programme of keratinocyte culture was supported by the grant No. 1184-2 and 3696-3 of the Internal Grant Agency of the Ministry of Health of Czech Republic and by the grant Keratinocyte, Ministry of Defense of the Czech Republic, the programme of chondrocyte culture is being supported by the grant No. ND/6042-3/00 Internal Grant Agency of the Minstry of Health.
The containers are placed in an air-conditioned room assuring stable ambient temperature during the year. The temperature in the vapour phase of nitrogen at the upper level of stored samples does not rise above -150°C.

DISCUSSION

The safe storage of serum or plasma is based on combination of rapid freezing with storage at adequate temperature. In small volumes as described above simple placing of vials into freezer with temperature –80°C or cooling by liquid nitrogen vapour is sufficient. If large volumes of plasma are to be stored like in transfusion medicine freezing in bags in special devices called shock freezers is necessary. Viable cells and tissues preserved in our practice are used clinically within months or years. The only exception is storage of cord blood in the frame of the Cord Blood Bank of the Czech Republic where the interval between collection and clinical use may be extended to decades. Stable temperature conditions inside freezers are essential for preventing recrystalization during storage. Continuous monitoring of temperatures inside and outside mechanical freezers and cryostorage tanks make early identification of technical breakdowns possible and the resulting damage of stored material can be thus prevented. If storage in liquid nitrogen is used, stable ambient temperature conditions lead to standard liquid nitrogen evaporation rate and /or filling the containers in regular intervals. This approach can be used also in other possible application of long-term storage of viable cells in biology such as collections of microorganism and cell culture cells that have been in operation for many years

both in national and international levels (Stacey and Doyle, 1998). Successful development of assisted reproduction techniques in breeding service and later in man led to the idea to organise long-term storage of germplasm necessary for conservation of biodiversity world-wide (Holt, 1998). Genetic resource banks (GRB) that store germplasm specifically spermatozoa, oocytes and embryos are now being organised in many countries and should serve for storage of genetic material from endangered and threatened species. The IIR is one of the international organisations ready to help in managing this issue in the global extent. The joined effort is necessary as there are still many issues to be solved. We are able now to preserve sperm or embryos of many species, cryopreservation of oocytes is, however, unsuccessful. Storage conditions used by us in preservation of solid tissues such as bones and fascia fully corresponds with the requirements of internationally accepted standards that declare the highest acceptable temperature –40°C. As alternative to deep freezing we perform also freeze-drying with subsequent sterilisation by ethylene oxide gas Achieving of such high level of biological safety, that is equal to the safety of medical devices is, however, possible only in tissue grafts not requiring the presence of viable cells. The efficient way how to improve biological safety in processing viable cells and tissues is strict application of the rules of aseptic work during collection and processing including the use of clean rooms with validated grade. In the new part of the tissue bank, that has been completing now we will be able to meet this requirement.

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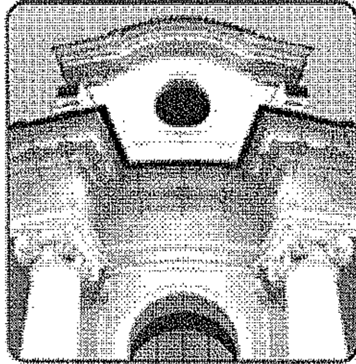

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BRIEF INTRODUCTION TO MECHANISMS OF FREEZING INJURY AND CRYOPROTECTION

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ABSTRACT

Understanding the fundamental processes causing injury during freezing and thawing is very important for cryobiologists, who preserve living cells in cryobanks. In freezing we can distinguish two cases. In crystallization molecules or atoms are arranged in regular structures. In vitrification solidification is not accompanied with formation of regular structures. Before the discovery of the cryoprotective effect of glycerol it was believed, that achieving vitrification using rapid cooling was the correct way to assure survival of cells and tissues. This way was, however, not successful. In the 60's Mazur explained the mechanism of avoidance of intracellular freezing in single celled organisms. Since that time slow cooling has been used in preservation of cell suspensions both in absence and presence of cryoprotectants. The attempts to freeze vascularised organs failed, however. The recent intensive research of properties of different vitrification solutions shows that vitrification is the promising approach to freezing of multicellular systems.

INTRODUCTION

Understanding the fundamental processes causing injury during freezing and thawing is important both for cryosurgeons, who try to destroy cells by application of cold and for cryobiologists, who try to preserve living cells at low temperatures. In the latter case prevention of the freezing and thawing injury and/or understanding the mechanisms of cryoprotection are of major importance. Cell and tissue cryobanks exist worldwide and are being established with various purposes. Many banks including the Tissue Bank of the University Hospital Hradec Králové (Klen, 1952, 1982, Straková et al., 1995, Měřička, 1999, 2000, 2002) store human cells and tissues suitable for therapeutic tissue transplantation or for experimental purposes (Kreuz, 1951, Sell, 1962, Joyce, 2000, Strong, 2000) (Fig. 1). Storage of genetic material of domestic animals has been widely used in breeding service since the 50's of the 20th century (Smith, 1962). The human gametes or early embryos are frequently stored in banks of centres of assisted reproduction (Gunasena and Critser, 1997). The most advanced tissue banks elaborate methods for preservation and storage of cell and tissue engineered products (Harringer et al. 1997, Sato et al., 1998). Many banks make collections of animal and plant cells or microorganisms used for diagnostic or experimental purposes. Establishing of a network of low temperature genome resource banks is very important for maintaining biodiversity on the earth (Wildt, 1997, Harnal et al., 2002).

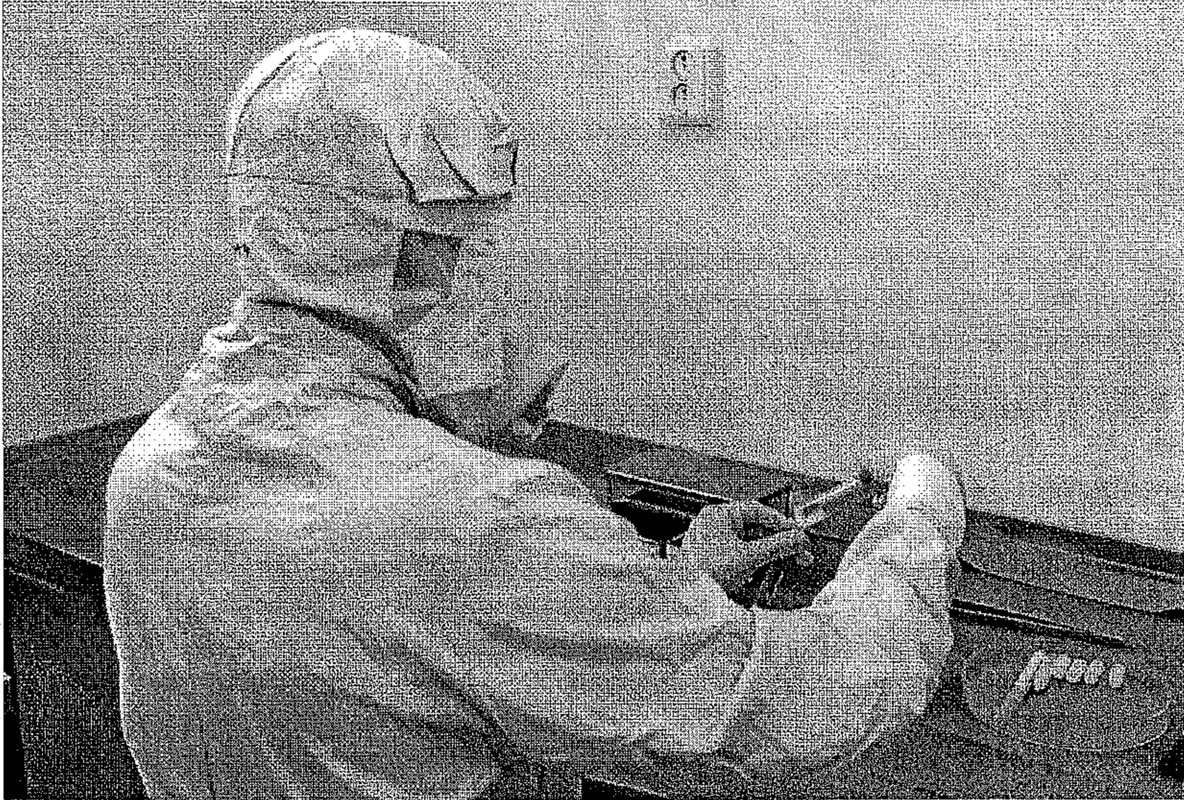

Figure 1: Advanced tissue banks preparing cryopreserved tissue grafts for clinical application use the clean room technology. The picture from the Tissue Bank of the University Hospital Hradec Králové shows taking the sample for sterility testing. The stainless steel table is placed below the laminar flow ceiling assurring the environment of the grade A, the background environment is of the grade B.

Freezing is the solidification of the liquid. We can distinguish two cases: 1/ Molecules or atoms are arranged in regular structures. 2/ Solidification is not accompanied with formation of regular structures. The former process is called crystallization and is accompanied with release of the latent heat of solidification. Under normal atmospheric pressure ice crystals are arranged in the hexagonal lattice. Cubic ice is formed under temperatures below -130°C (Meryman, 1966). Other crystallographic forms of ice occur under elevated pressure and are mentioned by de Quervain (1975). The latter process is called amorphisation or vitrification. Vitrification during cooling, however, does not exclude crystallization during warming. During this process called devitrification latent heat of solidification is released. Reviews on vitrification were published e.g. by Simatos and Turc (1975), MacFarlane et al. (1992) and Mehl (1996). Before the discovery of the cryoprotective effect of glycerol by Polge, Smith and Parkes (1949) it was believed, that achieving vitrification using rapid cooling was the correct way to assure survival of cells and tissues. This way was, however, not successful (Luyet and Gehenio, 1940). Later Mazur (1961, 1963, 1966a, 1966b) explained precisely the mechanism of avoidance of intracellular freezing in single celled organisms that led to good survival after thawing. Slow cooling enabling the cells to dehydrate and shrink and thus escape from intracellular ice formation has been used widely in preservation of cell suspensions both in absence (in single celled organisms) or presence of cryoprotective agents (e.g. in isolated mammalian cells). What was possible in cell suspensions was not successful, however, in complex multicellular systems, where formation of extracellular ice crystals is not innocuous. Also the role of intracellular ice formation may be different in organized tissues as explained recently by Acker and McGann (2000). The situation is even more complicated in organs, where freezing protocols based on using conventional cryoprotectants completely failed. This was the reason for intensive research of the physical and biological properties of different vitrification solutions in prominent cryobiology

centres in the world (Fahy et al. 1984, Fahy 1987, Rich and Armitage, 1991, Mac Farlane, 1992, Boutron, 1993, Mehl, 1993). Now it is well proved that vitrification of both intracellular and extracellular liquid is the promising approach to freezing of multicellular systems such as human tissues and may open the way to freezing whole vascularized organs (Fahy et al., 1991, 1994, Fahy and Ali 1997, Wusteman et al., 2002). In the following text we will mention briefly some particular issues.

1 CRYSTALLIZATION IN CELL SUSPENSIONS

The mechanisms of freezing injury and protection in suspensions of single celled organisms were described by Mazur in the sixties (Mazur, 1961, 1963, 1966a,b, 1970). In cell suspensions the crystals are formed first in the extracellular water. The intracellular water supercooles and the pressure gradient between higher water vapour tension above supercooled water inside the cell and lower tension above the ice crystallizing outside the cell is formed. This leads to outflow of water from the cell and to the crystal growth outside cells. Dehydration and shrinkage of cells is the result of this process. The ability of the cell to shrink and to escape from intracellular freezing is dependent on the cell size and/or initial volume and on the permeability of the cell membrane for water. This process was mathematically described by Mazur (1963, 1966a,b). Intracellular ice formation is thus more likely in large cells with thick membrane than in small cells with highly permeable membrane. This is the cause why it is easier to achieve good cell recovery in freezing cells of standard initial volume than in freezing of a mixture of cells of different initial volumes. Sufficient dehydration and/or shrinkage of cells can occur only in low freezing rates. In high freezing rates intracellular ice is formed and the cells do not shrink. The intracellular ice tends to recrystallization during warming which causes the cell death. The mechanisms of intracellular crystallization are still being discussed (Pitt, 1992). It starts at considerable lower temperatures than extracellular freezing, e.g. intracellular ice in isolated keratinocytes does not form until the temperature of −7°C is achieved (Zieger et al., 1997). In general, when external medium is frozen intracellular ice occurs between −5°C and −13°C, when external medium contains no ice freezing can occur at lower temperatures (Acker and McGann, 2000). The same authors (2000) identified 50% of intracellular ice formed for cells in suspensions at -9.4°C ± 0.3 and -8°C ± 0.3 for individual attached cells. Mazur (1966b) suggested the idea of seeding of the supercooled intracellular water by extracellular ice through natural cell membrane pores. Later Toner (1993) described two mechanisms of intracellular freezing: 1. Volume catalysed nucleation, where the nucleation is induced by foreign particles inside cells. 2. Surface catalyzed nucleation, where the nucleation is induced by the activity of internal surface of cells. In the contrary Muldrew and McGann (1990) expressed the idea that intracellular freezing is caused by seeding from extracellular ice through ruptures of cell membrane due to very rapid outflow of water from cells during the osmotic stress.

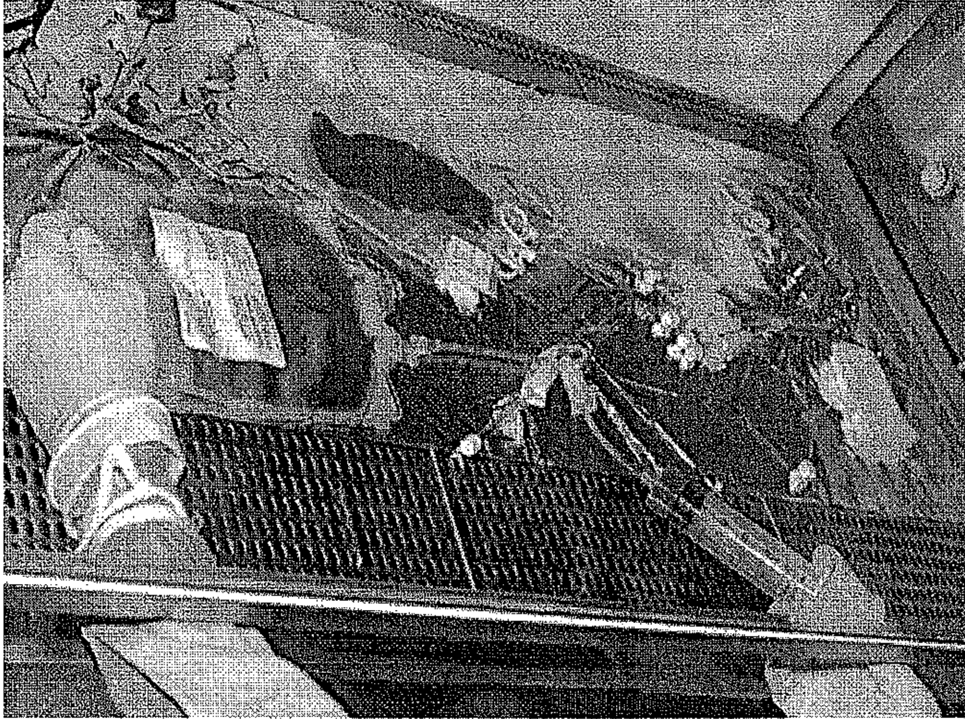

Figure 2: Preparing of the cell suspension for cryopreservation – transferring of the peripheral blood progenitor cell suspension from the collection bag into freezing bags made from plastic withstanding the liquid nitrogen temperature. Mixing with the cryoprotectant solution to final concentration of 10 % (v/v) of dimethylsulphoxide follows.

2 FREEZING OF MULTICELLULAR SYSTEMS

In these systems (tissues and organs) the damage is caused also by extracellular ice formation, as the ice causes disintegration of the tissues, e.g. large crystals can be detected by scanning electron microscopy in soft connective tissues submitted to slow freezing followed by freeze-drying (Měřička et al., 1989). Electron microscopy of frozen skin or cornea proofs damage of intercellular junctions (Zieger, 1997, Acker and McGann, 2000). In the contrary the presence of intracellular ice due to rapid freezing may not be associated with the loss of selective permeability of cells forming the tissue or the cell monolayer (Acker and McGann, 2000). In vascularized organs the water crystallizes in vessels, which causes the expansion of vessels and their ruptures, thus the haemorrhage can destroy the thawed organ after restoring the blood circulation (e.g. in kidney transplantation). In organs with cylindrical or spherical shape big differences between freezing rates occuring at the surface and in the centre of the organ are formed both in freezing and thawing. This causes injury by the solution effect as well as by volume expansion of the centre of the organ at the moment of the liquid-solid transition. This expansion may lead to organ rupture as the ice formed at the organ periphery breaks at the moment of solidification of the liquid in the centre (Karow et al., 1974). Introduction and removal of conventional cryoprotectant and resulting osmotic swelling of cells may lead to compression of the vessels of the organ and ischaemia leading to organ necrosis after transplantation. For the mentioned reasons the cryopreservation techniques based on classic equilibrium freezing and using conventional cryoprotectants can assure safe preservation and storage of cell suspensions and tissue samples having the flat geometry (e.g. skin). Successful cryopreservation of organs or complex tissues (such as corneas) probably needs to introduce the methodological approach based on using cryoprotective solutions enhancing vitrification, so-called vitrification solutions (Fahy and Ali, 1997, Brockbank et al., 2000, Meltendorf, 2002, Wustemann et al., 2002).

3 PRINCIPLES OF CRYOPROTECTION

Cryoprotection can be based either on avoidance of intracellular ice formation and solution effect or on extracellular and intracellular vitrification. The former situation can be achieved in the ideal case by simple manipulation of freezing rates, as described in yeasts by Mazur (1963, 1966a,b). In most cases, such as freezing of mammalian cells addition of cryoprotectants is necessary. The cryoprotectants can be divided to intracellular or penetrating cryoprotectants, e.g. glycerol or dimethylsulphoxide and extracellular or non-penetrating cryoprotectants, e.g. sucrose, dextran, polyvinylpyrolidon (PVP) or hydroxyethylstarch. The presence of intracellular cryoprotectants modifies the freezing process outside and inside of cells. They are used in high osmolar concentrations, which leads to considerable freezing point depression based on coligative properties of a cryoprotectant (Fig. 2). The introduction of a cryoprotectant into the cells before freezing is connected with considerable osmotic stress (Katkov, 2000). The presence of a cryoprotectant during freezing expands the unfrozen liquid fraction below the freezing point, which prevents excessive and rapid cell dehydration (Cocks and Brower, 1974). The mechanism of extracellular cryoprotection is not known precisely. Extracellular cryoprotectants are used in low osmolar concentrations, i.e. the depression of the freezing point is only minute. The possible mechanisms are: avoidance of nucleation, inhibition of crystal growth and binding of water which prevents extreme cell dehydration. In general the extracellular cryoprotectants are less effective than intracellular ones if used alone. The exception is e.g. successful cryopreservation of red blood cells with hydroxyethylstarch (Sputtek 1990, 1993). Extracellular cryoprotectants are frequently used, however, in combination with dimethylsulphoxide in freezing white blood cells and platelets. Another possible mechanism of cryoprotection is vitrification. It can take place even in using classic intracellular cryoprotectants, such as glycerol or dimethylsulphoxide. Vitrification in these solutions can be further enhanced by using synthetic ice binders (Wowk et al., 2000). Vitrification is regularly induced in presence of 1,2 propane-diol or 2,3 butane-diol (Boutron, 1991, 1993). These compounds can be used with good results, e.g. for freezing of red blood cells. It was proved that cocktails of compounds enhancing vitrification, so called vitrification solutions (Fahy, 1991, Mehl, 1996, Wusteman 2002, 2003), can be used with success in cryopreservation of complex tissues such as cornea, vessels or skin and probably also for cryopreservation of vascularized organs. In 1997 the group of G. Fahy successfully demonstrated immediate function of rabbit kidney after introduction and removal of vitrifiable 7.5 molar solution (Fahy and Ali, 1997). The solution called VS 4 contained dextrose, dimethylsulphoxide, dimethylformamide and L,2 propane-diol. Vitrification is used with success also in cryopreservation of plant cells and tissues (Steponkus, 1992), as well as for cryopreservation of embryos (Rall and Fahy, 1985).

4 IMMEDIATE INJURY AND STORAGE INJURY

The cells can be injured both by the freezing and thawing process itself or by storage under inadequate conditions. The immediate freezing and thawing injury can be minimized by combination of slow freezing and rapid thawing and application of cryoprotectants. It is the role of cell and tissue banks to decrease the storage injury to minimum, so that storage for years or decades without significant loss of viability could be achieved if necessary. The storage injury is avoided if complete solidification of the liquid is achieved and recrystallization and devitrification processes are minimized or avoided. The optimal storage conditions are assured at temperatures below -130°C, i.e. in vapour or liquid phase of nitrogen. At these temperatures the cells can be stored for years. Hunt and Pegg (1996) suggested to lower the safe temperature of the vapour phase of liquid nitrogen to −160°C using a copper heat shunt. At temperature of dry ice (-80°C) that can be achieved in commercially available mechanical freezers the recrystallization processes can take place and the possibility of existence of minute fraction of liquid can not be excluded as well. Safe storage for months is possible, however (Makino et al., 1991) (Fig. 3). Storage of viable cells and tissues at

higher temperatures cannot be recommended. The most detrimental are the temperatures near to the eutectic point for sodium chloride.

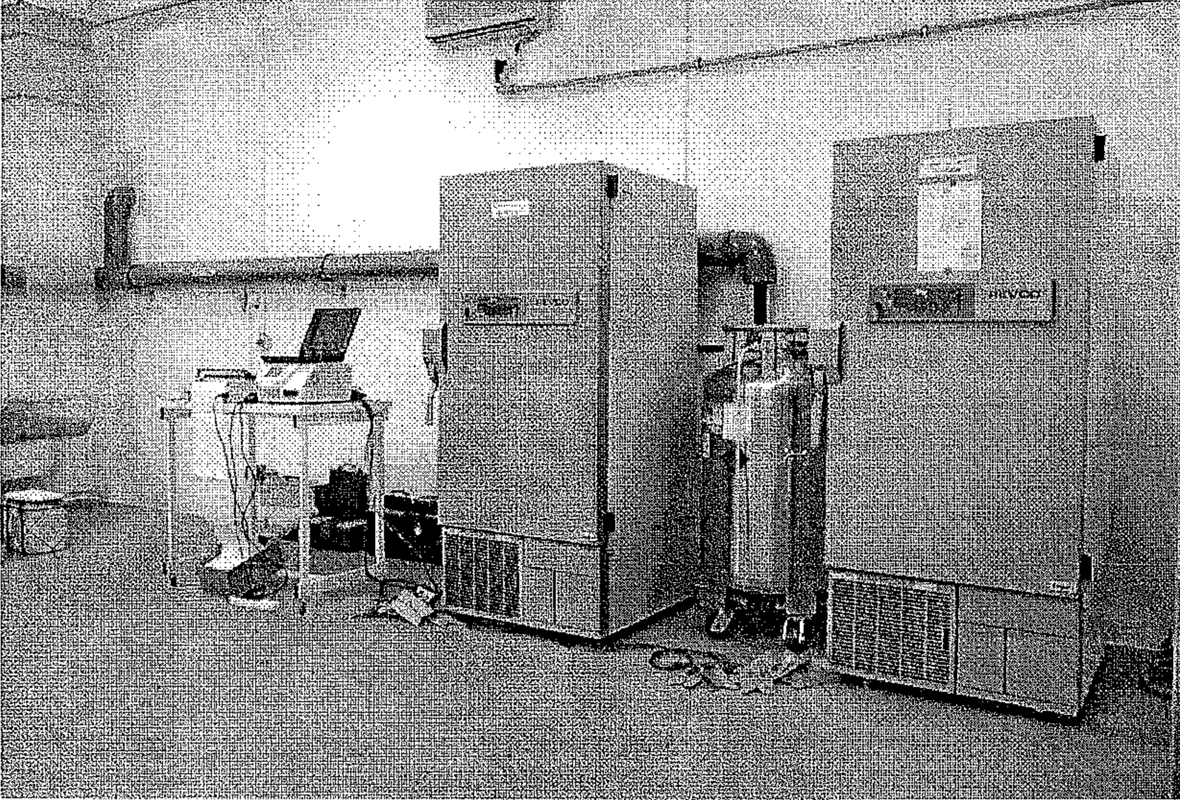

Figure 3: The temperature working and alarm limits in the mechanical freezer used for storage of cryopreserved cells and tissues at –80°C are set up during the validation process made by an independent authorized company. Multiple sensors are placed into the low-temperature cabinets during the validation process. The liquid nitrogen back-up cooling is set into operation if the alarm limits are crossed.

CONCLUSIONS

A substantial progress has been made in understanding the mechanisms of freezing injury and protection during the last 15 years. Although the classic cryoprotection models based on avoidance of intracelular crystallization by slow cooling and using conventional cryoprotectants are still the most frequent in practice of cell and tissue cryobanks the new approaches based on vitrification are used especially in freezing of complex tissues. This approach may open the way to freezing vascularized organs. Enhancement of vitrification by synthetic ice blocking agents may be the new contribution to solving of this problem. Establishing of freezing protocols applicable in current medical practice will still need, however, a lot of time and effort. In using classic freezing protocols computer simulation of cryobiological processes contributes to optimization of regimens used in freezing of cells.

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UČEBNÍ TEXTY UNIVERZITY KARLOVY V PRAZE

Vladislav Třeška
a kolektiv

TRANSPLANTOLOGIE PRO MEDIKY

alopecie, potransplantační lymfom, kožní a nekožní malignomy. Léčba těchto komplikací se uskutečňuje v rámci interdisciplinární spolupráce.

2.8.6 Ambulantní péče

Pacienti po transplantaci plic jsou po ukončení hospitalizace ambulantně sledováni v transplantačním centru. Smyslem těchto kontrol je včas rozpoznat a léčit:

- plicní komplikace – rejekce a infekce
- mimoplicní komplikace, které jsou nejčastěji spojeny s chronickou imunosupresivní léčbou

K ambulantním kontrolám, které se uskutečňují každých 4 – 8 týdnů, patří tato vyšetření: vyšetření plicních funkcí a krevních plynů, kompletní laboratorní screening, rtg plic, diagnostika CMV, vyšetření sputa, stanovení hladiny cyklosporinu A nebo tacrolimu. V pravidelných odstupech jsou provedena clearance kreatininu, kostní denzitometrie a CT hrudníku.

Z hlediska dlouhodobého sledování představuje spirometrie nejdůležitější vyšetření.

2.8.7 Výsledky a závěr

Transplantace plic představuje dnes etablovanou metodu léčby u pacientů v terminálním stadiu plicního onemocnění a navrací ztracenou kvalitu života. V posledním desetiletí došlo k rychlému rozvoji plicních transplantací, ale nedostatek vhodných dárců může brzo omezit očekávaný rozvoj. Střednědobé výsledky jsou dobré, ale chronická rejekce omezuje dlouhodobé přežití. Od pokroku v imunosupresivní terapii lze očekávat zlepšení celkových výsledků. Perspektivní je problematika xenotransplantací.

Zajímavá je i cena transplantace. V USA vlastní výkon i péče o nemocného v prvním roce po transplantaci stojí 150 000 USD a každý další rok 20 000 USD. V Rakousku je potřeba pro vlastní výkon a pooperační péči složit 1000 000 ATS. V ČR nemocnice účtuje pojišťovnám za výkon a pooperační péči 450–600 000 Kč.

2.9 Úvod do transplantace tkání

Pavel Měřička

Rozvoj klinických transplantací orgánů, jehož jsme byli svědky od poloviny 20. století, by nebyl možný bez předchozích experimentálních a klinických zkušeností s alotransplantacemi a xenotransplantacemi tkání. V této kapitole se budeme zabývat především společnou problematikou spojující transplantace jednotlivých tkání, tj. dárcovstvím tkání, kontraindikacemi odběru tkání, metodami odběru a konzervace tkání a jejich dlouhodobého uchovávání, včetně metod umožňujících modifikovat osud štěpu v těle příjemce. Zvláštní pozornost budeme věnovat prevenci přenosu infekčních chorob při transplantaci.

2.9.1 Historie a současná praxe transplantací tkání

I když zprávy o použití alogenních i xenogenních tkání lze vystopovat až do starověku, dokumentované případy pocházejí z 19. století. Výjimku tvoří zpráva z roku 1682 o náhradě defektu lebeční kosti vojáka, způsobeného poraněním šavlí, psí kostí. V 19. století byla pro-

vedena řada experir
zkušenosti. Hlavnín
chirurgických zákrc
úspěšnou lamelární
rozence pro klinick
o úspěšné perforati
kostí a chrupavek p
Naproti tomu zp
Girdner uvedl, že p
klinický program tr
20. století v bývalén
program rozšířil i n
Vědecký základ
Preservation of Tiss
rurgii), kterou předı
umožnily vznik tkář
kostní, kožní. Tepr
(v anglosaské litera
idea vznikla v USA
námořnictva (US N
od zemřelých dárců
mována Dr. R. Klen
ci Králové. V témže
V Hradci Králové s
tkání.
Podobný rozvoj
letech i v zahraničí,
gickou problematik
mechanismech ve s

2.9.2 Transplanta

S klasickou reje
málního štěpu, který
tomovanou plochu
rány. Po 14–21 dne
event. celý štěp pře
popálenina hluboká
chu autotransplantá
vu nahradit napěsto
transplantace podle
žití velmi tenkého a

vedena řada experimentálních studií s transplantacemi tkání a byly získány i první klinické zkušenosti. Hlavními zdroji tkání pro první klinické transplantace byly tkáně odstraněné při chirurgických zákrocích jiným nemocným. Tak např. v roce 1877 popsal von Hippel první úspěšnou lamelární keratoplastiku, v roce 1884 Lucas popisuje použití kůže předkožky novorozence pro klinickou transplantaci. V roce 1907 Zirm z Olomouce publikuje první zprávu o úspěšné perforativní keratoplastice, v roce 1911 Tuffier referuje o použití konzervovaných kostí a chrupavek pro klinickou transplantaci.

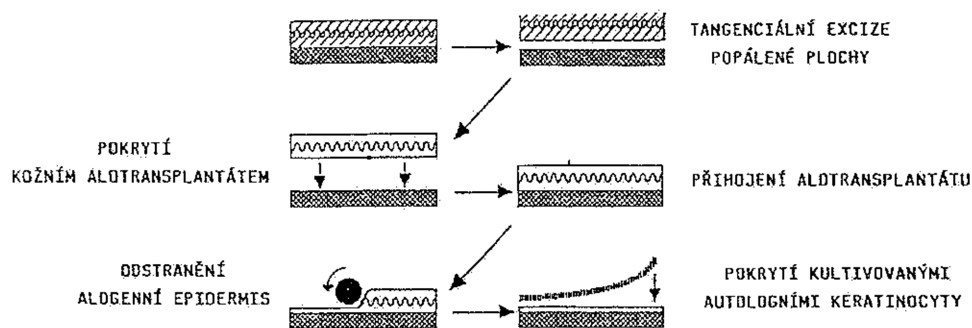
Naproti tomu zprávy o použití tkání zemřelých dárců jsou velmi vzácné, např. v roce 1881 Girdner uvedl, že použil jako první kadaverózní kůži pro klinickou transplantaci. Skutečný klinický program transplantace kadaverózní tkáně však zahájil teprve Filatov ve 30. letech 20. století v bývalém SSSR. První transplantovanou tkání byla rohovka, později Filatov tento program rozšířil i na další tkáně.

Vědecký základ **metodám konzervace tkání** dal již Alexis Carrel ve své přednášce The Preservation of Tissues and Its Application in Surgery (Konzervace tkání a jejich použití v chirurgii), kterou přednesl před Americkou lékařskou asociací v roce 1912. Carrelovy práce tak umožnily vznik tkáňových bank. Zprvu to byly banky specializované na jednotlivé tkáně: oční, kostní, kožní. Teprve po druhé světové válce vznikla idea **univerzálních tkáňových bank** (v anglosaské literatuře označovaných jako multitissue bank). Je všeobecně uznáváno, že tato idea vznikla v USA, kde byla v roce 1951 založena Tkáňová banka Amerického vojenského námořnictva (US Navy Tissue Bank, Bethesda, Maryland), která se orientovala na odběry tkání od zemřelých dárců. Prakticky současně v bývalém Československu byla totožná idea zformována Dr. R. Klenem, který v roce 1952 založil tkáňovou banku ve Fakultní nemocnici v Hradci Králové. V témže roce byla založena i kostní banka v Brně a v roce 1953 kožní banka v Praze. V Hradci Králové se v roce 1953 uskutečnila i první vědecká konference o transplantacích tkání.

Podobný rozvoj klinických transplantací alogenních i xenogenních tkání probíhal v 50. a 60. letech i v zahraničí, i když ještě nebyly plně dořešeny teoretické otázky spojené s imunologickou problematikou, resp. otázkou rejekce nebo přestavby štěpu v těle příjemce. O těchto mechanismech ve spojení s transplantacemi tkání se nyní stručně zmíníme.

2.9.2 Transplantace kůže

S klasickou rejekcí transplantátu se setkáváme při transplantaci kůže, resp. dermoepidermálního štěpu, který se používá především při léčbě popálených. Štěp se po přiložení na nekrektomovanou plochu nejprve rychle vaskularizuje a přihojí, takže dojde k dočasnému uzavření rány. Po 14–21 dnech však dojde ke spontánní rejekci epidermis. Proto je nutné epidermis, event. celý štěp před touto dobou chirurgicky odstranit a nahradit autologním štěpem. Je-li popálenina hluboká a rozsáhlá, takže není možné pro nedostatek odběrových ploch zakrýt plochu autotransplantáty, je výhodné alogenní dermální struktury ponechat a epidermální vrstvu nahradit napěstovanými autologními keratinocyty. Na tomto principu je založena metoda transplantace podle Cuona, vypracovaná v 80. letech v USA (*Obr. č. 36*). Alternativou je použití velmi tenkého autologního štěpu.



Obr. č. 36: **Metoda transplantace kůže podle Cuona**

2.9.3 Transplantace pojivových tkání

Rovněž **kostní tkáň** je vysoce antigenní, rejekce je však zaměřena pouze proti kostním buňkám, které po transplantaci rychle odumírají, resp. u spongiózní kosti proti buňkám kostní dřeně. Vlastní kostní hmota štěpu se nejprve odbourává činností osteoklastů a na tuto fázi navazuje postupná přestavba kosti, tzv. creeping substitution. Na její rychlost má vliv tzv. osteoinduktivní vlastnost štěpu, která závisí na obsahu kostního morfogenetického proteinu (Bone Morphogenetic Protein, BMP) v kostní matrix. Samotná rejekční reakce proti kostním buňkám, která vede k fibroprodukci v místě přestavby, však může přestavbu kostního štěpu výrazně zpomalit až zastavit. Transplantace kostí je v současné době ve světě nejčastější transplantací tkání, používá se nejen v ortopedii a traumatologii, ale i v řadě dalších oborů jako je neurochirurgie nebo stomatologie, včetně parodontologie a dentální implantologie.

Podobně jako kostní hmota se přestavují i **vazivové struktury**, jsou-li transplantovány do míst, která umožní vrůstání cév, jak jsme se zmínili výše u transplantace kůže. K náhradě dermis u hlubokých popálenin se používá deepidermizovaný kožní štěp, který je např. v USA k dispozici pod názvem Alloderm. Kombinuje se s tenkým autologním dermoepidermálním štěpem. Vazivové struktury jako fascie, tvrdá plena mozková nebo skléra, jsou často používány i heterotopicky, např. v neurochirurgii se fascie používají pro plastiky tvrdé pleny mozkové nebo v oftalmologii pro závěsné operace při vrozené nebo získané ptóze víček.

2.9.4 Transplantace rohovky

Při transplantaci rohovky dojde k náhradě předního epitelu dárce epitelem příjemce, který se vytvoří z kmenových buněk v limbu rohovky. Zadní epitel, resp. endotel rohovky dárce naproti tomu musí zůstat po transplantaci zachován, neboť jeho správná funkce je podmínkou zachování transparence štěpu. To je v ideálním případě zaručeno skutečností, že se nachází v přední oční komoře, která patří k imunologicky privilegovaným místům v těle příjemce. V praxi však v řadě případů k rejekci endotelu a následně k zakalení stromatu rohovky může dojít. Jednou z možností prevence je snaha o budování systémů výměny HLA typizovaných rohovek, které jsou transplantovány HLA typizovaným příjemcům, tedy podobnému systému jako je běžný u transplantace orgánů. Takové systémy existují např. ve Velké Británii nebo i v konti-

nentální západní Evropě. Na druhé straně stojí škola americká, která preferuje výměnu HLA netypizovaných rohovek a problém rejekce řeší výhradně použitím imunosupresiv.

2.9.5 Ostatní transplantace tkání

Zajímavým příspěvkem k řešení defektů kůže nebo i rohovky je **transplantace amnia**. Amnion v léčbě popálených i chronických kožních defektů slouží především ke stimulaci epitelizace ze zbývajících kožních adnex. Při léčbě defektů rohovky se používá jako náhrada struktur bazální membrány předního epitelu, na níž pak probíhá spontánní epitelizace migrací buněk z oblasti limbu.

Transplantace nervů se v klinice prakticky nepoužívá, i když metodika je experimentálně již dlouho vypracována. Transplantované nervy, tzv. interponáty, se používají k přemostění defektů a slouží pouze jako vodič pro prorůstání axonů z centrálního do periferního pahýlu. Současně však vzniká rejekční reakce v okolí nervového štěpu, která může interponát zničit dříve, než stačí axony štěpem prorůst.

2.9.6 Xenotransplantace tkání

Na rozdíl od transplantace orgánů se při transplantaci tkání běžně užívají i tkáně xenogenní. V ČR je nejčastější použití **prasečí kůže** jako dočasná náhrada kožního krytu, který se ovšem, na rozdíl od alogenního dermoepidermálního štěpu, nikdy nenechává přihojit a slouží tak jen jako biologický obvaz, který se pravidelně vyměňuje, dokud nedojde pod krytem ke spontánní epitelitizaci rány nebo není provedena autotransplantace kůže. V zahraničí se pro stejný účel používá i **kůže ovčí** nebo **psí** a v Brazílii dokonce kůže **tropických žab**. Kostní tkáň lze nahradit i speciálně upravenou **bovinní kostí**. Xenogenní kostní tkáň však nemá osteoinduktivní vlastnosti, takže přestavba může být výrazně horší než v případě alogenní dermis. Speciálně upravené telecí perikardy se používají v ČR i v zahraničí (pod názvem Dura–Guard) především pro plastiky tvrdé pleny mozkové. K náhradě vazivových struktur se používá i upravená prasečí dermis.

Současný rozvoj transplantací tkání v ČR lze dokumentovat na faktu, že v roce 1998 jen tkáňové banky, jejichž preparáty hradí pojišťovny, zaslaly pro klinické použití v různých lékařských oborech více než 1 700 alogenních tkáňových štěpů. Z xenogenních štěpů bylo pro klinickou potřebu vydáno 180 telecích perikardů a téměř 180 m2 prasečích dermoepidermálních štěpů. Tkáně byly transplantovány na více než 100 pracovištích. Řada dalších pracovišť, především ortopedických, si tkáňové štěpy pro transplantace připravovala sama.

Celkový úspěch transplantace tkáně však není jen výsledkem operační techniky a pooperační péče, ale celého procesu, který začíná odběrem tkáně a končí předáním použitelného štěpu na chirurgické pracoviště. Na rozdíl od transplantace orgánů, kdy interval mezi odběrem a transplantací počítáme v hodinách, může mezi odběrem a transplantací tkáně uběhnout několik měsíců i let. O jednotlivých krocích se v dalším textu zmíníme.

2.9.7 Etické a právní otázky dárcovství tkání od živých a zemřelých dárců

2.9.7.1 Odběry tkání od živých dárců

U **živých dárců** rozlišujeme 2 případy dárcovství:

1. Operační výkon je proveden výhradně za účelem získání tkáně k transplantaci, např. kůže, kosti nebo dnes nejčastěji kostní dřeně. Takový odběr může být proveden, podobně jako v případě odběru orgánu, pouze se souhlasem dárce.

2. Při operačním výkonu prováděném za léčebným účelem je odebrána tkáň, kterou lze použít k transplantaci, např. hlavice kyčelního kloubu při plastice kyčelního kloubu, véna saphena při operaci varixů, kůže při plasticko-chirurgických nebo esteticko- chirurgických výkonech. Podobně při porodu lze odejmout tkáně použitelné k transplantaci, konkrétně amnion a chorion, pupečníkové cévy a pupečníkovou krev.

I v tomto druhém případě je vhodné, aby lékař dárce požádal o souhlas, i když to současné zákony ČR přímo neukládají. O souhlas lze požádat před výkonem, např. před porodem nebo po skončené operaci, při níž se ukáže, že odebraná tkáň je skutečně použitelná k transplantaci. Současně se žádostí o souhlas k použití tkáně k transplantaci je nutné žádat dárce i o souhlas s opakovaným sérologickým vyšetřením na vyloučení pozitivity HIV a dalších infekcí.

Je nutno zdůraznit, že množství solidních tkání získané k transplantaci druhým způsobem, zdaleka převyšuje množství těchto tkání získané prvním způsobem. V zemích, ve kterých je silná opozice proti odběru tkání ze zemřelých dárců, např. Turecku, je pak tento postup dominantním způsobem získávání tkání k transplantacím.

2.9.7.2 Odběry tkání od zemřelých dárců

Pro odběr tkání od zemřelých dárců platí stejná pravidla jako pro odběr orgánů, tzn. předpokládá se souhlas s posmrtným odběrem, pokud zemřelá osoba neprojevila během svého života zákonem předepsanou formou svůj nesouhlas. V současné době platí v ČR jako předepsaná forma písemné prohlášení dárce, v praxi je však obvyklé respektovat jakoukoliv opozici projevenou proti odběru. Návrh transplantačního zákona předpokládá zachování písemné formy nesouhlasu a současně zřízení počítačového registru osob nesouhlasících s odběrem, v němž má každé pracoviště provádějící odběr povinnost hledat každého potenciálního dárce.

Na tomto místě je třeba se zmínit o vlivu, který má na postoje dárců a na použití alogenních i xenogenních tkání, **tradice a náboženství**. Lze konstatovat, že křesťanská náboženství v současné době dárcovství tkání podporují, s výjimkou mormonů. Přesto lze vypozorovat rozdíl mezi přístupem katolíků a protestantů. Zatímco protestanté kladou důraz na dárcovství jako individuální vědomý akt altruismu, katolíci se podřizují stanovisku církevních autorit, které, včetně papeže, dárcovství od zemřelých podporují. U řady jiných náboženství se snaha o provedení odběru tkáně může dostat do konfliktu s náboženskými předpisy týkajícími se manipulace s tělem zemřelého nebo požadavku na co nejrychlejší pohřeb. Např. japonské náboženství – šintoismus zakazuje jakékoliv úkony na těle zemřelého. Judaistické náboženství zdůrazňuje rychlost pohřbu, v praxi je však možné najít mezi těmito direktivami a praxí tkáňových bank, resp. odběrových týmů, pro obě strany přijatelný kompromis. Budhistické náboženství výrazně dárcovství tkání podporuje. U islámu se setkáváme se širokým spektrem postojů církevních autorit, které se liší podle jednotlivých náboženských škol. Lze se tak setkat s poměrně výraznou podporou, ale i s výrazným odmítáním dárcovství tkání od zemřelých. Proti transplantaci tkáně odebrané od zemřelých dárců neislámského náboženství nemá islám žádné námitky.

Podobně je třeba brát v úvahu náboženské zákazy týkající se použití xenogenních tkání. Např. v arabských zemích se místo prasečích dermoepidermálních štěpů používají při léčbě popálených dermoepidermální štěpy ovčí, nebo se dává přednost amniu. Turečtí autoři popisují velmi dobré výsledky s použitím amnia impregnovaného dusičnanem stříbrným. Ani náboženské zákazy však neplatí absolutně, pokud jde o život zachraňující výkony, jak svědčí např. práce Ben Hurovy o úspěšném použití prasečích dermoepidermálních štěpů u popálených izraelských vojáků při arabsko-izraelské válce.

2.9.8 Praktické p

Odběr tkáně od
chirurgická residua
získávat tkáně třem

1. Během tzv. mno
u dárců s prokáz
2. Zorganizováním
3. Provedením nea

První způsob se
nutno zdůraznit, že
kem celkového mn
dějí od dárců zemře
ňových bank prov
vybavených sálech
kých nebo soudně-
dříve za 2 hodiny p
duje klinickou dok
Na tomto místě se
dárce. V anamnéze
ření na HIV, HBsA
chování. Mezi ně pa
hodobé pobyty v ze
infekcí, jako je anar
rozeným růstovým
cí popsán. Samozř
nebo sepse či pyen
gicky vyléčeného b
soustavy. Výjimkou
připouštějí odběr i u
má být odebrána i
Odběr se provád
dezinfekci a zarouš
Tkáň se ukládá
třeba vzhled dárce
z vena saphena, ve
množství, aby moh
rozdíl od praxe při
se při provádění sér
tkání, setkáváme č
a testy je proto čast
logické testy je sam
se nespotřebuje při
ní panel představuj

2.9.8 Praktické provádění odběrů tkání a jejich kontraindikace

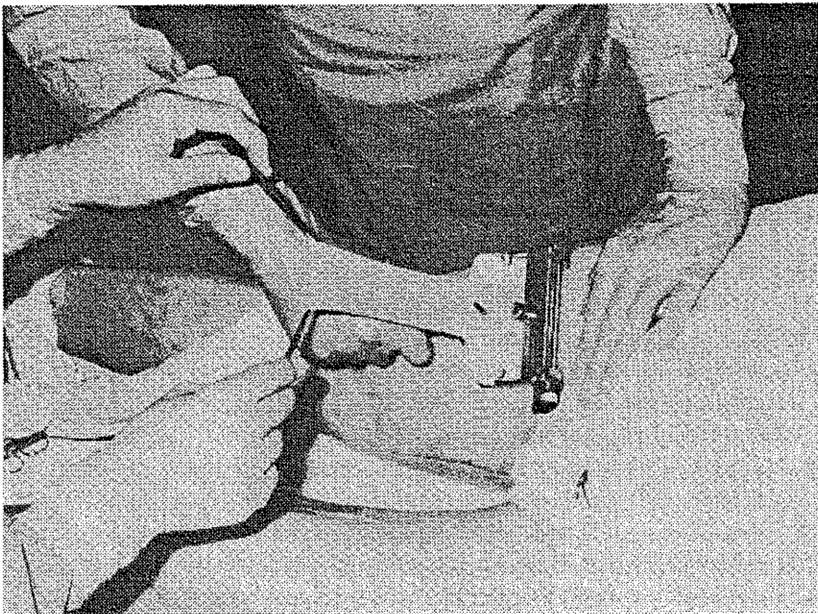
Odběr tkáně od živého dárce se provádí jako normální operační výkon. Podobně jsou i tzv. chirurgická residua získávána při operačních výkonech nebo porodu. U zemřelých dárců lze získávat tkáně třemi způsoby:

1. Během tzv. mnohotného odběru tkání a orgánů odběrovými týmy transplantačních center u dárců s prokázanou smrtí mozku.
2. Zorganizováním aseptického odběru před pitvou.
3. Provedením neaseptického odběru během pitvy.

První způsob se opět neliší od odběru orgánů popsaných v předchozích kapitolách. Je však nutno zdůraznit, že množství tkání získaných od dárců s prokázanou smrtí mozku je jen zlomkem celkového množství tkání získaného od zemřelých dárců. Ostatní odběry tkání se provádějí od dárců zemřelých na srdeční zástavu. Odběrové týmy chirurgických pracovišť nebo tkáňových bank provádějí aseptické odběry u těchto dárců buď ve zvlášť k takovému účelu vybavených sálech nebo v improvizovaných podmínkách, většinou na patologicko-anatomických nebo soudně-lékařských odděleních. Takový odběr lze zahájit, podobně jako pitvu, nejdříve za 2 hodiny po smrti. Samozřejmostí je, že lékař odpovědný za odběr podrobně prostuduje klinickou dokumentaci nemocného a vyloučí eventuální **kontraindikace odběru** tkáně. Na tomto místě se zmíníme pouze o hlavních kontraindikacích, které platí pro zemřelé i živé dárce. V anamnéze a klinické dokumentaci dárce pátráme po pozitivitě sérologického vyšetření na HIV, HBsAg, HCV nebo syfilis i anamnéze hepatitidy, dále po projevech rizikového chování. Mezi ně patří především homosexualita, promiskuita, intravenózní aplikace drog, dlouhodobé pobyty v zemích s vysokou incidencí HIV infekce, dále pátráme po riziku prionových infekcí, jako je anamnéza neurologických a psychiatrických onemocnění, předchozí terapie přirozeným růstovým hormonem nebo předchozí transplantace tkání, u nichž je přenos těchto infekcí popsán. Samozřejmou kontraindikací jsou pak projevy akutního přenosného onemocnění nebo sepse či pyemie. Kontraindikací odběru tkání jsou i maligní nádory s výjimkou chirurgicky vyléčeného basocelulárního karcinomu kůže a primárních novotvarů centrální nervové soustavy. Výjimkou jsou odběry rohovek, u kterých standardy některých organizací očních bank připouštějí odběr i u nemocných s karcinomy. Kontraindikací je rovněž onemocnění tkáně, která má být odebrána i vysoký věk dárce, např. u šlach nebo menisků se doporučuje limit 55 let.

Odběr se provádí vysterilizovanými chirurgickými nástroji po řádném oholení, omytí, dezinfekci a zarouškování příslušného odběrového pole (*Obr. č. 37).*

Tkáň se ukládá do vysterilizovaných skleněných nebo plastikových obalů. Po odběru je třeba vzhled dárce dokonale rekonstruovat. Součástí odběru tkáně je i odběr krve, obvykle z vena saphena, vena femoralis nebo vena subclavia. Krev je nutno odebrat v dostatečném množství, aby mohly být provedeny předepsané sérologické testy včetně konfirmačních. Na rozdíl od praxe při odběru orgánů, kdy se krev odebírá při ještě zachovalém krevním oběhu, se při provádění sérologických testů u dárců zemřelých na srdeční zástavu, tedy u většiny dárců tkání, setkáváme častěji s falešně pozitivními reakcemi (v důsledku postmortální hemolýzy) a testy je proto často nutno konfirmovat. Nemožnost získat dostatečné množství krve pro sérologické testy je samozřejmě indikací k vyřazení tkáně z klinického použití. Sérum dárce, které se nespotřebuje při provádění testů, se archivuje pro eventuální pozdější retestování. Základní panel představují testy na průkaz HIV, HBsAg, HCV a syfilis. Pozitivita v jakémkoli uve-



Obr. č. 37: **Aseptický odběr dermoepidermálního štěpu u zemřelého dárce dermatomem na stlačený vzduch**

deném testu je kontraindikací použití tkáně. Z dalších testů se doporučují testy na CMV, kde je průkaz aktivní infekce relativní kontraindikací. (U živých dárců tkání se ponechávají tkáně v karanténě a testy se opakují po 6 měsících.).

Odběr tkáně se provádí co nejdříve po smrti, jakmile je k dispozici odběrový tým, nejde však o tak urgentní situaci jako při odběru orgánů. Jako horní časový limit pro odběr se obvykle uvádí 12 hodin, pokud tělo zemřelého je uchováno při teplotě zevního prostředí nebo do 24 hodin, pokud tělo bylo uloženo při teplotě 4°C.

V řadě zemí se užívá i neaseptický odběr tkání během pitvy a veškeré tkáně se finálně sterilizují. Odběry tkání mimo zdravotnická zařízení, tj. např. v prostorách pohřebních ústavů, které jsou praktikovány i v některých vyspělých zemích, se v ČR neprovádějí.

2.9.9 Zpracování tkání

Zpracováním tkání rozumíme nejrůznější úpravy odebrané tkáně provedené ještě před vlastní konzervací štěpu. Patří sem např. **mechanické odstranění zbytků tukové tkáně** odebrané spolu s vazivovými strukturami, odstranění periostu nebo perichondria. Je-li tkáň získána z tzv. chirurgických residuí, je nutno odstranit i patologickou tkáň, např. nekrotické části hlavice kosti kyčelní. Do metod úpravy patří i **snižování celularity štěpu** a tím i jeho imunogenicity, např. u spongiózních kostních štěpů se vymývají zbytky buněčných elementů kostní dřeně nebo se extrahuje tuk, je-li dřeň tukově přeměněna. Pokud si chirurg připravuje tkáň k transplantaci sám, obvykle se omezuje na prostou mechanickou preparaci a úpravu štěpu na konečný tvar vhodný pro plánovaný operační výkon. Složitější metody úpravy jsou vyhrazeny tkáňovým bankám. K těmto metodám patří i **demineralizace kostních štěpů**, která demaskuje mezibuněč-

nou hmotu obsahují
Uristem, působí oste
současně s deminera
při teplotě 37°C, vzn
navíc k výše popsan
její přestavbě tudíž n
cím v konečné fázi k

Jak jsme uvedli,
ce osudu štěpu v tě
i opačného efektu, tj
tomu např. při tzv. s
genu. Tato metoda se
ní nebo telecích peri

2.9.10 Konzervac

Při konzervaci tká
i konzervace normo

Metody nízkoteploti

Tkáně můžeme u
pod bodem tuhnutí p
zervací hypotermní.

Tkáně v hypoterm
komůrka je jakákoli
roztoku nebo jiného

Obr. č. 38: **Prasečí de**

nou hmotu obsahující kostní morfogenetický protein. Tento protein, objevený a izolovaný Uristem, působí osteoinduktivně na buňky příjemce a urychluje tak přestavbu kosti. Pokud je současně s demineralizací provedena **deplece kostních buněk** několikadenní inkubací tkáně při teplotě 37°C, vznikne tzv. AAA-kost (Allogeneic Autolysed Autodigested Bone), která má navíc k výše popsaným vlastnostem i výrazně nižší imunogenicitu než neupravená kost. Při její přestavbě tudíž nedochází k rejekci kostních buněk a tím k zánětlivým pochodům vedoucím v konečné fázi k fibroprodukci, která ohrožuje zdárný průběh přestavby kosti.

Jak jsme uvedli, můžeme vhodnou metodou úpravy tkáně dosáhnout podstatné modifikace osudu štěpu v těle příjemce ve smyslu urychlení jeho přestavby. Lze však dosáhnout i opačného efektu, tj. zpomalení až úplného zastavení přestavby tkáně v těle příjemce. Tak je tomu např. při tzv. **stabilizaci tkáně glutaraldehydem**, který vytvoří příčné vazby v kolagenu. Tato metoda se používá pro přípravu xenogenních biologických protéz srdečních chlopní nebo telecích perikardů používaných k plastikám tvrdé pleny mozkové.

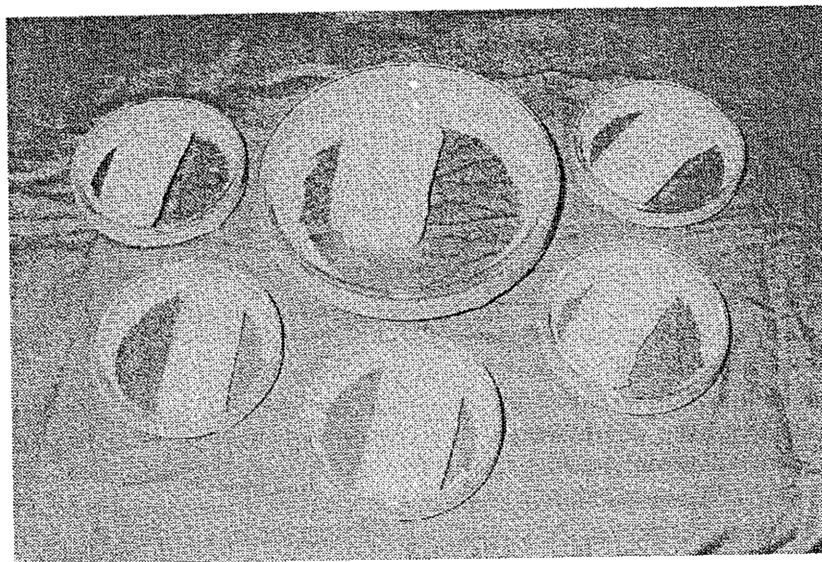
2.9.10 Konzervace tkání

Při konzervaci tkání dominují metody nízkoteplotní konzervace. Poměrně rozšířená je u tkání i konzervace normotermní, tj. použití tkáňové, resp. orgánové kultury.

Metody nízkoteplotní konzervace

Tkáně můžeme uchovávat jak v nadnulových, tak podnulových teplotách, resp. nad nebo pod bodem tuhnutí použitého media. Konzervaci v oblasti nad bodem tuhnutí nazýváme konzervací hypotermní. Nejčastěji používaná teplota je teplota +4°C.

Tkáně v hypotermii uchováváme buď v tzv. vlhké komůrce nebo v různých médiích. *Vlhká komůrka* je jakákoliv sterilní nádobka, na jejíž dno nalijeme malé množství fyziologického roztoku nebo jiného vhodného média, vložíme do ní tkáň, nádobku uzavřeme a uložíme při



Obr. č. 38: **Prasečí dermoepidermální štěpy konzervované ve vlhké komůrce – Petriho miskách**

teplotě +4°C. Tím vytvoříme podmínky bránicí vysychání tkáně a současně zpomalující metabolické pochody. Metoda se dosud používá pro konzervaci alogenních i xenogenních dermoepidermálních štěpů (*Obr. č. 38*), ale i očních bulbů, resp. neexcidované rohovky. Zatímco u dermoepidermálních štěpů zaručuje metoda použitelnost do 10–14 dnů, u neexcidované rohovky pouze do 48 hodin. Po této době rychle dochází k zakalení stromatu rohovky.

Druhým způsobem hypotermní konzervace je *konzervace v různých médiích*. Jsou to především média odvozená od médií tkáňových kultur nebo roztoků na promývání buněk, ale lze použít i roztoky pro konzervaci orgánů, např. UW roztok, Eurocollins nebo Custodiol. Velký pokrok při konzervaci rohovky byl dosažen použitím médií MK (McCarrey a Kaufman) a později Optisolu pro konzervaci excidované rohovky. Použitelnost se tak podařilo prodloužit na 4 dny u MK média, resp. na 14 až 21 dnů u média Optisol. Konzervace v těchto mediích je nejvíce rozšířena v USA a v sítích očních bank koordinovaných americkými odborníky. Na síť IFEB (International Federation of Eye Banks) se sídlem v Baltimore je napojena i Mezinárodní oční banka v Praze, která tuto metodu zavedla v ČR na počátku 90. let.

Hypotermní konzervaci dáváme přednost tam, kde potřebujeme pro výsledný efekt transplantace zachovat tkáň živou, tj. u rohovek, u dermoepidermálních štěpů nebo při rekonstrukci kloubního povrchu alogenním chrupavkovým štěpem. Jak u rohovek, tak u chrupavky tato metoda stále předčí svým výsledkem kryokonzervaci. U dermoepidermálních štěpů jsou hypotermní konzervace a kryokonzervace prakticky rovnocenné.

V podnulových teplotách můžeme konzervovat tkáň dvěma způsoby:

1. *Prostým zmrazením* na teplotu nižší než -40°C, bez kryoprotektiv, tj. látek zabraňujících letálnímu účinku procesu zmrazení–rozmrazení na buňky a tkáně. Použití tohoto způsobu, tzv. hlubokého zmrazení (deep freezing) je samozřejmě omezeno jen na tkáně, u nichž zachování vitality buněk není podmínkou úspěšné transplantace, tzn. na pojivové tkáně. Tkáně, vložené do vhodného sterilizovaného obalu se vloží do zmrazovacího zařízení dosahujícího výše uvedené nebo nižší teploty a při téže teplotě se pak skladuje až do použití. Je běžně uznáváno, že za uvedených podmínek lze skladovat tkáně do 5 let.

2. *Kryokonzervací*, tj. postupem, kdy použitím kryoprotektiva a definovaného režimu zmrazení dosáhneme zachování vitality buněk štěpů. Tento způsob se používá především pro dermoepidermální štěpy a buněčné suspenze (např. buňky kostní dřeně nebo pupečníkovou krev), ale i pro srdeční chlopně a cévy. Méně úspěšný je u rohovek a chrupavek s výjimkou suspenze kultivovaných chondrocytů. Jako kryoprotektiva se používají především glycerol v koncentraci 10–15% nebo dimetylsulfoxid v koncentraci 5–10%. Úspěšná kryokonzervace je stále možná jen u tkání, u nichž je možno provést zmrazení v tenké vrstvě (několika mm) a tak dosáhnout uniformního teplotního režimu zmrazení, resp. rozmrazení v celém objemu konzervované tkáně. Kryokonzervace orgánů zatím úspěšná není.

Alternativou hlubokého zmrazení je *lyofilizace*, tj. postup, při kterém tkáň nejprve zmrazíme a poté z ní sublimací odstraníme vodu, takže zbytkovou vlhkost snížíme pod 5%. Lyofilizované tkáně jsou uchovávány ve skleněných nebo plastikových obalech a jsou dostupné z řady tkáňových bank v USA i v západní Evropě. V bývalém Československu byla zavedena již v 60. letech. Výhodou je možnost skladovat štěpy při pokojové teplotě, takže chirurg si může určitou zásobu štěpů vytvořit na svém oddělení. Rovněž transport lyofilizovaných štěpů je velmi jednoduchý. Lyofilizované tkáně je nutno před použitím rehydratovat.

Kryokonzervova
-80° C v mechanick
fázi dusíku (-196° C
buňky tkáně minimá
tuje možnost časově

Normotermní konz
Tento druh konze
vložena do kultivač
sféře s 5 % oxidu uhl
da je rozšířena zejm
Ve výčtu používa
centrovaném glycer
ním krytem na rozdí
ského popáleninové
i *konzervace v konc*

2.9.11 Opatření p

Transplantace tká
ce. Pokud jde o mož
odběr nevylučuje **ba**
zovat do postupů zp
alkoholu nebo roztok
štěpů, které jsou urč
nické použití tkáně,
midis. Při zachování
V literatuře exist
nost je především vě
ní době se do popře
popsán po transplan
stenci původce této
ní osob s nevyjasně
spojené s histologic
V poslední době
transplantaci. Ta b
transplantace, u níž
riální i virové infek
s nálezem Listerie
sických patogenů n
výstupní bakteriolo
V současné době
sečí endogenní retro
současných poznatk
tkáně s tkání příjemc
liv běžně používaný

Kryokonzervované buňky a tkáně dlouhodobě uchováváme při teplotách nižších než -80° C v mechanických zmrazovacích zařízeních, v parách (-150° C) nebo přímo v kapalné fázi dusíku (-196° C). Podle zkušeností autora lze při teplotě -80° C bezpečně skladovat živé buňky tkáně minimálně po dobu 1 roku, při teplotě kapalného dusíku se všeobecně konstatuje možnost časově nelimitovaného skladování.

Normotermní konzervace

Tento druh konzervace, který se technicky neliší od praxe tkáňových kultur, tzn. tkáň je vložena do kultivační nádobky s vhodným médiem a uchovávána při teplotě 37° C v atmosféře s 5 % oxidu uhličitého, je vhodnou alternativou hypotermní konzervace rohovek. Metoda je rozšířena zejména v západní Evropě. Uvádí se, že rohovka je použitelná do 3–4 týdnů.

Ve výčtu používaných konzervačních metod nesmíme zapomenout na *konzervaci kůže v koncentrovaném glycerolu*. Alogenní glycerolizovaný štěp je v západní Evropě dominujícím kožním krytem na rozdíl od ČR, kde převažuje použití xenotransplantátů, které zavedl tým pražského popáleninového centra a kožní banky v 70. letech. Mezi další používané metody patří i *konzervace v koncentrovaném etanolu*.

2.9.11 Opatření pro snížení rizika přenosu infekce transplantovanou tkání

Transplantace tkání s sebou vždy nese určité riziko přenosu bakteriální nebo virové infekce. Pokud jde o možnost přenosu bakteriální infekce, je nutno si uvědomit, že ani aseptický odběr nevylučuje **bakteriální kontaminaci** odebraných tkání. Je proto zcela oprávněné zařazovat do postupů zpracování tkání dekontaminační postupy s použitím jódových preparátů, alkoholu nebo roztoků antibiotik. Následně se provádí bakteriologická výstupní kontrola. U všech štěpů, které jsou určeny k implantaci, vylučuje jakýkoliv pozitivní mikrobiologický nález klinické použití tkáně, u dermoepidermálních štěpů se připouští nález Staphylococcus epidermidis. Při zachování lege artis postupů lze riziko bakteriální infekce snížit na minimum.

V literatuře existuje řada údajů o přenosu virové infekce transplantovanou tkání. Pozornost je především věnována popsaným případům přenosu HIV a hepatitidy B a C. V poslední době se do popředí dostává i problematika Creutzfeld-Jakobovy choroby, jejíž přenos byl popsán po transplantacích tvrdé pleny mozkové, rohovky i skléry. Vzhledem k známé resistenci původce této choroby na dekontaminační postupy je třeba zdůraznit význam vyloučení osob s nevyjasněnou neurologickou symptomatologií z dárcovství a význam pitvy dárce spojené s histologickým vyšetřením mozku zaměřeným na průkaz této choroby.

V poslední době je zvýšená pozornost věnována i možnosti **přenosu infekce při xenotransplantaci**. Ta byla v minulých letech považována paradoxně za bezpečnější než alotransplantace, u níž bylo zdůrazňováno riziko HIV infekce. Riziko rovněž zahrnuje bakteriální i virové infekce. Z původců klasických antropozoonóz se např. autor této statě setkal s nálezem Listerie při odběru prasečích dermoepidermálních štěpů. Z důvodů nálezů klasických patogenů nebo podmíněných patogenů se na pracovišti autora vyřazuje na základě výstupní bakteriologické kontroly v průměru 10 % zhotovených prasečích štěpů.

V současné době se považuje za největší potenciální riziko použití prasečích orgánů prasečí endogenní retrovirus, označovaný zkratkou PERV (porcine endogenous retrovirus). Podle současných poznatků by se však mohl uplatnit pouze při dlouhodobém kontaktu xenogenní tkáně s tkání příjemce a při imunosupresi, což je případ klasické orgánové transplantace, nikoliv běžně používaných transplantací tkání.

Při použití bovinní tkáně je samozřejmě nutno vzít v úvahu v současné době diskutovanou možnost přenosu bovinní spongiformní encefalopatie (BSE). Toto riziko je však minimalizováno již samotným faktem, že tkáně pro transplantační účely jsou odebírány od telat.

Na tomto místě je třeba se stručně zmínit i o další možnosti jak snížit rizika přenosu bakteriální i virové infekce, a to o **sterilizaci tkáně**. Ta je samozřejmě možná jen u tkání, u nichž není vitalita nutná pro úspěch transplantace, je vhodná proto především pro tkáně pojivové. Lze ji však použít i pro kožní štěpy nebo amnion.

Sterilizační metody, které umožňují sterilizaci v celém objemu tkáně, jsou buď ozáření gama paprsky nebo sterilizace etylénoxidem. K povrchové sterilizaci lze použít i tzv. plazmu, tj. peroxid vodíku.

Sterilizaci gama zářením lze provádět u zmrazených i lyofilizovaných štěpů a je rozšířena především v USA, ale i v západní a střední Evropě. V ČR se používá od sedmdesátých let. Nevýhodou je, že zhoršuje mechanické vlastnosti tkání, především jsou-li ozářeny v lyofilizovaném stavu. S ohledem na inaktivaci virů se v současné době doporučují dávky kolem 35 kGy, místo dříve používaných dávek 25 kGy.

Při sterilizaci etylénoxidem riziko zhoršení mechanických vlastností nehrozí, nevýhodou je však jeho kancerogenita a z toho vyplývající nutnost stanovení residuí etylénoxidu a jeho reakčních produktů ve sterilizované tkáni před jejím uvolněním pro klinické použití. Metoda je rutinně používána jak v USA, tak v západní Evropě i v ČR.

Ani jedna z metod však není rovnocenná sterilizaci nasycenou vodní parou pod tlakem, kterou lze pro tkáně užít skutečně jen ve výjimečných případech.

Z uvedeného vyplývá, že jak při alotransplantaci, tak při xenotransplantaci tkání určité riziko přenosu infekce vždy existuje a že toto riziko lze správnou praxí snížit na minimum, nikoliv však zcela eliminovat. Riziko použití alogenní nebo xenogenní tkáně k transplantaci není možné podceňovat, ale ani přeceňovat. Vždy je nutno je porovnávat s předpokládaným přínosem pro operovaného i s možnými riziky alternativních postupů.

REJSTŘ

A
ABO kompatibilita
Akcelerovaná akut
Akutní rejekce 14,
Akutní tubulární ne
Algoritmus výběru
Alografty 131
Alokace odebraněh
Alotransplantace 1
Alotransplantáty 1
Arytmie 36
Autotransplantace
Azathioprin 60

B
Bakteriální kontam
Basiliximab 84
Biologická dostupn
Biologická chlopeň
Biologická imunos
Blok srdce-plíce 1
Bronchiolitis oblite

C
Centrální koordina
Cévní steh 14
Clearance 54
Cross – match 13
Cushingův reflex 3
Cyclosporin A 65

Č
Čekací doba 12
Čekací listina (wait

D
Dárce 11
Dárce ledvin 41
Dárce s „bijícím sr

CHAPTER 2 - APPENDIX 1

1a SPECIFICATIONS OF COLLECTED CELLS AND TISSUES

Tkáňová ústředna Fakultní nemocnice v Hradci Králové

Název pracoviště: Banka progenitorových hematopoetických buněk
Specifikace č. 1/2004
Vypracoval: MUDr. Pavel Měřička
Platí od: 1.1.2004
Datum plánované revize: 1.1.2005

Lidská autologní kostní dřeň neseparovaná kryokonzervovaná

Specifikace vstupního produktu - kostní dřeň filtrovaná
Kostní dřeň asepticky odebraná odběrovým týmem (2 odebírající lékaři, 2 sestry, 1 lékař nebo VŠ pro manipulaci s odebranou dření) opakovanými punkcemi z pánevních kostí od pacienta do vaku z odběrové soupravy fy Baxter (1 000 ml, z toho 200 ml roztoku ACD) na aseptickém sále. Následně je provedena filtrace dřeně odběrovou soupravou fy Baxter na sálech ortopedické kliniky – provádí tým Tkáňové ústředny, označení odběrového vaku daty pacienta (jméno, příjmení, rodné číslo), zkratkou KD, datem odběru.
Vložení odběrového vaku do zevního obalu, jeho přenesení do materiálové propusti kryokonzervační části nového pracoviště tkáňové ústředny, dočasné uchování do doby zpracování při teplotě +4°C.

Předepsaný způsob odběru vzorků a jejich zkoušení:
Odběr vzorku periferní krve nemocného na sérologické vyšetření – provádí OKH FN (anti-HIV 1,2, HIV –1 Ag, anti- HTLV, EBV, HSV, VZV, HBsAg, anti-HCV, CMV, RRR, TPHA, Toxo), vyšetření provádí ÚKM a ÚKIA FN. Tabulka s výsledky testů se zašle Tkáňové ústředně.

Průběžný odběr vzorků odebrané dřeně na hematologické (OKH FN), kultivační (CFU-GM) (TÚ FN) a imunologické vyšetření (CD 34) (ÚKIA FN) – provádí tým Tkáňové ústředny.

Označení :

<table>
<tr>
<td>Tkáňová ústředna FN HK
C 2089-03 01
BM Odběr: 12.11.2003 14 00
Dárce/Příjemce

rč:</td>
<td>Autotransplant.
KS O Rh(D) poz.
Odebraný objem:1200 ml
Obsahuje 210 ml ACD-A roztoku
Skladovat při teplotě +4°C
Použitelnost do 13.11 14 00</td>
</tr>
</table>

Podmínky skladování:
Teplota + 4°C, meze 1-10°C

Doba použitelnosti produktu: 24 hodin

Název biologického materiálu: Alogenní vazivový štěp typu kost-šlacha-kost

Popis materiálu:
Tkáň odebraná asepticky na operačním sále nebo odběrovém sále Tkáňové ústředny vysterilizovanými nástroji po omytí, desinfekci a zarouškování těla zemřelého dárce odběrovým týmem (lékař a.sestra nebo laborant či jiný VŠ specialista).
Obalový materiál: sáček Steriking sterilizovaný radiačně–dvojitý nebo dvojitý polyetylénový sáček v plastovém kelímku - vše radiačně sterilizováno.
Obal označen číslem dárce, názvem odebrané tkáně a datem odběru - vzor štítku přiložen:

Tkáňová ústředna FN HK Odběrové centrum D 10/04, Dg.: S 06.2 odběr dne 23.6. 07:51 tkáň: **LIG** 2x

Předepsaný způsob odběru vzorků:
Odběry: krev dárce na serologické vyšetření podle vyhlášky MZ ČR č. 437/2002.
Bakteriologický stěr z odebrané tkáně.

Kvalitativní a kvantitativní požadavky včetně jejich limitů:
Negativita v serologických testech předepsaných vyhláškou. Absence kontraindikací odběru předepsaných vyhláškou v klinické i anatomické diagnóze.

Podmínky skladování:
U štěpů určených ke kryokonzervaci + 4°C do doby finálního zpracování (max. 24 hodin).

Požadavky na asepsi:
Roušky, operační pláště, operační nástroje - sterilizace vodní parou pod tlakem (centrální sterilizace FN).
Obalové materiály: radiační sterilizace, Bioster Veverská Bítýška
Desinfekční roztoky: Betadine, Egis Pharmaceuticals, Maďarsko

Tkáňová ústředna Fakultní nemocnice Hradec Králové
Název pracoviště: Odběrové centrum
Specifikace č5./2004
Vypracoval MUDr Pavel Měřička
Platí od 1.7 2004
Datum předpokládané revize:1.1. 2005

Název biologického materiálu: Alogenní dermoepidermální štěp

Popis materiálu:
Tkáň odebraná asepticky na operačním sále nebo odběrovém sále Tkáňové ústředny vysterilizovanými nástroji, po omytí, desinfekci a zarouškování těla zemřelého dárce odběrovým týmem (lékař a sestra nebo laborant či jiný VŠ specialista).
Obalový materiál: dvojitý polyetylénový sáček v plastovém kelímku - vše radiačně sterilizováno nebo sterilizovaný skleněný obal.
Obal označen číslem dárce, názvem odebrané tkáně a datem odběru - vzor štítku přiložen:

Tkáňová ústředna FN HK Odběrové centrum D 10/04, Dg.: S 06.2 odběr dne 23.6. 07:51 tkáň: **Ků** 2x

Předepsaný způsob odběru vzorků:
Odběry: krev dárce na serologické vyšetření podle vyhlášky MZ ČR č. 437/2002.
Bakteriologický stěr z odebrané tkáně.
Kvalitativní a kvantitativní požadavky včetně jejich limitů:
Negativita v serologických testech předepsaných vyhláškou. Absence kontraindikací odběru předepsaných vyhláškou v klinické i anatomické diagnóze.

Podmínky skladování:
Uchování při teplotě + 4°C do doby finálního zpracování (max. 24 hodin).

Požadavky na asepsi:
Roušky, operační pláště, operační nástroje - sterilizace vodní parou pod tlakem (centrální sterilizace FN).
Obalové materiály: radiační sterilizace, Bioster Veverská Bítýška
Desinfekční roztoky: Betadine, Egis Pharmaceuticals, Maďarsko

1b SPECIFICATIONS OF FINAL PRODUCTS

Tkáňová ústředna Fakultní nemocnice v Hradci Králové

Název pracoviště: Banka progenitorových hematopoetických buněk
Specifikace č. 2/2004
Vypracoval: MUDr. Pavel Měřička
Platí od: 1.1.2004
Datum plánované revize: 1.1.2005

Lidská autologní kostní dřeň neseparovaná kryokonzervovaná

Specifikace konečného produktu

Kostní dřeň odebraná asepticky odběrovým týmem a následně filtrovaná – viz specifikace výchozího produktu: kostní dřeň filtrovaná.

Výchozí produkt je přepuštěn do kryokonzervačních vaků Cryocyte TM (Baxter) (zpravidla 15-20 vaků s 50 ml koncentrátu), následuje smísení koncentrátu s 50 ml předchlazeného (+4°C) roztoku 20% DMSO v 6 % roztoku dextranu s přidáním 5 ml 20% lidského albuminu do každého vaku za stálého třepání vaky na třepačce. K přípravě smí být použit pouze DMSO s atestem SÚKL. Vaky označeny daty pacienta (jméno, příjmení, rodné číslo), zkratkou KD, datem odběru a pořadím vaku (A,B,C, atd.):

Tkáňová ústředna FN HK **C 2089-03 0H** BM Odběr: 12.11.2003 Dárce/Příjemce rč:	Autotransplant. KS O Rh(D) poz. 100 ml Kryokonzervace: 12.11.2003 Skladovat při teplotě <-80°C

Následuje vložení vaků do zevních kovových kazet s okénkem, kterým lze vidět štítek na vaku a řízené zmrazování -1K/min do -90°C, -5K/min do -150°C. Při ukládání do nízkoteplotní skříně REVCO je možno program ukončit po dosažení teploty -100°C v kontrolním vaku.

Předepsaný způsob odběru vzorků a jejich zkoušení:

Odebrání vzorků na hematologické vyšetření a vyšetření sterility (ÚKM FN – metoda Ústavu Paula Ehrlicha, SRN) z každého vaku a odebrání vzorků do 2 kryoampulí (1,6 ml) z každého vaku pro hematologické (OKH FN) a kultivační vyšetření CFU-GM (TÚ FN) po rozmrazení.

Kvantitativní a kvalitativní požadavky včetně limitů:

Zhodnocení výsledků všech vyšetření, rozhodnutí o vhodnosti preparátu k aplikaci nebo o nutnosti kombinovat odběr se separací periferních kmenových buněk. Za optimální hodnotu se považuje 2.10^4 CFU-GM/kg hmotnosti pacienta při stanovení z rozmrazených kontrolních vzorků. Za minimální hodnotu se považuje 1.10^4/kg při stejném způsobu stanovení. Produkt má vyhovovat zkoušce na sterilitu. V případě pozitivního mikrobiologického nálezu se provede identifikace mikrobů a vyšetření na citlivost a o použití se rozhodne na transplantačním semináři OKH.

V případě úmrtí pacienta se kryokonzervovaný materiál i kontrolní vzorky vyřadí na základě písemného potvrzení vystaveného ošetřujícím lékařem. Toto potvrzení se archivuje.

Podmínky skladování:

Kazety s vaky se ukládají do kontejneru s kapalným dusíkem do par dusíku nebo do zařízení stejného typu do kapalné fáze dusíku nebo nízkoteplotní skříně REVCO (-80°C) s havarijním dochlazováním kapalným dusíkem při vzestupu teploty nad -70 °C, uložení kontrolních vzorků ve stejných teplotních podmínkách jako kazety.

Skladování kazet s vaky a kontrolních vzorků ve výše uvedených teplotách, pravidelné automatické nebo manuální doplňování dusíku, monitorování teplot v mechanickém zařízení REVCO pomocí software Netcom a Read 95 (ReguCon, s.r.o., Praha), zajišťujících trvalý zápis formou tabulky i grafu. Akustický signál alarmu je nastaven na těchto hodnotách: horní mez –60°C, dolní mez –100°C.

Doba použitelnosti produktu:

Skladování při teplotě –80°C 1 rok bez dodatečného přezkoušení viability vzorků.
Skladování v parách dusíku 2 roky bez dodatečného přezkoušení viability vzorků.
Skladování v kapalné fázi dusíku 3 roky bez dodatečného přezkoušení viability vzorků.

Tkáňová ústředna Fakultní nemocnice Hradec Králové
Název pracoviště: Tkáňová banka
Specifikace č. 07/2004
Vypracoval: MUDr. Pavel Měřička
Platí od: 1.1.2004
Datum předpokládané revize: 1.1.2005

Název produktu: Alogenní dermoepidermální štěp kryokonzervovaný

Kód VZP: 43106

Složení produktu:
Dermoepidermální štěp o tloušťce cca 0,3 mm odebraný z dolních končetin nebo dorsální strany trupu po předchozím oholení. Způsob odběru aseptický - viz specifikace výchozího produktu.

Popis štěpu a obalu:
Tkáň odebraná od zemřelého dárce, splňujícího kritéria způsobilosti podle vyhlášky MZ ČR č. 437/2002, mechanicky zpracovaná na štěpy o různých rozměrech, dekontaminovaná 5% Betadinem v prostorách třídy čistoty A. Jako kryoprotektivní roztok použit 10% dimetylsulfoxid v 6% roztoku dextranu s přidáním 5% dvacetiprocentního lidského albuminu. Jeden obal obsahuje jeden nebo více štěpů s definovanými rozměry. Jako transparentní obalový materiál použit dvojitý sáček Sterking, Wipak Medical, Finsko sterilizovaný gama zářením (Bioster Veverská Bítýška) - vzor protokolu o obdržené dávce a výsledcích kontrolních testů přiložen. Po obdržení výsledků všech testů, na jejichž základě je provedeno propuštění produktu, přiložen transparentní třetí obal téhož výrobce a definitivní označení produktu štítkem:

Tkáňová ústředna Fakultní nemocnice v Hradci Králové
Člen Evropské asociace tkáňových bank, Sokolská 534, 500 05 Hradec Králové

Alogenní dermoepidermální
kryokonzervovaný štěp
230 03 KU 001 07 435 cm^2

kód VZP: 43106
Dárce vyhovuje v předepsaných testech
DIAGNÓZA dárce S 06.2
DEKONTAMINOVÁNO 5% betadinem
KONZERVOVÁNO 10% DMSO
SKLADOVAT při teplotě -80°C a nižší
SPOTŘEBOVAT do 5.8.2004
Bakteriologický nález: ST. EPIDERMIDIS
Upozornění: ZEVNÍ OBAL NENÍ STERILIZOVÁN. ASEPTICKY MANIPULUJTE POUZE S OBSAHEM VNITŘNÍHO ZATAVENÉHO SÁČKU

Předepsaný způsob odběru vzorků a jejich zkoušení:
4 bakteriologické a 1 mykologický stěr z tkáně před dekontaminací.
4 bakteriologické a 1 mykologický stěr z tkáně po dekontaminaci a oplachu fyziologickým roztokem.
V obou případech provedeno aerobní a anaerobní bakteriologické a mykologické vyšetření podle přiloženého schématu – provádí ÚKM FN.
Odběr 10 ml oplachového fyziologického roztoku do sterilní zkumavky - bakteriologické a mykologické vyšetření.

Kvalitativní a kvantitativní požadavky včetně limitů:
Údaj o rozměrech štěpu.
Bakteriologický nález po dekontaminaci připouští výskyt Staphylococcus epidermidis nebo nepatogenních sporulujících mikrobů, negativní mykologický nález.
U obalu – sterilita zajištěna radiační sterilizací, namátkově prováděna kontrola předsterilizační zátěže v akreditované laboratoři – kopie certifikátu o akreditaci a vzor nálezu přiložen.

Podmínky skladování:
Teplota –80°C v mechanickém zmrazovacím zařízení s jištěním kapalným dusíkem.

Doba použitelnosti produktu:
2 roky.

Tkáňová ústředna Fakultní nemocnice Hradec Králové
Název pracoviště: Tkáňová banka
Specifikace č. 11/2004
Vypracoval: MUDr. Pavel Měřička
Platí od: 1.1.2004
Datum předpokládané revize: 1.1.2005

Název produktu: Alogenní štěp typu kost-šlacha-kost kryokonzervovaný

Kód VZP: 43110

Složení produktu:
Vazivový štěp připravený z ligamentum patellae s přilehlými kostními bloky. Způsob odběru aseptický - viz specifikace výchozího produktu. Z jednoho ligamenta se připraví 2 až 3 štěpy.

Popis štěpu a obalu:
Tkáň odebraná od zemřelého dárce, splňujícího kritéria způsobilosti podle vyhlášky MZ ČR č. 437/2002, mechanicky zpracovaná, dekontaminovaná 5% Betadinem a zabalená v prostorách třídy čistoty A. Jako kryoprotektivní roztok použit 10% dimetylsulfoxid v 6% roztoku dextranu s přidáním 5 % dvacetiprocentního lidského albuminu. Jeden obal obsahuje vždy 1 štěp s definovanou délkou šlachy. Jako transparentní obalový materiál použit dvojitý sáček Sterking, Wipak Medical, Finsko sterilizovaný gama zářením (Bioster Veverská Bítýška) - vzor protokolu o obdržené dávce a výsledcích kontrolních testů přiložen. Po obdržení výsledků všech testů, na jejichž základě je provedeno propuštění produktu, přiložen transparentní třetí obal téhož výrobce a definitivní označení produktu štítkem:

Tkáňová ústředna Fakultní nemocnice Hradec Králové
Člen Evropské asociace tkáňových bank, Sokolská 534, 500 05 Hradec Králové

Alogenní vazivový štěp
zmrazený (ligamentum patellae)
230 04 Lig 033 09 4,5 cm

Dárce tkáně vyhovuje v předepsaných testech
Kód VZP: 43110 DIAGNÓZA DÁRCE: I 63.0
DEKONTAMINOVÁNO 5% BETADINEM
KRYOKONZERVOVÁNO 10% DIMETYLSUFOXIDEM
BAKTERIOLOGICKÝ NÁLEZ: negativní
SKLADOVAT při teplotě -80°C a nižší
SPOTŘEBOVAT do 5.6.2005
Upozornění: ZEVNÍ OBAL NENÍ STERILIZOVÁN, ASEPTICKY MANIPULUJTE POUZE S OBSAHEM VNITŘNÍHO ZATAVENÉHO SÁČKU

Předepsaný způsob odběru vzorků a jejich zkoušení:
4 bakteriologické a 1 mykologický stěr z tkáně před dekontaminací.
4 bakteriologické a 1 mykologický stěr z tkáně po dekontaminaci a oplachu fyziologickým roztokem.
V obou případech provedeno aerobní a anaerobní bakteriologické a mykologické vyšetření podle přiloženého schématu – provádí ÚKM FN.
Odběr 10 ml oplachového fyziologického roztoku do sterilní zkumavky, vyšetření sterility metodou Ústavu Paula Ehrlicha, SRN – provádí ÚKM FN - schéma přiloženo.

Kvalitativní a kvantitativní požadavky včetně limitů:
Údaj o rozměrech štěpu.
Negativní bakteriologický nález, negativní mykologický nález, vyhovuje ve vyšetření na sterilitu.
U obalu – sterilita zajištěna radiační sterilizací, namátkově prováděna kontrola předsterilizační zátěže v akreditované laboratoři – kopie certifikátu o akreditaci a vzor nálezu přiložen.

Podmínky skladování:
Teplota –80°C v mechanickém zmrazovacím zařízení s jištěním kapalným dusíkem.

Doba použitelnosti produktu:
2 roky.

CHAPTER 2 - APPENDIX 2

STANDARD OPERATING PROCEDURES FOR TISSUE DONATION

2a Living donors

Tkáňová ústředna Fakultní nemocnice Hradec Králové
Název pracoviště: Odběrové centrum
Informovaný souhlas dárce č. 1/2004
Vypracovali: MUDr. M. Lutonský, Ph.D., MUDr. P. Měřička
Platí od 1.1.2004
Datum předpokládané revize: 1.1.2005

Ortopedická klinika Fakultní nemocnice Hradec Králové

Informovaný souhlas dárce

Potvrzuji, že jsem byl ošetřujícím lékařem informován, že hlavici kosti stehenní, která mi bude při operaci nahrazena endoprotézou, je možno použít pro léčbu jiných nemocných. Rovněž jsem byl seznámen se skutečností, že k tomu, aby přenos tkáně byl pro příjemce co nejbezpečnější nebo bylo možno ji dlouhodobě uchovávat v klinicky použitelném stavu, je v některých případech zapotřebí ji různými způsoby upravit, eventuálně vysterilizovat a použít ve formě tzv. bioimplantátu. Na základě těchto informací dávám svůj souhlas k takovému použití tkáně, která mi bude odstraněna. Rovněž dávám souhlas k opakovanému odběru krve na serologické vyšetření.

Jméno a podpis dárce...

Tkáňová ústředna Fakultní nemocnice Hradec Králové
Název pracoviště: Odběrové centrum
Informovaný souhlas dárce č. 2/2004
Vypracovali: MUDr. M. Lutonský, Ph.D., MUDr. P. Měřička
Platí od 1.1.2004
Datum předpokládané revize: 1.1.2005

Ortopedické oddělení Nemocnice s poliklinikou.................................

Informovaný souhlas dárce

Potvrzuji, že jsem byl ošetřujícím lékařem informován, že hlavici kosti stehenní, která mi bude při operaci nahrazena endoprotézou, je možno použít pro léčbu jiných nemocných. Rovněž jsem byl seznámen se skutečností, že k tomu, aby přenos tkáně byl pro příjemce co nejbezpečnější je zapotřebí provést serologické vyšetření z odebraného vzorku krve v době operace a za 6 měsíců po ní. Na základě těchto informací dávám svůj souhlas k použití tkáně, která mi bude odstraněna, k transplantaci. Rovněž dávám souhlas k opakovanému odběru krve na serologické vyšetření.

Jméno a podpis dárce...

Tkáňová ústředna Fakultní nemocnice Hradec Králové
Název pracoviště: Odběrové centrum
Informovaný souhlas dárce č. 3/2004
Vypracovali: MUDr. P. Kopecký, Ph.D., MUDr. P. Měřička
Platí od 1.1.2004
Datum předpokládané revize: 1.1.2005

Porodnická a gynekologická klinika Fakultní nemocnice Hradec Králové

Informovaný souhlas dárce

Potvrzuji, že jsem byla ošetřujícím lékařem informována, že součásti plodového lůžka, plodových obalů, pupečníkové cévy a pupečníková krev mohou být použity pro léčbu jiných nemocných. Rovněž jsem byla seznámena se skutečností, že k tomu, aby přenos buněk nebo tkáně, získané při porodu mého dítěte, byl pro příjemce co nejbezpečnější, je zapotřebí provést serologické vyšetření z odebraného vzorku krve v době porodu a za 6 měsíců po něm. Na základě těchto informací dávám svůj souhlas k použití buněk nebo tkání, které byly získány při porodu mého dítěte, k transplantaci. Rovněž dávám souhlas k opakovanému odběru krve na serologické vyšetření.

Jméno a podpis dárkyně...

Tkáňová ústředna Fakultní nemocnice Hradec Králové
Název pracoviště: Odběrové centrum
Informovaný souhlas dárce č. 4/2004
Vypracovali: MUDr. Z. Talábová, CSc., MUDr. P. Měřička
Platí od 1.1.2004
Datum předpokládané revize: 1.1.2005

Oddělení plastické chirurgie a popálenin, Chirurgická klinika, Fakultní nemocnice Hradec Králové

Informovaný souhlas dárce s použitím tkáně pro experimentální účel

Potvrzuji, že jsem byl ošetřujícím lékařem informován, že kůži, která mi bude odstraněna při esteticko-chirurgickém výkonu, je možno použít pro léčbu jiných nemocných nebo pro výzkum, který přispívá k rozvoji nových léčebných a diagnostických metod. Na základě těchto informací dávám svůj souhlas k takovému použití tkáně, která mi bude odstraněna. Rovněž dávám souhlas k opakovanému odběru krve na serologické vyšetření.

Jméno a podpis dárce..

Tkáňová ústředna Fakultní nemocnice Hradec Králové
Název pracoviště: Odběrové centrum
Informovaný souhlas dárce č. 5/2004
Vypracovali: MUDr. J. Pavlata, Ph.D., MUDr. P. Měřička
Platí od 1.1.2004
Datum předpokládané revize: 1.1.2005

Ortopedická klinika Fakultní nemocnice, 500 05 Hradec Králové

Informovaný souhlas s použitím tkáně pro experimentální účel

Vážený pane/paní .... ,

během Vaší operace Vám bude nahrazen kyčelní kloub protézou. Hlavice kosti kyčelní, kterou Vám odejmeme, by byla za normálních okolností znehodnocena. Moderní lékařské metody však umožňují, abychom část Vaší kosti a části chrupavky, které nejsou postiženy chorobnými změnami, uchovali a použili po patřičné úpravě pro léčbu nemocných, kteří trpí podobným onemocněním jako Vy. U kostní tkáně jsou příslušné metody již vypracovány a v lékařství běžně používány, u chrupavky je třeba použít složitých metod, při nichž jsou buňky chrupavky rozmnoženy ve zkumavce. Vypracováním takové metody se zabýváme na naší klinice. Prosíme Vás proto o souhlas s bezplatným darováním Vašich odňatých tkání pro léčebné a výzkumné účely. Dále Vás prosíme o souhlas s odběrem krve pro virologické vyšetření, které je nutné k tomu, aby Vaše tkáň mohla být použita pro jiného nemocného. Toto vyšetření bude provedeno před operací a poté za 180 dnů po Vaší operaci. Pokud s těmito podmínkami dárcovství a vyšetření souhlasíte, potvrďte souhlas svým podpisem.

S díky za spolupráci

Doc. MUDr. K. Urban, CSc.

Ortopedická klinika FN Hradec Králové

Jméno, příjmení: ....

Rodnéčíslo:
....

bydliště:
....

telefon: ....

datum .... podpis ....

2b Deceased donors

Tkáňová ústředna Fakultní nemocnice Hradec Králové
Název pracoviště: Odběrové centrum
Informace rodině zemřelého dárce č. 1/2004
Vypracovali: MUDr. P. Měřička, JUDr. L. Rožnovská
Platí od 1.1.2004
Datum předpokládané revize: 1.1.2005

Fakultní nemocnice Hradec Králové

Vážená paní,
Vážený pane,

jednou z moderních metod léčby řady onemocnění, která se provádí ve Fakultní nemocnici Hradec Králové, ale i v ostatních velkých nemocnicích v ČR, je transplantace tkání. Transplantací rohovky je možné navrátit zrak, transplantací kůže zachránit život těžce popálenému, transplantací kostí, šlach či jiných vazivových struktur léčit následky úrazů spojených se ztrátami tkání.

K transplantaci lze v některých případech použít tkáně odebrané od živého dárce, ve většině případů je však zapotřebí odebrat tkáně zemřelému dárci. Tyto odběry se v naší fakultní nemocnici rovněž provádějí.

V souladu se zákonem o darování, odběrech a transplantacích tkání a orgánů č. 285/2002 Sb. dle ustanovení § 15 jste byli zástupcem kliniky, kde je Váš blízký příbuzný, ústně informováni o eventuálním odběru tkání.

Dovolujeme si Vás ještě touto cestou požádat o podepsání tohoto dokumentu o podané informaci. Pokud je Vám známa informace, že Váš blízký příbuzný nesouhlasil s dárcovstvím tkání, prosíme Vás o laskavé sdělení této skutečnosti ošetřujícímu lékaři Vašeho blízkého.

Děkujeme Vám, že jste v této, pro Vás jistě nelehké chvíli, věnovali pozornost této informaci.

....
Přednosta příslušné kliniky

CHAPTER 2 - APPENDIX 3

STANDARD OPERATING PROCEDURE FOR PROCESSING OF CRYOPRESERVED TISSUE GRAFT

Tkáňová ústředna Fakultní nemocnice Hradec Králové
Název pracoviště: Tkáňová banka
Obecný standardní operační postup č. 3
Vypracoval: MUDr. P. Měřička
Platí od 1.1.2004
Datum plánované revize: 1.1.2005

Obecný standardní operační postup přípravy kryokonzervovaného tkáňového štěpu – varianta pro štěpy určené k implantaci

Informace rodině zemřelého dárce o možném odběru nebo informovaný souhlas živého dárce – ošetřující lékař příslušné kliniky

Aseptický odběr na odběrovém sále Tkáňové ústředny nebo na operačním sále

Sérologické vyšetření dárce podle Vyhláška MZ ČR č. 437/2002 (ÚKM a ÚKIA FN)

Vstupní mikrobiologická kontrola (ÚKM FN)

Uložení do sterilní skleněné nádoby nebo sterilního sáčku- skladování při teplotě +4°C

Dekontaminace 5 % Betadinem v prostředí s prokázanou třídou čistoty A s pozadím B (validace 1 x ročně)

Oplach fyziologickým roztokem

Inkubace se sterilním kryoprotektivním roztokem (10% dimetylsulfoxid v roztoku dextranu)

Výstupní mikrobiologická kontrola (ÚKM FN), 4 bakteriologické stěry a 1 mykologický stěr z každého štěpu, vyšetření na sterilitu (u štěpů určených k implantaci)

Vložení do radiačně sterilizovaného obalu Steriking Cover Bag

Zatavení, označení

Zmrazení na teplotu –80°C do doby získání výsledků všech testů – karanténa

Propuštění a definitivní označení

Skladování při teplotě –80°C do doby výdeje

Platnost pro specifikace č. 11/2004

Tkáňová ústředna Fakultní nemocnice Hradec Králové
Název pracoviště: Tkáňová banka
Obecný standardní operační postup č. 4
Vypracoval: MUDr. P. Měřička
Platí od 1.1.2004
Datum plánované revize: 1.1.2005

Obecný standardní operační postup přípravy kryokonzervovaného tkáňového štěpu – varianta pro kožní štěpy

Informace rodině zemřelého dárce o možném odběru nebo informovaný souhlas živého dárce – ošetřující lékař příslušné kliniky

Aseptický odběr na odběrovém sále Tkáňové ústředny nebo na operačním sále

Sérologické vyšetření dárce podle Vyhláška MZ ČR č. 437/2002 (ÚKM a ÚKIA FN)

Vstupní mikrobiologická kontrola (ÚKM FN)

Uložení do sterilní skleněné nádoby nebo sterilního sáčku - skladování při teplotě +4°C

Dekontaminace 5 % Betadinem po dobu 20 minut u alogenních štěpů a následná dekontaminace v roztoku antibiotik u xenogenních dermoepidermálních štěpů

Oplach fyziologickým roztokem

Inkubace se sterilním kryoprotektivním roztokem (10% dimetylsulfoxid v roztoku dextranu u alogenních, 15% glycerol v roztoku dextranu u xenogenních dermoepidermálních štěpů)

Výstupní mikrobiologická kontrola (ÚKM FN), 4 bakteriologické stěry a 1 mykologický stěr z každého štěpu

Vložení do radiačně sterilizovaného obalu Steriking Cover Bag

Zatavení, označení

Zmrazení na teplotu –80°C do doby získání výsledků všech testů – karanténa

Propuštění a definitivní označení

Skladování při teplotě –80°C do doby výdeje

Platnost pro specifikace č. 07/2004, 08/2004

CHAPTER 2 - APPENDIX 4

STANDARD OPERATING PROCEDURES FOR BACTERIOLOGICAL CONTROL

Standardní operační postup pro mikrobiologickou kontrolu tkáňových štěpů – provádí ÚKM FN Hradec Králové

Pomnožení v kapalných půdách

2 bakteriologické stěry
thioglykolátová půda +36°C: 1 stěr aerobní podmínky 24 hodin
1 stěr anaerobní podmínky 48 hodin

2 bakteriologické stěry
thioglykolátová půda +21°C: 1 stěr aerobní podmínky 24 hodin
1 stěr anaerobní podmínky 48 hodin
1 mykologický stěr
kapalná Sabouraudova půda - aerobní podmínky 24 hodin

<u>Vyočkování na pevné půdy</u>
Bakteriologické kultury
krevní agar aerobní podmínky +36°C 24 hodin
anaerobní podmínky +36°C 48 hodin

Mykologická kultura
Sabouraudův agar, aerobní podmínky +36°C 48 hodin

Standard operating procedure for sterility testing.

Schéma 2

Standardní operační postup pro testy sterility – metodika Ústavu Paula Ehrlicha, SRN – verze pro solidní tkáňové štěpy - provádí ÚKM FN Hradec Králové

- odběr 10 ml kryokonzervačního roztoku z vaku se štěpem do sterilní zkumavky
- vzorky o objemu 5 ml jsou vloženy do automatického systému pro hemokultury s použitím certifikovaných kultivačních půd.
- 1 vzorek za aerobních podmínek 37°C 7 dnů.
- 1 vzorek za anaerobních podmínek 37°C 7 dnů.
- jakýkoliv růst je považován za pozitivní výsledek testu

CHAPTER 2 - APPENDIX 5

TRACEABILITY ASSURANCE

a) LABELS USED FOR SOLID TISSUE GRAFTS

Tkáňová ústředna Fakultní nemocnice v Hradci Králové

Člen Evropské asociace tkáňových bank, Sokolská 534, 500 05 Hradec Králové

Allogenní dermoepidermální štěp kryokonzervovaný

230 99 KU 008 02 18 x 5 cm

kód VZP: 43024

Dárce tkáně vyhovuje v předepsaných testech

DIAGNOSA DÁRCE: I 71.2

DEKONTAMINOVÁNO 5% betadinem

KONZERVOVÁNO 10% roztokem dimetylsulfoxidu v mediu MEM a 6% roztoku dextranu

SKLADOVAT při teplotě –80°C a nižší

SPOTŘEBOVAT do 1.1.2002

Bakteriologický nález: NEGATIVNÍ

Upozornění: ZEVNÍ OBAL NENÍ STERILIZOVÁN. ASEPTICKY MANIPULUJTE POUZE S OBSAHEM VNITŘNÍHO ZATAVENÉHO SÁČKU

B) LABELS USED FOR HAEMATOPOIETIC CELL GRAFTS

AUTOLOGOUS BONE MARROW TRANSPLANTATION

Tissue Bank Faculty Hospital Hradec Králové
C 2089-01 0A - 0Q
BM Collection date: 10/7/2001
Donor/Recipient
Novák Josef
520705/050

AUTOTRANSPLANT

BG B Rh(D) pos
100 ml
Cryopreservation date: 10/7/2001
Store at temperature -80°C or below

AUTOLOGOUS PERIPHERAL BLOOD PROGENITOR CELL-TRANSPLANTATION

Tissue Bank Faculty Hospital Hradec Králové
C 2090-01 0A - 0C
PBPC Collection date: 10/7/2001
Donor/Recipient
Novák Jaroslav
530705/050

AUTOTRANSPLANT

BG A Rh(D) pos
100 ml
Cryopreservation date: 10/7/2001
Store at temperature -80°C or below

ALLOGENEIC PERIPHERAL BLOOD PROGENITOR CELL-TRANSPLANTATION

Tissue Bank Faculty Hospital Hradec Králové
C 2095-01 0A - 0C
PBPC Collection date: 12/7/2001
Donor: Nováková Anna
555701/2254
Recipient:Nováková Jaroslava
555224/250

ALLOTRANSPLANT
BG Donor: A Rh(D) pos
BG Recipient: B Rh(D) pos
100 ml
Cryopreservation date: 12/7/2001
Store at temperature -80°C or below

CORD BLOOD

CZCB-HK-00052
Cord blood
Bag A, vol. 100 ml
Collection date: 9/5/2002
Cryopreservation date: 9/5/2002

NEWSVIEW Friday DECEMBER 21st, 2001

A daily in-depth look at current events in the Czech Republic.

[December 20th] [December 19th] [December 18th] [December 17th]
[December 14th] [December 13th] [December 12th] [December 11th] [December 10th]

Czechs exporting body parts?

To hear the following story in Real Audio, click here:

Streaming RA / RA Download

The field of medical research has developed substantially since the fall of Communism, but in some areas, it seems, the law hasn't been able to keep up. Claims were recently made in the press that several Czech hospitals were exploiting a legal loophole that allows them to harvest human tissue without the patient's consent - which is legal - but then sell it abroad - for profit - which is not illegal, but not very ethical either. Radio Prague's ***Nicole Klement*** *has more.*

For the last decade the Czech Republic has been struggling to update and amend laws dealing with scientific research. A new law on transplants is still being discussed in parliament, and the papers claimed this week that several Czech hospitals and a private export firm have been using a legal loophole to create a market where human body tissue is harvested and sold abroad for profit. By law hospitals are allowed to remove tissue from dead patients without the patient's or their relatives' consent. But the current law doesn't explicitly ban or regulate the sale of human tissue.

Pavel Meriska is the head of the tissue bank at Charles University's affiliated hospital in the town of Hradec

Kralove.

"There are two systems in the world concerning transplant legislation. First is the so called opting-in system where the donor must say yes I would like to be a donor after my death and this is expressed by a donor card. The other system is called opting-out and it is typical for Austria and Belgium and it is based on the fact that someone can say that they would not like to become a donor after death. With this system the person would have to put themselves on a computerised list of people rejecting donation. This is the system that is prepared in the new transplantation law in the Czech Republic."

But the new law has not yet passed through the Upper House. So hospitals are left to work with the old one, which is not exact enough to cover all aspects of donor rights.

"We have a very old guidelines that says that the patient is a potential donor unless they write a letter that says otherwise. But in my 20 years of practice I have never seen such a letter. So, in my practice I accept any opposition to autopsy or harvesting that is expressed by the family."

Human tissue from the Czech Republic is quite sought after, because testing of body parts is above international standards, and post mortems are performed on 80% of bodies. The figure is far lower abroad - across the border in Austria, autopsies are performed in only one death in ten.

Many doctors say selling body parts for profit is simply unethical. The deceased patient has not usually given their consent, and - of course - there is money involved. It remains to be seen what will remain of this unusual trade once the new transplant law has been passed.

Related links:

NEW SAFETY ASSURANCE FOR BIOLOGICAL SKIN COVERS

Měřička P.[1], *Straková H.*[2], *Čermák P.*[2], *Štěpánová V.*[1], *Hradecký Z.*[3], *Drahošová M.*[1]

[1]Tissue Bank,
[2]Department of Clinical Microbiology,
[3]Department of Clinical Immunology and Allergology,
Faculty Hospital Hradec Králové, Czech Republic

SUMMARY

The described system of safety assurance of cryopreserved allogeneic and xenogeneic dermoepidermal transplants comprises serological examination of deceased tissue donors, long-term storage of sera of all donors, microbiological control of prepared allogeneic and xenogeneic dermoepidermal grafts, labelling of released tissue grafts and monitoring of temperatures inside the mechanical freezer (-80 °C). From a total number of 76 donors from whom tissues were collected for transplantation during 1999-2001, tissues were discarded in two instances. One because of a positive HBsAg test, the others second one because of presence of anti-HTLV antibodies. In xenogeneic dermoepidermal grafts, out of a total number of 1,203 grafts prepared during the same period, 84 (6,9 %) were discarded because of the presence of pathogeneic or potentially pathogeneic microbes. The system of labelling released grafts makes unequivocal identification of the pathway from recipient to donor possible, while at the same time respecting the anonymous character of the donor's data. In xenogeneic grafts it ensures the identification of the appropriate batch. Storage of cryopreserved biological skin covers at a temperature of -80 °C in low temperature cabinets with emergency back-up cooling with liquid nitrogen and a supplementary source of electric power, proved very useful. The system responds to the gradual implementation of the principles of Quality Management System ISO 9000 and Good Manufacturing Practice into the activities of tissue banks. Further tightening of the demands for the safety of allo- and xeno- transplantation is foreseen in conjunction with the occurrence of transmissible spongiform encephalopathy and porcine retroviruses.

ZUSAMMENFASSUNG

Neues System der Sicherung von Hautdeckungen.

Měřička P., Straková H., Čermák P., Štěpánová V., Hradecký Z., Drahošová M.

Das beschriebene System der Sicherung von kryokonservierten allogenen und xenogenen dermoepidermalen Transplantate umfasst die serologische Untersuchung der verstorbenen Gewebespender, langfristige Aufbewarung von Spenderseren, die mikrobiologische Untersuchung der allogenen und xenogenen dermoepidermalen Transplantate, Markierung von freigegebenen Gewebekonserven und Monitorüberwachung der Temperatur innerhalb des mechanischen Gefriergerätes (-80 °C). In den Jahren 1999-2001 wurden Gewebe zum Transplantationszweck insgesamt von 76 Spendern abgenommen. Aus dieser Gesamtzahl wurden die Gewebe von zwei Spender ausgeschlossen, im ersten Fall aufgrund eines positiven HBsAg Tests, im zweiten Fall wegen des Befundes der HTLV Antikörper. Von den 1203 xenogenen dermoepidermalen Transplantaten wurden wegen des Befundes der pathogenen, oder potentiell pathogenen Mikroben 84, d. h. 6,98 % der Konserven ausgeschlossen. Das System der Markierung von freigegebenen Gewebekonserven ermöglicht eindeutige Identifizierung des Weges vom Spender bis zum Empfänger, bei gleichzeitiger Anonymitäthaltung der Spenderangaben. Bei xenogenen Transplantaten ermöglicht es die Identifizierung entsprechender Charge. Die Aufbewahrung von kryokonservierten Hautdeckungen bei einer Temperatur -80 °C im Gerät mit einer Unfallnachkühlung mittels flüssiges Stickstoffes und einer alternativen Stromquelle hat sich vollstästandig bewährt. Unseres System reflektiert das stufenweise Einführen von ISO 9000 Normen und sachgerechten Herstellungsverfahren in die Gewebebanken. Wir sehen daher eine nachfolgende Verschärfung der Sicherungsforderungen im Zusammenhang mit dem Vorkommen der übertragbaren spongiförmigen Enzephalopathie und der schweinischen Retroviren voraus.

Key words: biological skin covers, treatment of burns, cryopreservation, safety of tissue transplantations, serological control of tissue donors

The problem of safety assurance for cell and tissue transplantations in the Czech Republic as well as abroad is very urgent. At present, recommendations are elaborated on this issue are being made at a pan-European level as well as in individual countries. At the beginning of 2001, all member countries of the Council of Europe (EC) received extensive documentation called "Safety and Quality Assurance for Organs, Tissues and Cells" with request for comments. This material

was produced by a group of EC specialists (Group EC specialists, 2001). Further to a review of the comments, it is expected that this document will become the general recommendation for EC member countries. At the XXIIIrd Congress of the Transplantation Society held in Rome in 2000, a request was also made to draw up European Union regulations governing this problem. To demonstrate an example of a conceptual solution within the framework of one country, we may use the guidelines of the British Ministry of Health's guidelines - "A Code of Practice for Tissue Banks Providing Tissues of Human Origin for Therapeutic Purposes" which is based on the principles of Quality Management System ISO 9000 and Good Manufacturing Practice (GMP) (Ministry of Health, United Kingdom, 2001). The principles of quality management according to ISO 9000 standards can be found in Fiala's publication (Fiala et al., 2000). The principles of Good Manufacturing and Distribution Practice were published in the SUKL Newsletter (Pospíšilová, 1998). In the Czech Republic (CR), the draft of the Transplantation Act (Ministry of Health CR, 2001) has already passed through its first reading in Parliament. In addition, individual decrees of the Ministry of Health (MofH) that should include the problem of tissue transplantation safety.

The problem of safety assurance for tissue transplantations is very broad and includes several specific issues such as setting the criteria for donor screening and testing, examination of the collected and processed tissues, producing standard operating procedures (SOP), establishing a system for tracking the tissue from donor to recipient, developing a system for monitoring physical parameters during low temperature preservation, quality control of solutions, medical devices and chemicals used, continuous training of the staff and regular testing of quality of the environment (Pospíšilová, 1998). In the submitted paper, attention will be focused only on particular issues:

- serological examination of tissue donors
- microbiological control of prepared allogeneic and xenogeneic grafts
- ensuring traceability of the tissue from donor to recipient
- monitoring physical parameters during storage at subzero temperatures.

METHODS AND RESULTS

Serological examination of donors.

As a basic panel for donor testing that was introduced in the eighties, the authors considered tests for anti-HIV 1 and 2, HBsAg and syphilis (Měřička et al., 1990, Měřička et al., 1991). Gradually the panel's terms of reference was extended to include anti-HCV tests (Měřička et al., 1996-97) and from 2000 by HIV 1 Ag (Měřička and Klein, 2000), anti-HTLV I and II, and anti-CMV IgG and IgM. During the last three years the authors have used third generation tests, i.e. Elisa (Murex Diagnostics ABBOTT Ax Sym, EIA). For detection of the HIV antigen, they use a fourth generation test (bio-Rad Laboratories). For the diagnosis of syphilis they use the TPHA reaction (Welcosyph, Welcome, Great Britain) and RRR (Immutrep, RPR, Omega Diagnostics, Great Britain). Indications for exclusion of potential donors and/or the discarding of collected tissues are positive tests for anti-HIV, HTLV and HCV antibodies, evidence of HBsAg, as well as positive results for syphilis. A positive result for anti-CMV IgM antibodies is indicated on the cover of the graft by a biohazard label (Fig. 1).

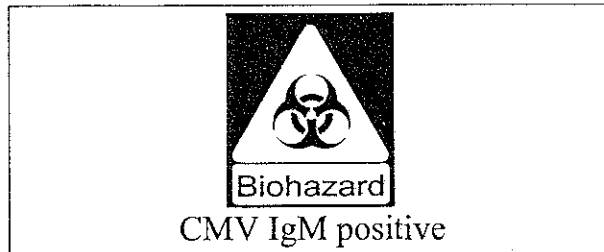


Fig. 1. Biohazard label used in case of positive tests for CMV

In living donors the tests are made at the time of collection and after a 6-month quarantine period. The tests are arranged by the attending physician for the patient. In dead donors with confirmed cerebral death, the blood sample is obtained during the interval between confirmation of brain death of the donor using angiography and discontinuation of the circulation during organ collection. The blood collection and tests are arranged by the transplantation co-ordinator in the Regional Transplantation Centre at the Department of Urology of the Faculty Hospital Hradec Králové. In tissue donors who died from cardiac arrest, blood is collected during the tissue harvest by means of a catheter inserted into the vena cava inferior via the dissected vena saphena magna or vena femoralis. Contrary to the practice in the eighties, all blood sera are frozen and stored for an unspecified period of time. Criteria for the exclusion of donors during the past three years are given in Table 1.

Table 1. Causes of discard of tissues collected from donors in 1999-2001

year	total no. of donors	positive test	no. of donors whose tissues were discarded
1999	28		0
2000	26	HBsAg, HTLV	2
2001	22		0
total	76	HBsAg, HTLV	2

Bacteriological control of prepared grafts.

Bacteriological examination of collected and processed tissues also underwent some technical

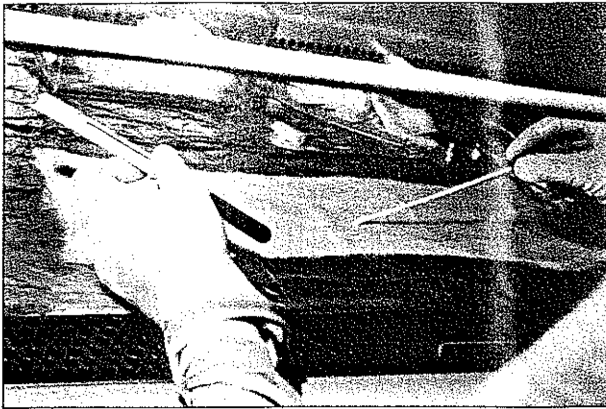


Fig. 2a. Preparation of bacteriological smears from a xenogeneic dermoepidermal graft

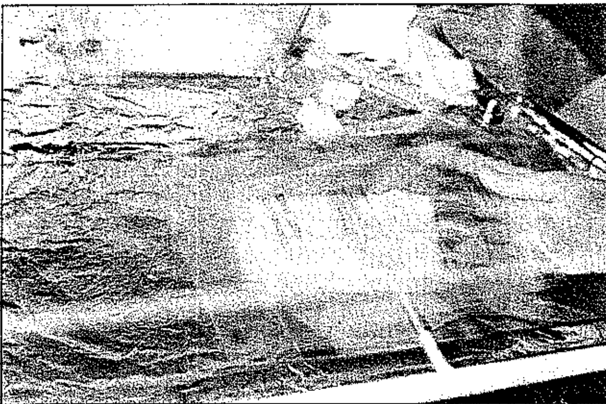


Fig. 2b. Packing the same graft into a plastic cover sterilised by irradiation. Both operations are performed in a laminar flow equipment.

changes. When selecting the method, we always used as a basis the pharmacopeial requirement of cultivation under aerobic and anaerobic conditions at two different temperatures (ČSL 4, 1987, ČL 97,1997). Since 1998 the authors have used a standard procedure that is illustrated in diagram. From each graft four smears are made for bacteriological examination (Transsystem Amies, Copan, Brescia, Italy) and one smear for mycological examination (Fungiquick-abouraud medium, Copan, Brescia, Italy) (Fig. 2a, b). Contrary to the common practice of some 10 years ago (Měřička et al., 1990, Měřička et al., 1991), the period of propagation in liquid media under anaerobic conditions has been extended from 24 to 48 hours. The indication for graft discard is the finding of any bacteriological contamination except Staphylococcus epidermidis. The results of the output bacteriological control in dermoepidermal grafts are presented in Tables 2 and 3.

Table 2. Results of bacteriological control in allogeneic dermoepidermal grafts

year	no. of grafts	negative	with St. epidermidis
1998	7	4	3
1999	2	0	2
2000	4	4	0
total	13	8	5

Table 3. Results of bacteriological control of xenogeneic dermoepidermal grafts

year	no. of grafts	no. of grafts discarded	% of eliminated
1999	455	24	5
2000	433	16	4
before Aug. 1 2001	315	44	14
total	1203	84	6.98

DIAGRAM - STANDARD OPERATING PROCEDURE OF BACTERIOLOGICAL CONTROL

1) **Propagation in liquid media**
a) 2 bacteriological smears
thioglycolate medium +36 °C
- 1 smear aerobic conditions 24 h.
- 1 smear anaerobic conditions 48 h

b) 2 bacteriological smears
thioglycolate medium +21 °C
- 1 smear aerobic conditions 24 h
- 1 smear anaerobic conditions 48 h

c) 1 mycological smear
liquid Sabouraud's medium - aerobic conditions 24 h

2) **Inoculation on solid media**
a) bacteriological cultures
blood agar aerobic conditions +36 °C 24 h.
anaerobic conditions +36 °C 48 h.
b) mycological culture
Sabouraud's agar, aerobic conditions +36 °C 48 h

Traceability assurance

The demand for traceability of the pathway of the tissue from donor to recipient while respecting the confidential character of the donor's data is another issue that must be resolved by collaboration between the tissue bank and the clinical department. The authors use a system that was developed as a result of a modification of the labelling standard of released transfusion preparations (MofH CR, 1997). Each graft is labelled by an identification mark, which starts with the code 230 allotted to the authors department by the

Tissue Bank Faculty Hospital Hradec Králové
Member of the European Association of Tissue Banks, Sokolská 534, 500 05 Hradec Králové

Allogeneic dermoepidermal cryopreserved graft
230 99 KU 008 02 18x5 cm
VZP code: 43024

Tissue donor meets prescribed tests
Donor's diagnosis: I 71.2
Decontaminated with 5 % betadine
Preserved with 10 % solution of dimethylsulphoxide in medium MEM and 6 % dextran solution
Store at temperature -80 °C or below
Use before Jan. 1, 2002
Bacteriological finding: negative
Attention: The outer cover is not sterilised. Handle aseptically only contents of the inner sealed bag

Fig. 3. Label used for allogeneic dermoepidermal grafts.

Tissue Bank Faculty Hospital Hradec Králové
Member of the European Association of Tissue Banks, Sokolská 534, 500 05 Hradec Králové

Xenogeneic dermoepidermal cryopreserved graft
230 01 KUE 283 15 50x7 cm

VZP code: 43027

Decontaminated with 5 % betadine and solution containig chloramphenicol, streptomycin and furantoin

Preserved with 15 % glycerol solution in 6 % dextran

Store at -80 °C or below

Use before: Aug. 2, 2002

Bacteriological finding: Staphylococcus epidermidis

Attention: The external cover is not sterilised. Handle aseptically only contents of the inner sealed bag

Fig. 4. Label used for xenogeneic dermoepidermal grafts.

General Health Insurance Co. (VZP, 1998), and the year of graft preparation. This mark also includes a description of the graft and the identification number of the donor during the particular year (Fig. 3). The label also contains the international code of the donor's diagnosis, a statement on the negative results of serological examinations of the donor and the result of the bacteriological examination of the graft. There are also data on the method of preservation and /or decontamination of the tissue used, and instructions for handling the graft before use.

In xenogeneic grafts, instead of the number of the donor there is the number of the batch (Fig. 4) in the particular year.

The labels are added to the allogeneic or xenogeneic graft in the protective, i.e. third outer cover, only after completing the output control (Fig. 5). The authors recommend to clinical colleagues that these labels be stuck into the recipient's clinical documentation.

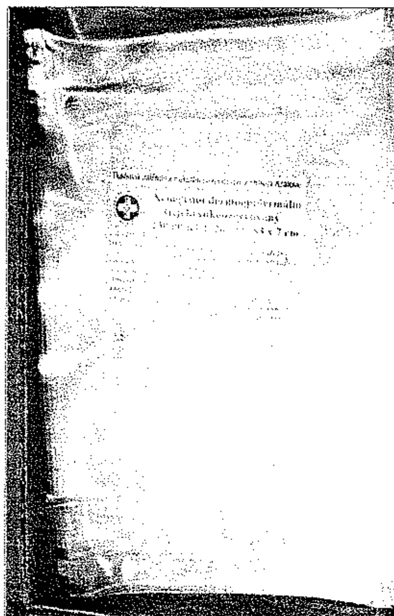
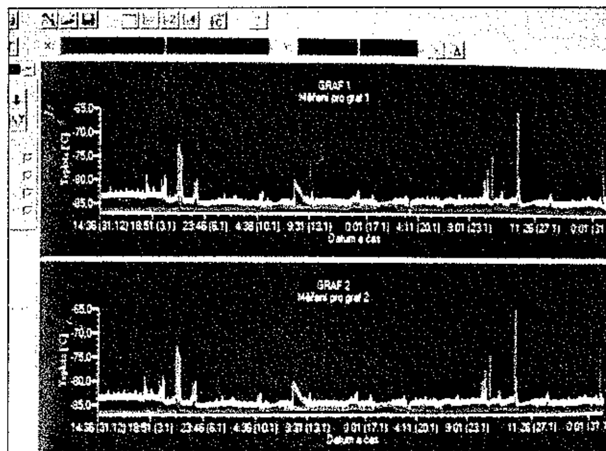


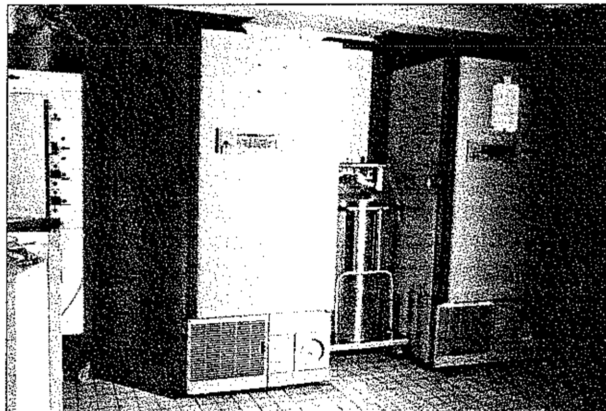
Fig. 5. Xenogeneic dermo- epidermal grafts prepared to be released for clinical use

Follow-up of physical parameters during tissue storage.

In similar fashion to the practice carried out by the Skin Bank of the Department of Burn Medicine at the Faculty Hospital, Královské Vinohrady, Prague, the authors also prepare cryopreserved covers (Praus et al., 1980, Böhm and Dvořák, 1981, Moserová and Houšková, 1989, Königová, 1990, Měřička et al., 1995, Měřička et al., 1998). In xenogeneic grafts they use glycerol as the cryoprotective agent, while in allogeneic grafts dimethylsulphoxide is used. Contrary to the practice employed by the Prague department, the authors prefer storage in mechanical freezers. Initially, they used equipment that reached a temperature of -70 ° to -75 °C (Měřička et al., 1987, Klein et al., 1988), and later used equipment that reached a temperature of -80 °C connected with an emergency back-up cooling with liquid nitrogen (Měřička et al., 1995, Měřička et al., 1998). The equipment has an acoustic alarm. Dealing with possible emergencies and moving



6a)



6b)

Fig. 6a. Temperature record of four low temperature freezers used for storage of biological material. The temperature inside the equipment used for the storage of tissue grafts is recorded in yellow - equipment in Fig. 6b on the right.

the material to other equipment is up to the staff on duty.

The temperature record made by the monitoring system Digiterm using the programmes Netcom and Read 95 (ReguCon, Ltd, Prague), with temperature recording at 5-minute intervals, indicates that the temperature inside the equipment is below -80 °C; the monitoring system is able to record the rise of temperature every time the door is opened (Fig. 6). In addition to the temperature inside the equipment, the external temperature and relative humidity of the air is also continually recorded.

DISCUSSION AND CONCLUSIONS

From the presented data it may be concluded that the described elements of our system of safety assurance meet the demands stipulated by international standards. The spectrum of serological tests made in donors is even wider than described in the FDA guidelines (FDA, 1997). The small number of donors from whom tissues are discarded because of positive serological finding not detected during hospitalization is ascribed by the authors to the exclusion investigation of the clinical documentation before tissue collection and the exclusion of all potential donors with suspected contact with infectious diseases (case-history of hepatitis, dialysis treatment). Clinically unsuspected HBsAg positivity in a deceased donor is no surprise. This was already encountered in the past (Měřička et al., 1990). Similarly as the finding of anti-HCV antibodies (Měřička et al., 1991, Měřička et al., 1996-97). The finding of positive anti-HTLV antibodies very shortly after the introduction of this test into our practice was a surprise. No haematological disease was not found either in the case-history of the donor nor in his necropsy, although a positive test result in the presence of the disease is described in the literature (Yamamoto and Hinuma, 1999). To date, the authors have not encountered positive tests for syphilis as a cause for discarding tissues. With regard to safety assurance of transplants, and also to rule out suspicion that an infectious disease developed in the recipient was caused by tissue transplantation, the authors feel that it is important to store the sera for an unlimited period. These stored sera can also be retested in the event that the testing method undergoes marked changes and some grafts from a given donor have not yet been released for clinical use.

No allogeneic dermoepidermal graft was discarded as a result of the final bacteriological output control. However, the group is too small for general conclusions. The discard percentage in xenogeneic dermoepidermal grafts is still considered acceptable and it is within the limits defined during validation of the method in 1998 (mean discard rate 10.13 % with a confidence limit for P 0.95 of 3.45 to 16.8 %).

The authors ascribe great importance to the new way of labelling, which unequivocally identifies the route from tissue donor to its recipient, so-called traceability. This labelling system is used for all tissue grafts provided by our department to different clinical branches. Furthermore, it is useful from the practical aspect that the final external cover with the inserted label is attached to the graft only after the completing the output control. The cover of grafts for clinical use is thus unequivocally different from the cover of grafts kept in quarantine up to the time that the final result of all bacteriological tests is known, i.e. cca 14-21 days after tissue collection.

Our experience with temperature monitoring inside and outside mechanical mechanical freezers revealed that the freezing equipment is able to keep the set temperature regardless of the marked fluctuation in external temperature in the course of a year. The temperature in the non-airconditioned room where the freezers are situated, occasionally ranged above +30 °C (Měřička et al., 2000). The temperature of -80 °C used nowadays for storage for dermoepidermal grafts is successfully used in our country and worldwide for the storage of vital cells for clinical transplantations, e.g. concentrates of haematopoietic cells (Bláha et al., Makino et al., 1991, Wats et al., 1998, Měřička et al. 2000). Standards of the American Red Cross Tissue Service (Campagnari and O Malley, 1994) report that it is possible to store cryopreserved dermoepidermal grafts at a temperature of -60 to -100 °C for a period of 2 years, a period not usual in common practice. The monitoring of the temperature with electronic recording has fully replaced the former system used in our department in the seventies and eighties which was based on mechanically registering the temperature.

In the near future we will also have to accept further requirements associated with the safety of tissue grafts and tissue transplantations. In allogeneic tissues, this is above all a question of preventing the transmission of spongiform encephalopathy, either as classical Creutzfeldt-Jakob disease (CJD) or its new variant described in conjunction with bovine spongiform encephalopathy (BSE) (Prichard, 1987, Starke and Mohring, 1987, Hogan et al., 1999, FDA, 1999). So far, no serological test is known for detecting this disease in man, and it is recommended that histological examination of the brain of dead donors is used for detection (FDA, 1999). This examination has been performed for the authors since the beginning of 2001 by Fingerland's Institute of Pathology of the University Hospital Hradec Králové.

More intense supervision of the sources of xenogeneic tissue is also associated with the BSE epidemic. Although this disease has not been demonstrated in pigs, possible transmission to pigs cannot be ruled out completely. As to other possible infectious agents potentially transmissi-

ble from pigs to man, retroviruses have been discussed recently using the abbreviation PERV (porcine endogenous retroviruses) (Cunningham et al., 2001, Herring et al., 2001, Tacke et al., 2001). Possible transmission is assumed to be a risk, however, it is more likely to occur in cases of classical transplantation where the circulation of the graft is connected to that of the recipient and not in the case of skin covers where vascularization should not occur. In any case, however, these facts will increase the demands on veterinary controls of slaughtered animals. Another requirement in our time will most probably be the storage of serum or tissue specimens of every animal for possible later testing.

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Oznámení

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I. KONGRES NEMOCÍ Z POVOLÁNÍ S MEZINÁRODNÍ ÚČASTÍ,

který se uskuteční ve dnech 15.–16. 11. 2002 v přednáškovém sále Společenského domu v Luhačovicích.

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THE IMPORTANCE OF STORED SUPPLIES OF BIOLOGICAL SKIN COVERS IN TERRITORIAL MANAGEMENT OF MASS BURN CASUALTIES

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SUMMARY

This study provides supportive evidence of a possible role played by planning supplies of biological covers needed in fire disaster, based on the experience of the authors. The major steps to be taken are these: 1. Providing a technically and technologically adequate base for collection and long-term storage of cells and tissues ready for use in case of catastrophe. 2. Developing a method for estimating the amount of reserved tissue grafts. 3. Solution of logistical problems associated with providing supplies for operating theatres treating disaster casualties. 4. Organisation of national and international network of graft exchange capable of supporting local skin banks in times of need. In contrast to the situation in the 1970s and 1980s, nowadays the Czech Republic can deliver any required amount of biological covers without having to face technological difficulties. The idea of collaborating in the management of a fire disaster emerged from experience gained by the authors during the Bashkir disaster in 1989, relating particularly to an inadequate reserve stock of skin grafts and the impossibility of increasing their production. Intensified demands for the safety of grafts and recent experience from the US emphasise the need for an immediate conceptual solution concerning production of reserves of biological covers that should be ready for transportation to wherever needed. Another urgent necessity is the establishment of conditions enabling effective international collaboration at a disaster event.

ZUSAMMENFASSUNG

Ist die Reservebildung der biologischen Hautdeckungen wichtig für Sicherstellung der Verbranntenbetreuung im Falle unerwarteter Umstände?

Měřička P., Hošek F.

Die Autore rekapitulieren eigene Erfahrungen mit der Planung der Verwendung von biologischen Hautdeckungen im Falle massenhaftes Aufkommen von Verbrannten. Die Hauptsaufgaben solcher Planung sind: 1. Die Bildung einer adäquaten technischen und technologischen Grundlage für die Abnahme und langfristige Bewahrung der Zellen und Gewebe, die im Falle einer Katastrophe benutzt werden können. 2. Entwicklung einer Methode zur Abschätzung der Reserven von Gewebepropfen. 3. Die Lösung logistischer Probleme, vornehmlich der Versorgung von chirurgischen Arbeitsstätte, die an der Folgenbeseitigung einer Katastrophe teilhaben. 4. Organisierung eines nationalen und internationalen Austauschnetzes der Propfen, die die lokale Gewebebanke im Falle einer Katastrophe unterstützen können. Die Sicherstellung der zureichenden Reserven der biologischen Hautdeckungen stellt in der Tschechischen Republik zur Zeit kein technologisches Problem dar. Eine Idee von Eingliederung eigener Arbeitsstätte in die Lösung einer katastrophenartigen Situation ging von Erfahrungen der Autore während der Katastrophe in Bashkir im 1998 aus. In Hinsicht auf höhere Forderungen nach der Sicherheit der Hautdeckungen und gleichzeitige Erkenntnisse aus der USA, ein Konzept für Bildung der Reserven von Hautdeckungen ist von großer Bedeutung. Andere wichtige Aufgabe ist eine effektive internationale Kooperation in den Katastrophenfälle.

Key words: fire disaster, reserves of biological skin covers, allotransplantation of skin, xenotransplantation of skin, skin bank

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The availability of an adequate store of temporary skin covers to meet the needs of a mass burn disaster is of utmost importance (Gunn, 1992, Masellis et Gunn, 1995). Moreover, the store should contain a good supply of both allogenic and xenogenic biological covers (Masellis et Gunn, 1995, Klein, 2000). For example, Tosinska-Okroj et al. (1994–1995) described the use of glycerolized allogenic dermo-epidermal grafts from the Euroskin Bank at Beverwijk (Holland) (van Barre1994) in treating mass bum casualties injured at a concert in the area of the Gdansk shipyard (320 casualties).

Recently, similar grafts have been used in treating victims of the terrorist attack on the World Trade Center in New York on September

11, 2001. These grafts (hypothermal or deep freeze conservation) were obtained from the American Association of Tissue Banks (AATB, 2001).

We witnessed the use of both lyophilized xenotransplants (preparation Suiderm, Bioveta, Terezín, Czech Republic) and chemically preserved, radiation-sterilised xenotransplants prepared by a local skin bank (Laboratory for a production of transplants, Alloplast, Ufa, SSR) (Měřička et al.,1990, 1995) during the Bashkir train gas-pipeline disaster (gas explosion with 700 victims) (Herndon, 1990, Kulyapin et al., 1990). Despite considering the use of either allogenic dermo-epidermal grafts or cultured keratinocytes, neither were used at this occasion.

All these facts justify contemporary programmes emphasising the necessity of setting up stores of tissue grafts – which in fact existed in the past in the Czech Republic. Within the framework of these programmes financed by the former Federal Ministry of National Defense, numerous methods were developed, e.g. cryo-conservation of allogenic dermoepidermal grafts (Böhm et Dvořák, 1981), xenogenic dermo-epidermal grafts (Praus, Böhm, Dvořák, 1980, Moserová et Houšková, 1989), lyophilisation of xenogenic dermo-epidermal grafts (preparation Suiderm produced by Bioveta, Terezín), lyophilisation of allogenic and chorioamniotic grafts (Klen et Skalská, 1976). After the break-up of the Czechoslovak Federation, financing of these programmes by the army was stopped completely, and at present all services of tissue banks are paid for by health insurance companies. This system is not interested in supporting the idea of storing biological material; on the contrary, it restricts it.

According to the available literature, a similar situation seems to occur all over the world except in Israel, where skin banks store supplies of deep-frozen allotransplants with an expiration time of 5 years (assumed duration of graft viability). At the end of this time grafts are given to the clinic for use, and stocks are replenished regularly.

OUR APPROACH TO A SOLUTION OF THE PROBLEM

Since the late 1980s systematic attention has been given to the role of tissue banks in a fire disaster. Our ideas and experience have repeatedly been discussed at various occasions (Congress of European Burn Association, Prague 1989, Second International Conference: The Management of Burns and Fire Disasters: Perspectives 2000, Palermo, 1992) and also published (Měřička et al. (1990), Měřička et al. (1995)).

A textbook for military physicians called „Preparation and Use of Biological Covers in the Treatment of Burns in War Surgery and Disaster Medicine“ was published by Klein, Měřička et al. (2000) at the Military Medical Academy of Jan Evangelista Purkyně in Hradec Králové. Our objectives were to familiarise the wider, professional public with these specific problems. The textbook deals only with classical biological covers and not with cultured skin substitutes (keratinocytes).

In the early 1990s we formulated 4 conditions as guidelines for tissue banks in the event of fire disaster (Měřička et al., 1995):

1) establishing an adequate technical and technological base for collection and long-term storage of cells and tissues to be ready for use in case of disaster

2) designing methods capable of assessing volume of stored grafts

3) solving logistic problems associated with supplies for operating theatres in disaster areas

4) organising exchange of grafts on a national and international scale in support of local skin banks exposed to disastrous conditions

METHODS OF COLLECTING AND PROCESSING SKIN GRAFTS

In contrast to technologies used in the 1970s in processing xenogenic and allogenic dermo-epidermal grafts (storage in liquid phase or nitrogen vapours) (Praus et al.,1980, Böhm et Dvořák, 1981), our present practice includes sampling with a dermatome, deposition in plastic covers and dry-freeze storage at a maximum temperature of -80 °C (Měřička et al, 1995, 2001, 2002).

Using a dermatome enables standardisation of samples, the use of a uniform lyophilisation technology for both xeno- and allogenic dermo-epidermal grafts and subsequent sterilisation with gamma radiation (Měřička et al., 2000, 2001). Recently sterilisation with gamma radiation has been replaced by sterilisation with ethylene oxide or the so-called plasma (hydrogen peroxide).

The method of sampling with a Humby knife (blade) is justified under field conditions. We used it in preparing xenogenic dermo-epidermal grafts at the Ufa laboratory after the disaster in 1989, using an emergency set of sterilised tools which we always carry with us during a disastrous situation (Měřička et al.,1995) (Figs. 1,1a). Grafts were processed aseptically in a laminar box and preserved in Petri dishes at +4° C. They served to complete the amount of grafts produced by the Ufa laboratory on a Czech-made machine identical to that used in producing Suiderm. Chemical conservation and sterilisation was followed by gamma radiation. These grafts were used mainly at the burn casualty centre, Ufa. Total production of biological cover material was 30 m^2. We learned from this catastrophe that providing there is an effective organisation of work, production of grafts may be increased to meet demands for temporary covers of necrotomies even under exceptional conditions.

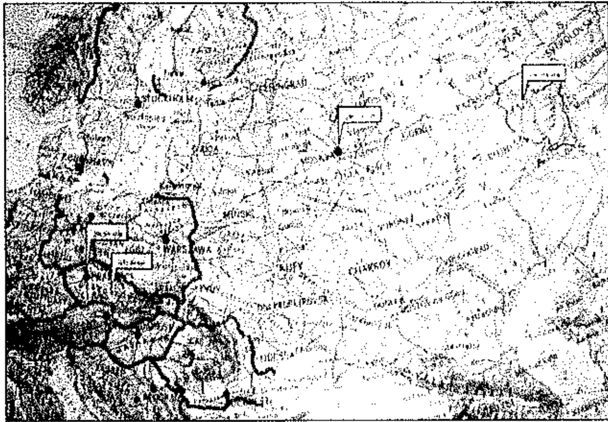

Fig. 1a. Map of road to Ufa, with dates

Fig. 1. Collection of a xenogenic dermo-epidermal graft performed by the author in the laboratory for production of transplants, Ufa, June 1989. Further processing of grafts in laminate box (left corner) for use in the Republican Children Hospital, Ufa

METHOD OF CALCULATING THE VOLUME OF RESERVES

In assessing anticipated amount of stored grafts and necessary quantity of stored material there are two issues to be resolved:

1. identifying average consumption of covers per casualty depending on degree of injury and pertinent confidential intervals. Our own values (Měřička, Klein et al, 1995, Měřička et al. 2000) indicated that we needed approximately between 6 and 11 m^2 of xenotransplants for 10 seriously injured adult patients.

2. identifying the current and security reserves of a tissue bank. Here we may well use our method designed for identifying pharmacy stocks (Měřička, Klein et al., 1995, Měřička et al. 2000). We distinguished between current stock, changing at regular intervals (e.g., by one graft collection of pig butt a week), and reserve stock, consisting of grafts deposited permanently in the bank and issued only in case of emergency or close to expiration time (see e.g. Israel tissue banks). The volume of reserve stock can be calculated with equation $S_1 = K.s$ whereby S = standard deviation of demands, K differs in agreement with chosen deficit of probability e.g., K = 3 stands for probability deficit 0.001. Equation 2 may be used in calculating current stock: $C_b = C \cdot t : T$ whereby C = number of days within supply cycle, t = turn-over within period under consideration T = number of days covered by pertinent period.

Establishment of reserves may clearly be influenced by individual technologies. In contrast to the situation in the 1990s, when grafts were conserved in Petri dishes and stored in mechanical freezing equipment, and the volume of emergency stores was 2.5 m^2 at maximum, our present technologies (using space-saving plastic bags for storage in deep-freezer) have almost doubled the volume of stored material (4 m^2).

Collaboration between Skin Banks on both National and International Level

Consideration should be given to the establishment of a national or international network of skin banks for graft exchange capable of supporting local skin banks or burn casualty centres under conditions of fire disaster.

Table 1. Size of biological skin covers issued in 1996 by skin banks in the Czech Republic as compared with size of skin covers issued by the Euroskin Bank (Beverwijk, Holland).

Area covered by issued grafts in cm^2			
Tissue bank	Xenogenic dermoepidermal graft	Allogenic dermoepidermal graft	Culture keratinocyte
Prague – Vinohrady	1 972 200	0	6 450
Brno – Bohunice	0	0	10 455
Hradec Králové	121 526	745	770
CZ total	2 093 726	745	17 675
Euroskin Bank		1 500 000	

Data from report of committee for collection and transplantation of tissue, Český transplant and yearbook of Bioimplant Service, Leiden, Holland 1997.

Table 1 presents information about the size of biological covers issued for clinical use in the Czech Republic in 1996 (Czech transplant 1996, Měřička and Klein, 2000). Data is missing from the Ostrava centre, which did not have an independent skin bank at that time and is focussing its activities on preparing allogenic dermo-epidermal grafts, using for conservation a method designed by the Euroskin Bank (conservation in concentrated glycerol at +4 °C). The size of issued grafts compares well with Euroskin Bank production in Beverwijk, Holland (Bioimplant, Service 1997). The table shows the number of skin covers delivered to clinics. It does not present informa-

tion about the reaction of individual teams to demands for increased production of skin covers under exceptional circumstances.

Table 2. Possibility of increasing delivery of xenogenic dermo-epidermal grafts from skin tissue centre, Medical School Hospital Hradec Králové under exceptional conditions.

Number of heavy burn casualties	Issue from reserve stock (m^2)	Required no. of standard collections	No. of weekly collections (calendar week)
5	4	1	1
10	4	4	2 or 4
15	4	8	4
20	4	12	6

Table 2 presents information about both the number of burn casualties supplied with skin covers from our own reserves and an increase in production. As can be seen coping with up to 10 heavy burn casualties is not difficult. We were able to prepare simultaneously grafts for 5 heavy casualties. On the other hand, coping simultaneously with 20 heavy cases may just be beyond our capacity.

TRANSPORTATION OF BIOLOGICAL SKIN COVERS

Exceptional situations may require collaboration with all Czech tissue banks in preparing skin covers for transportation to individual burns centres. Hypothermally-preserved grafts may be transported in polystyrene or other boxes used for transporting blood, using simple ice or ice in plastic boxes as a cooling medium. Hypothermal conditions are suitable for transporting both allogenic and xenogenic dermo-epidermal grafts. Transportation of deep frozen grafts is more demanding, because the temperature inside the transport box should not rise much above -80 °C. This requires perfect heat-insulating cover with either solid carbon dioxide or liquid nitrogen as a cooling medium. Fig 2 (2, 2a) shows the type of box suitable for transporting dermo-epidermal grafts on dry ice. We use either cars or the services of Czech Railways for transportation.

If we were to request international help in case of a catastrophe, we should be prepared to return it. This involves securing long distance transportation of grafts. For civil transportation abroad, we employ authorized removal companies which are also licensed for air transport by IATA. We should make sure that the company has a license for carrying dry ice to the country of destination. Generally, very few companies possess such a license to countries outside the EU. In this case, a classic cooling medium such as frozen saline solution would have to be used – which is what we did last year to transport tissue grafts to the Ukraine (Figs 3, 3a). A great disadvantage of this cooling medium is its considerable weight. Another aspect to be remembered is the system of transportation. DHL places different consignments in the same transport, while World Courier guarantees individual transport. In both cases, the routes are generally far from direct, i.e. from door to door. For instance, DHL transports a consignment from Hradec Králové to Bratislava by way of Prague, Brussels and Cologne. Some companies take full responsibility for temperature conditions inside the consignment by using data loggers. Table 3 surveys transportation times. It may be advisable to complete each graft with serum from its donor, owing to rules observed by several countries which require repeated serological tests at their own laboratories. Frozen sera may be transported in cryovials.

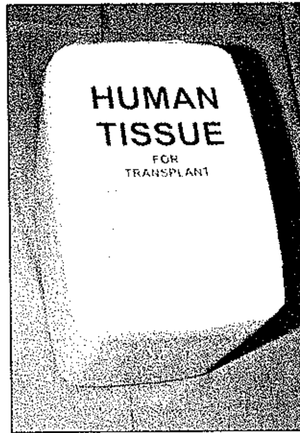

Fig. 2a.

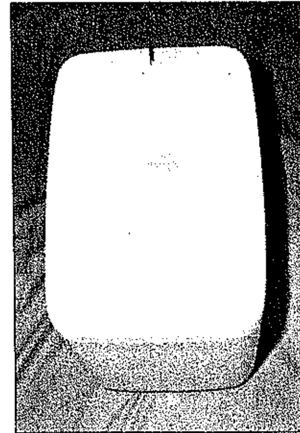

Fig. 2b.

Figs 2a, 2b. Polystyrene box with dry ice for air transport of allogenic dermo-epidermal grafts to Berlin. Cooling medium – 6 kg dry ice

Fluid nitrogen has to be transported by air in „dry shippers“, which are Dewar vessels filled with porous material that soaks up liquid nitrogen. Owing to their bottle-necked shape, this type of vessel is suitable for transporting smaller, cylindrically shaped samples.

The easiest material for transportation are lyophilised grafts at room temperature. Therefore, they ought to be made part of the equipment carried by a rescue team.

DISCUSSION AND CONCLUSIONS

Our description of using biological covers in case of disaster demonstrates differences between various countries. While allotransplants conserved either hypothermically or by deep-freeze are in common use in the States (Hansbrough, 1992), western European countries conserve allo-

Table 3. Examples of air transport of frozen tissue grafts from Tissue Centre, Medical School Hospital, Hradec Králové to other countries.

Destination	Transporter	Cooling medium	Transport Time	Tissue type
Bratislava	DHL	Dry ice	24 hrs	Allogenic fascial graft
Berlin	DHL	Dry ice	24 hrs	Allogenic dermoepidermal graft
Ivanofrankovsk	DHL	Frozen saline sol.	96 hrs	Autologous bone graft

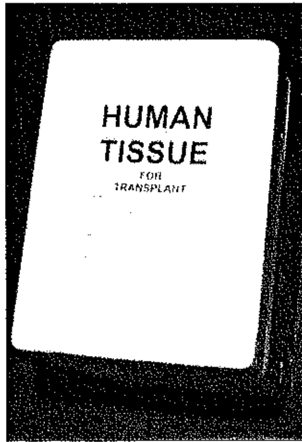

Fig. 3a.

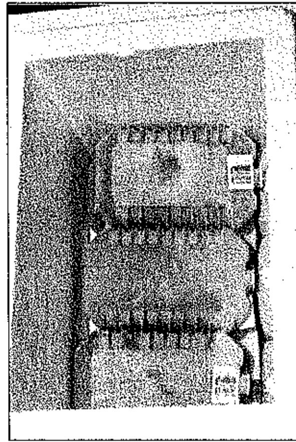

Fig. 3b.

Figs 3a, 3b. Large polystyrene box (60 x 40 x 50 cm) plastic bricks filled with concentrated saline solution pre-cooled to -80 degree C, used for transporting autologous bone graft for replantation (reconstruction of calva after neuro-surgery in Hradec Králové) Ukrainian patient. Weight of consignment more than 20 kg, size of inside wrapping of graft 20x32x3cm.

transplants in concentrated glycerol (van Baare, 1994), and the Czech Republic uses either heat- or cryoconserved xenotransplants (Moserová et Housková, 1989, Königová, 1989, Klein, 1989, 1995). Although a small quantity of allotransplants is stored in skin banks of Ostrava, Brno, Hradec Králové, this amount would hardly be enough to cover the requirements of a fire disaster, and therefore production would have to be increased considerably. Experience from the Bashkir disaster also indicates the possibility of using chemically conserved and lyophilized xenotransplants. When comparing the amount of xenotransplants issued in the Czech Republic with that of allotransplants prepared by the Euroskin Bank (Table 1), it has to be remembered that in contrast to allotransplants, xenotransplants generally require repeated application. The amount of allotransplants prepared by the Euroskin Bank is substantially lower than that prepared in the US and Canada over an approximately identical period. Kagan (1998) reports that production of 44 skin banks existing in these countries in 1995, issued 8 732 880 cm^2 of hypothermically or cryo-conserved allogenic dermo-epidermal grafts. However, just 20 of these skin banks were accredited at that time to the American Association of Skin Banks.

Calculation of consumption of biological covers per 10 patients depends naturally on methods and procedures employed in practice (these may be either radical or conservative). Under circumstances of an extensive catastrophe, conservative procedures will evidently be preferred although this means a potential increase in demands for biological covers.

All published data including those concerning the attack on September 11 (Tosinska Okroj, 1994–1995, Měřička et al., 1990, 1995, AATB, 2001) indicate that efforts to produce reserves or increase production of skin grafts in skin banks are focused on types of covers that are currently used in practice.

Our idea about participating in the management of an exceptional situation was based on the fact that we possessed a small reserve of cryoconserved xenotransplants and were ready to increase production of biological covers whenever needed (Měřička et al.,1995, Měřička et Klein, 2000). We are not certain that our approach is still justified and whether it would not be more appropriate to try to increase reserves of biological materials by involving in their production several institutions of the Czech Republic, so as to be ready for increased demand in the event of a fire disaster. An increase in transplant production at the time of an outbreak of disaster may be impeded by the availability of auxiliary services at, for instance, sterilisation plants or control laboratories – which are available solely to clinical institutions in case of an extensive disaster. Another argument would favour stricter safety measures and subsequently a protraction of the technological cycle of graft production (Měřička et al., 2002). In addition, we cannot be certain that all grafts produced will answer to the criteria of microbiological control.

Apart from increasing reserve stocks for which technical conditions are available, an exceptional situation requires absolutely perfect collaboration among tissue banks. A good example is the reaction of American tissue banks to the September disaster (AATB, 2001). Following the attack, the AATB immediately set up a special service calling upon all accredited banks to announce the amount of grafts they had available for dispatch. The first call was answered immedi-

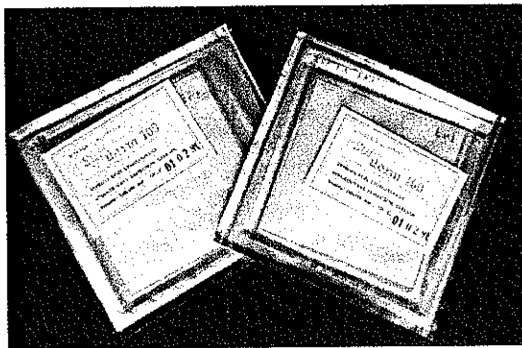

Fig. 4a.

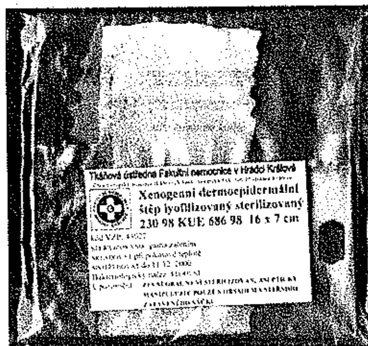

Fig. 4b.

Figs 4a, 4b. Lyophilised xenogenic dermo-epidermal grafts sterilised with gamma radiation. Prototype of Suiderm, Bioveta, Terezín, Czech Republic (Fig. 4a). Graft prepared in 1998 by Tissue Centre, Medical School Hospital, Hradec Králové (Fig. 4b).

ately by 20 banks offering a total of 1,500 square feet of allotransplants (approx. 1,383,545 cm^2, roughly equalling the annual production of the Euroskin Bank in 1997) (Table 1). The New York Center received biological covers from skin banks in geographically remote areas (Ohio, Virginia, Texas) indicating a considerable mobility in dispatching reserve stocks. It is this mobility that should be given utmost attention in designing plans for coping with disastrous situations. In our opinion, air transport of grafts on dry ice is an easier alternative than in vapours of liquid nitrogen (dry shippers).

The least suitable alternative appears to be transportation in pre-cooled bricks filled with concentrated saline solution. However, if there was no other alternative and the distance to be covered is great, temperature conditions inside the bricks should always be validated in advance in order to avoid an increase in temperature above the permitted limit.

When considering setting up a reserve stock of skin covers, we should not forget to include lyophilised grafts as a possibility worth considering. Although production of Suiderm was stopped some time ago, we are familiar with the know-how involved (Figs. 4, 4a). Their transportation is very easy, but the clinic has certain reservations, such as inadequate adherence to excised skin, a worse barrier function than that of vital grafts. In addition, they are ineffective in preventing microbial growth (Pruitt et Levine, 1984, Kagan, 1998).

Recent experience in the States has shown that safety rules of transplants are strictly observed even under the most exceptional conditions. This was demonstrated by the fact that only accredited skin banks were asked for help (these offered more skin covers than requested- AATB, 2001). This example underlines the importance of a uniform system of accreditation under exceptional conditions. The situation in Europe is quite different. Transportation of biological covers from one country to another is impeded by differences in legislation betwen individual countries or even an absence of individual rules ensuring safety of tissue transplants. A change may be brought about by introducing and accepting uniform EU directives concerned with high-quality standards and safety measures to be observed in collecting, testing, preparing, storing and distributing human cells and tissues for clinical transplantation. Negotiations are under way, and among various suggestions is one for the establishment of a network of skin banks accredited throughout Europe.

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C7

PREVENTION OF INFECTION TRANSMISSION IN LOW-TEMPERATURE PRESERVATION AND STORAGE OF BIOLOGICAL MATERIALS BELOW -80°C

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ABSTRACT

The potential risk of infection transmission during storage was evaluated in 40 patients and 5 donors undergoing collection of haemopoietic progenitor cells for transplantation. Serological tests included proofs of retroviruses, hepatitis viruses, herpesviruses, syphilis and toxoplasmosis. Acute infection requiring treatment was found in 2 patients. Laboratory signs of active infection with CMV were detected in 6, with VZV in 2, with HSV in 2 and with toxoplasmosis in 1 patient. Although contamination during storage in liquid nitrogen described by Tedder and Hawkins was apparently caused by technical failures, our results showed that the infection rate in patients undergoing collections was relatively high. As the prophylactic measure we established quarantining of all cryopreserved concentrates in the vapour phase of liquid nitrogen until the results of all tests were completed. Only bags from patients without any signs of acute infection can be stored in the liquid phase of nitrogen.

INTRODUCTION

We demonstrate use of different temperatures for storage of haematopoietic progenitor cells for clinical transplantation with regard to potential risk of infection transmission. Makino *et al.* (1991) described, that the hematopoietic recovery after transplantation remained almost unchanged if the concentrates were stored for 5-18 months at the temperature of –80°C in mechanical freezers. We use storage at this temperature in many cases of autologous or allogeneic transplantations as the interval between collection of progenitor cell concentrates and transplantation does not usually exceed one year in our practice (Měřička *et al.*, 1999, Měřička *et al.*, 2000). Some authors described even 5 year successful storage of progenitor cells at -80°C (Katayama *et al.*, 1997), however a common practice is to choose vapour or liquid phase of nitrogen if storage for years is needed. The liquid phase of nitrogen can be recommended especially if storage for decades is required. This can happen if the patient's own progenitor cells are used as so called back-up, ready for use in cases of graft failure in allogeneic progenitor cell transplantations or in storage of cord blood for unrelated transplantation in paediatric haematology (Gluckmann *et al.*, 1993, Kobylka, 1997, Měřička *et al.*, 1999). Storage of biological materials in the liquid phase of nitrogen is, however, considered hazardous due to the possibility of contamination among damaged bags with the stored material.The liquid serves as a medium for dissemination of infectious agent released from damaged bags in this case. Hepatitis B transmission by this mechjanism was exactly proven by

Tedder *et al.* (1995) and Hawkins *et al.* (1996). The possibility of contamination can be lowered both by technical measures, e.g. elimination of use of low quality plastic bags (Tedder *et al.*, 1995), as well as by meticulous testing of patients and donors undergoing progenitor cell collections for signs of acute infection diseases (Serke and Johnsen, 2001). Secondary contamination with bacteria can be prevented by using laminar flow equipment for processing of concentrates before cryopreservation. In this paper we deal only with prevention of contamination caused by infection of donor detectable by serological tests. To evaluate the potential risk of infection transmission the groups of 40 patients and 5 donors treated at the Dept. of Haematology and Paediatric Haematology of the University Hospital Hradec Králové were screened according to common standards of the European Group for Blood and Marrow Transplantation (EBMT) and the International Society for Hemotherapy and Graft Engineering - Europe (ISHAGE-Europe).

1. METHODS

Blood samples were taken in donors and patients in the first day of collection. In most cases the peripheral blood progenitor cell concentrate was obtained by single or repeated haemaphereses by the separator Cobe Spectra in the Haemapheresis Unit of the Department of Clinical Haematology (Bláha *et al.*, 2001, Měřička *et al.*, 1999). Bone marrow was obtained under general anaesthesia by aspiration from iliac bone at the Department of Orthopaedic Surgery using a bone marrow collection kit (Baxter Healthcare Corporation, Fenwal Division, U.S.A.) (Měřička et al.1999).

1.1. The panel of serological tests used

Serological tests were performed at the Department of Clinical Microbiology and Department of Clinical Immunology and Alergology of the University Hospital, Hradec Králové. The panel of tests included the proof of following infections: retroviruses (HIV, HTLV), hepatitis (A,B,C) and herpesviruses: Epstein-Baar virus, cytomegalovirus (CMV), varicella-zoster virus (VZV), herpes simplex virus (HSV). Tests for syphilis and toxoplasmosis were performed as well. The results of tests indicating severe infections (HIV, HBsAg, HCV) were available at the day of collection, the results of other tests within one week.

1.2. Processing and cryopreservation conditions

Processing of collected concentrates was made in the laminar flow equipment (Herasafe, Heraeus, FRG) with the validated air quality grade A. Collected concentrates were mixed with an equal volume of 20% dimethylsulphoxide (B.Braun,Medical,France)diluted in 6% dextran precooled to +4°C. Human serum albumin was added to the final concetration of 1%. The suspension was frozen in 100 ml plastic disposable bags (Cryocyte TM, Baxter, Fenwal Div., U.S.A.), that were heat sealed and closed in metal cassettes. The cassettes were frozen in the Kryo-10 programmable freezer (Planer Biomed, Sunbury on Thames, England). Freezing rate was adjusted to -1K/min till -90°C, -5°C/min till -150°C. Storage in -80°C in the mechanical freezer with emergency back-up cooling system (REVCO, U.S.A.) or in vapour or liquid phase of nitrogen (container BK 200 A, Ferox Děčín, Czech Republic) followed (Měřička et al 1999,Měřička et al 2000). All bags were stored closed in metal cassettes until thawing at the patient's bedside.

2. RESULTS

Acute infection at the time of collection, that required treatment at the Department of Infection Diseases was proved in 2 cases (1 varicella, 1 toxoplasmosis). In the case of varicella in 11-year old boy the frozen concentrate was discarded at the time of the new successful collection

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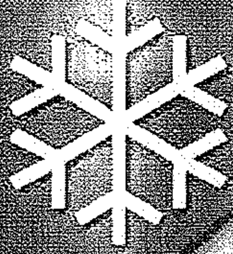

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Date & Time: Thursday, August 21, 2003
3:30 pm - 5:30 pm - Technical Sessions

Session Chair: Dr. Yaguang Luo, USDA-ARS, Produce Quality and Sa
Laboratory, PSI, USA

Paper Title: **Our System of Cross-Contamination Prevention Storage of Haematopoietic Progenitor Cells**

Paper Number: ICR0302

Author(s): Pavel Mericka , Blaha Vavra , V. Stephanova

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ICR0302

OUR SYSTEM OF CROSS-CONTAMINATION PREVENTION DURING STORAGE OF HAEMATOPOIETIC PROGENITOR CELLS

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ABSTRACT

The possibility of cross-contamination among damaged plastic bags stored in the liquid phase of nitrogen and containing cells for clinical application represents an urgent issue after publication of Tedder´s paper describing transmission of hepatitis B via this mechanism. The preventive measure applied by us include: 1) Meticulous testing of all patients and donors. 2) Bacteriological testing of collected concentrates. 3) Decision on safe method of storage (liquid or vapour phase of nitrogen or mechanical freezer) on basis of the result of the serological tests. 4) Use of high-quality plastic disposable bags and external metal cover preventing mechanical damage during storage Our study performed in 93 patients and donors showed that the potential source of cross contamination is a relatively high active virus infection, herpesviruses being the most common finding. Bacterial contamination occurred only in 0.42% of 479 bags tested for sterility and represents a negligible risk in our centre.

INTRODUCTION

Safety of cell and tissue transplantation including transplantation of haematopoietic progenitor cells of different origin (bone marrow, peripheral blood, cord blood) has become an urgent issue due to large-scale application of the method in the modern medicine. Many governmental and non-governmental organisations have been involved in preparing standards or guidelines that should increase safety of this medical procedure. The Council of Europe (EC) issued last year an extensive material called "Safety and Quality Assurance for Organs, Tissues and Cells" that should become the general recommendation for the EC member countries. As an example of a conceptual solution within the framework of one country, we may use the guidelines of the British Ministry of Health: A Code of Practice for Tissue Banks Providing Tissues of Human Origin for Therapeutic Purposes, based on the principles of Quality Management System ISO 9000 and good manufacturing practice. The haematopoietic tissue banks in the Czech Republic including ours are using now the common standards of EBMT and ISHAGE Europe as basis for elaboration their own quality assurance programmes. The transplantation centre of the Department of Haematology of the 2nd Department of Internal Medicine of the University Hospital Hradec Králové and co-operating tissue bank have more than 15-year experience in cryopreservation of haematopoietic progenitor cells (Bláha et al.1990, Mericka et al., 1991). Our experimental, laboratory and clinical experience was presented in the IIR congresses in the Hague (Mericka et al., 1995) and Sydney (Mericka et al, 1999), in the IIR conference Cryogenics in Prague (Mericka,. et al 2000) as well as at the C 1- Commission meeting in Hradec Králové, Czech Republic in 2002 . For storage of cryopreserved cell concentrates we have been used plastic disposable bags since 1991, when they replaced stainless steel containers of own design used since 1986 (Bláha et al., 1990, Mericka et al., 1991, Mericka et al., 1995). The possibility of cross-contamination among damaged plastic bags stored in the liquid phase of nitrogen and containing cells for clinical application has become an urgent issue after publication of Tedder´s paper (Tedder et al. 1995),describing transmission of hepatitis B via this mechanism. This issue was described in cryopreservation of donor red blood cells, nevertheless in cryopreservation of haematopoietic progenitor cells the issue is likely to be more urgent as the cells are often collected in non-healthy persons. Our tissue centre was elaborating system of prophylactic measures for several years and this system has been introduced now into current practice. The system described in this paper includes: 1) Meticulous serological testing of all patients and donors. 2) Microbiological testing of collected concentrates for presence of bacteria and fungi. 3) Decision on safe method of

storage (liquid or vapour phase of nitrogen or mechanical freezer) on basis of the result of the serological tests. 4) Use of high-quality plastic disposable bags and external metal cover preventing mechanical damage during storage.

1 METHODS

The study evaluating the potential risk of infection transmission was performed on the group of 71 patients and 22 donors undergoing autologous or allogeneic transplantations. The panel of serological tests recommended by standards of EBMT and ISHAGE–Europe was used.

1.1 Collection of haematopoietic progenitor cells

In most cases the peripheral blood progenitor cell concentrate was obtained by single or repeated haemaphereses by the separator Cobe Spectra in the Haemapheresis Unit of the Department of Clinical Haematology (Bláha et al., 2001, Mericka et al., 1999). Bone marrow was obtained under general anaesthesia by aspiration from iliac bone at the Department of Orthopaedic Surgery using a bone marrow collection kit (Baxter Healthcare Corporation, Fenwal Division, U.S.A.) (Mericka et al., 1999). The blood sample collections for serological testing were performed in the date of bone marrow harvest or in the first day of peripheral progenitor cell harvest. The donors were all clinically healthy persons (Table 1). The patients suffered from different haematology conditions (Table 2).

Table 1: Haematopoietic progenitor cell collections in donors

	Number
Related donors	20
Unrelated donors	2
Bone marrow harvest	1
Peripheral progenitor cell harvest	21
Cryopreservation performed	20
Preservation at room temperature	2
Total number of donors	22

Table 2: Group of patients

	Number
Non-Hodgkin lymphoma	20
Hodgkin lymphoma	8
Multiple myeloma	30
Acute myeloid leukaemia	10
Other conditions	3
Total number of patients	71

In cases of unrelated transplantations, where collections were performed abroad, the tests were repeated at reinfusions.

1.2 The panel of serological tests used

Serological tests were performed at the Department of Clinical Microbiology and Department of Clinical Immunology and Alergology of the University Hospital, Hradec Králové. The panel of tests included the proof of following infections: retroviruses (HIV, HTLV), hepatitis (A,B,C) and herpes viruses: Epstein-Barr virus,

cytomegalovirus (CMV), varicella-zoster virus (VZV), herpes simplex virus (HSV). The tests and methods used for virus detection are listed in the Table 3.

Table 3: Test and methods used for virus detection

Group of viruses	Tests - serology markers	Method
Retroviruses		
HIV 1,2	anti-HIV 1,2 HIV 1 Ag	EIA
HTLV I,II	anti-HTLV I,II	EIA
Hepatitis viruses		
HAV	anti-HAV, anti-HAV IgM	EIA
HBV	HBsAg anti-HBc, anti-HBc IgM	EIA EIA
HCV	anti-HCV	EIA
Herpes viruses		
VZV	anti-VZV IgG, IgM	EIA
HSV 1,2	anti-HSV 1,2 IgG, IgM	EIA
CMV	anti-CMV IgG, IgM	EIA
EBV	anti-VCA IgG, IgM	EIA

Tests for syphilis and toxoplasmosis were performed as well (Table 4). The results of tests indicating severe infections (HIV, HBsAg, HCV) were available at the day of collection, the results of other tests within one week.

Table 4: Tests and methods used for detection of toxoplasmosis and syphilis

	Tests	Method
Syphilis	RRR TPHA	TPHA Welcosyph RRR Immutrep, RPR
Toxoplasmosis	anti-toxo IgG, IgM	EIA Toxoplasma IgG EIA Toxoplasma IgM

1.3 Processing and cryopreservation conditions

Collected concentrates were mixed with an equal volume of 20% dimethylsulphoxide (B.Braun Medical, France) diluted in 6% dextran precooled to +4°C. Human serum albumin was added to the final concentration of 1%. The suspension was frozen in 100-ml plastic disposable bags (Cryocyte TM, Baxter, Fenwal Div., U.S.A.), that were heat sealed and closed in metal cassettes with a window for viewing the label of the bag. This label assures the traceability through all processing steps as well as clear identification during storage and it is put into the patient's documentation after reinfusion of the concentrate. Each collection is given a specific number. Our label template used for bone marrow is demonstrated:

AUTOLOGOUS BONE MARROW
TRANSPLANTATION
Tissue Bank Faculty Hospital Hradec Králové
C 2089-01 0A - 0Q
BM Collection date: 10/7/2001
Donor/Recipient
Novák Josef

The cassettes were frozen in the Kryo-10 programmable freezer (Planer Biomed, Sunbury on Thames, England). Freezing rate is adjusted to −1K/min till −90°C, -5°C/min till −150°C. Storage conditions are described below.

1.4 Sterility testing of progenitor cell concentrates

Processing of the concentrate was performed in the laminar flow equipment (Herasafe, Heraues, FRG) with the validated grade A placed in the environment with undefined grade. Sealing of the bag is performed inside this equipment using a sealing machine Hematron (Baxter, U.S.A.). The samples for microbiological control, performed at the Dept. of Microbiology, are collected from the collection bag and all cryopreservation bags before freezing (after mixing with the cryoprotectant solution) and at the bedside of the patient from each thawed bag. The sterility testing method according to the Ehrlich Institute (FRG) is used. Standard operating procedure for testing sterility of cryopreservation media is the following: Any growth is regarded as positive result of the test and particular bag is discarded.

Paul Ehrlich Institute, FRG

2 ml of the collected sample of the cryopreservation medium are placed to the sterile jar and supplemented with sterile physiological saline solution to the volume of 10 ml.
5-ml samples are taken, for culturing in automatic hemoculture system using certified culture media
1 in aerobic conditions, 37°C, 7 days
1 in anaerobic conditions, 37°C, 7 days

1.5 Storage conditions

The frozen concentrates are stored in mechanical freezers, vapour phase or liquid phase of nitrogen under well-defined and continuously monitored temperature conditions. Monitoring of temperature inside mechanical freezers (Revco Ultima II, U.S.A.) adjusted to -85°C is assured by the system Digiterm with programmes Netcom and Read 95, (ReguCon Prague Ltd.), that records data in 5-minute intervals. The uncertainty of temperature measurement is 0.3°C . For monitoring temperature inside liquid nitrogen storage tanks XLC 1200 (Chart, U.S.A., Planer Biomed, U.K.) the system Tec2000 with records in 30-minute intervals is used. The tanks are filled automatically from the Cryocyl 230 LP (Chart, U.S.A., Planer Biomed, U.K.) container, connected with the tubing with an external liquid nitrogen container (Aga Cryo, AB Sweden) containing 6,000 litres of liquid nitrogen. One of the containers is used for storage in vapour phase exclusively, the other one is used for long-term storage of material immersed into the liquid nitrogen. The precise adjusting of working liquid nitrogen levels and alarm conditions was made by ReguCon Prague (Ltd) and validation by (GM Project, Opava, Czech Republic). Temperature data measured at two levels in the container used for storage in liquid nitrogen vapours are shown in the figure 1 . The working and alarm limits for storage in mechanical freezer and liquid nitrogen summarised in the Table 5.

Table 5: Storage conditions

	Working limits (°C)	**Alarm limits (°C)**
Mechanical freezer	-80 to -86	-51 to -100
Vapour phase of liquid nitrogen	-161 to -183	-130 to -195
Liquid phase of nitrogen	-177 to -195	-150 to -199

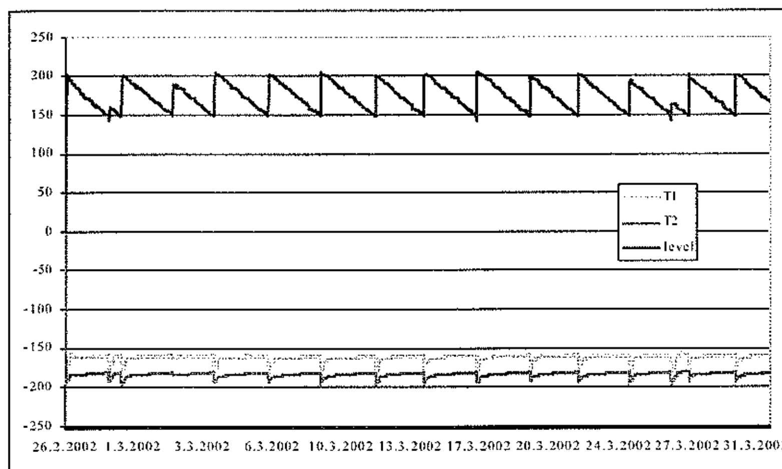

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Figure 1 Temperature conditions in the vapour phase of liquid nitrogen and liquid nitrogen level fluctuations

2.1 Serology of donors and patients

No laboratory signs of active infection were found in 15 donors (13 related, 2 unrelated), i.e. in 68.2 % and in 55 patients (77.5 %). The cases of positivity of active virus infection in donors and patients are summarised in the Table 6, the cases of positivity of other infection are summarised in the Table 7.

Table 6: Positivity of serological markers of active virus infection in donors and patients

Virus/positivity of the test	Donors	Patients
HIV	0	0
HTLV	0	0
HAV	0	0
HBV/HBsAg	0	1
HCV	0	0
VZV/anti-VZV IgM	3	3
HSV IgM	1	2
CMV IgM	3	6
EBV/anti-VCA IgM	0	2

Table 7: Positivity of serological markers of other active infection in donors and patients

Positivity of the test	Donors	Patients
Toxo IgM	0	2
TPHA, RRR	0	0

It can be summarised that in both groups active infection with herpes viruses was the most common (in patients 13, in donors 7). Hepatitis B was found in one case only. The CMV IgG test was the most common marker of previous infection, it was found in 14 donors and 55 patients.

2.2 Microbiological control of cell concentrates after collection and during reinfusion

In the period from 1 April 2001 till 1 February 2002 the total of 479 bags with bone marrow or peripheral blood stem cell concentrates were tested before freezing as well as 28 bags with cord blood. In 2 cases of peripheral blood stem cells the bacterial growth was identified. These two bags were discarded. In cord blood the bacterial growth was found in 1 case. This collection was not yet included in the donor register. The sterility testing is repeated from each bag during reinfusion at the patient's bedside. In any case the bacterial growth was found during the observed period.

2.3 Decision on optimal storage conditions

The decision on optimal storage method is made as follows: The bags originating from patients with no signs of active infection can be immersed into the liquid nitrogen Bags originating from patients with signs of active infection can be stored in the vapour phase of nitrogen only. The bags originating from patients with markers of hepatitis infection must be the vapour phase of nitrogen only. The bags originating from patients with markers of hepatitis infection must be stored separately in a mechanical freezer (-80°C). In September 2002 we store haematopoietic progenitor cells from 30 patients and donors. The proportion among different ways of storage is the following: Storage in the liquid nitrogen 18 persons (60 %), storage in the vapour phase 10 persons (33 %), separate storage in a mechanical freezer 2 persons (7 %).

3 DISCUSSION

Successful cryopreservation of haematopoietic progenitor cell concentrates leads also to good preservation of pathogenic viruses, bacteria or even protozoa if collection is performed in an infected donor or patient. The described cases of contamination during storage in liquid phase of nitrogen were limited to hepatitis B virus (Tedder et al., 1995), nevertheless even viruses that are very common in population such as CMV or VZV can cause severe complications after transplantation that can result in patient's death. The percentage of persons with no signs of active infection was lower in the group of patients, the control group of donors is, however, still small. The results of serological screening are also of high importance for the patient himself as two cases of clinically unsuspected acute infection were discovered and prophylactic measures preventing possible complications after transplantation could have been performed. Acute infection at the time of collection, that required treatment at the Department of Infection Diseases was proved in 2 cases (1 varicella, 1 toxoplasmosis). In the case of varicella in 11-year old boy the frozen concentrate was discarded and the new successful collection was performed after completing the treatment of the acute disease. In the second patient with manifested toxoplasmosis the concentrate was reinfused under prophylactic administration of Daraprim, after completing the treatment of the acute phase of the disease. The positive finding of active toxoplasma infection was a surprise and the case was thoroughly discussed among our group of haematology transplant specialists as survival of toxoplasma gondii in presence of DMSO is well described (Lin, D.B. et al., 1995). For this reason the prevention of contamination during storage of haematopoietic progenitor cells is of high importance. The cases of contamination described by Tedder (Tedder et al., 1995) and Hawkins (Hawkins et al., 1996) were apparently caused by technical failures in particular by using Hemofreeze bags that were noted to fracture sometimes in thawing. This factor was avoided in our practice as we have been using Cryocyte bags since 1993. We can confirm extremely low cracking rate of these bags that was described already by

Warkentin in 1993 (Warkentin et al, 1993). This is a contrast to practice of some centres. Our Standard Operating Procedure also includes testing of airtightness of the sealing of each bag before closing into the cassette. No manipulation with the bag after freezing or during storage is performed and the bags are removed from the cassettes during thawing at the patient's bedside. This is a contrast to practice of some centres that remove the bags from cassettes after completing controlled rate freezing and store them in paper envelopes. Our new system seems to be more safe than using stainless steel containers in the past as there was always problem with assuring airtightness of the openings (Mericka et al, 1991). Although the mentioned measures lower the possibility of infection transmission during storage the demonstrated results of serological screening showed that the rate of positive findings in collected progenitor cells concentrates is relatively high. and approximately one third of our group of patients and donors requires storage in the vapour phase of liquid nitrogen. In contrast the potential risk of infection transmission due to secondary contamination of concentrates during collection and processing is extremely low in our group. The danger of secondary contamination during processing is now lowered also by using clean room technology (laminar equipment of the grade A in the environment (grade B) that is just being introduced to our daily practice.

CONCLUSIONS

The most probable source of cross-contamination in our conditions is active infection in a patient or donor that was identified in approximately one third of our donors and patients.
This infection is not limited to hepatitis B, in our group herpesvirus infection prevailed
Active infection by toxoplasmosis may be a source of cross contamination.
Cross contamination caused by secondary bacterial contamination of collected concentrates during processing is much less likely in our condition and occurred only in 0.42% of tested bags.

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LE SYSTÉME DE LA PREVENTION DE CONTAMINATION PENDANT LE STOCKAGE DES CELLULES SOUCHES HÉMATOPOIETIQUES.

RESUME: La probabilité de la contamination entre des poches plastiques endomagées pendant le stockage dans l azote liquide et contenants des cellules pour l aplication clinique a ete devenue une probléme urgent aprés publication de Tedder qui a decrit la transmission de l hepatite B par cette mechanisme. Notres mesures de prevention contients: 1.Applications de tests serologiques chez tous les malades et donoeurs. 2.L examination bacteriologique des concetrates cellulaires . 3. decision optimalconcernant le stockage (congélateur mechanique, la phase vapoureuse ou liquide de l azote liquide) sur la base des resultates des test serologiques.4. L application des poches plastiques de la qualité superiuere fermés dans les casetes metaliques Notre étude effectuée sur 93 malades et doneurs a montrée que la source potentielle de la contamination la plus importante est representée par infection active viral. La contamination bacteriel qui a été decouverte chez 0.42% de 479 poches examinées a representée le risk negligible dans notre centre.

C04-08

THE IMPORTANCE OF OXYGEN LEVEL MONITORING IN THE CRYOSTORAGE FACILITIES

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ABSTRACT

Experience with two-year operation of the cryostorage facility of a cell and tissue bank of the University Hospital Hradec Králové is demonstrated. The total of 52.4 l of liquid nitrogen is transformed daily to the gas phase in the room with forced ventilation. Wall mounted oxygen monitors are used for continuous monitoring of atmospheric oxygen. The data are recorded in 5-minute intervals. Two alarm levels identified by acoustic signals are adjusted. The first level of 20% is valid for women according to the Czech legislation, the second level of 18% is the recommendation of the manufacturer. During operation of the system the oxygen level may sink below 20% in case of automatic filling of high capacity containers or during filling of back-up vessels. The oxygen level below 18% does not occur if the forced ventilation works well. The ventilation breakdown can lead to lowering the oxygen level below 18%.

INTRODUCTION

Although the possibility of lowering the atmospheric oxygen level in cryostorage facilities due to excessive evaporation of liquified gases is well known, this problem is sometimes neglected both by working staff of tissue banks and companies involved in designing or building such facilities. Fatal cases can be caused by ignorance of properties of liquified gases or underestimation of the danger in the oxygen deficiency hazard areas.The lack of continuous oxygen monitoring, breakdown or absence of sufficient ventilation as well as breakdown of the monitoring system itself can lead to dangerous situations. Theilacker (Theilacker, 2003) described recently also the possibility of indicating false high oxygen levels in presence of gases with lower molecular weight than nitrogen, such as helium or argon. In this paper we describe own two-year experience with continuous oxygen level monitoring in the cryostorage facility of a cell and tissue bank of the University Hospital Hradec Králové.

1 DESCRIPTION OF THE SYSTEM

The cryostorage department of this bank (Fig. 1) is equipped with two liquid nitrogen containers XLC 1200 (Chart, Europe, Planer Biomed, UK) filled automatically from the Cryocyl 230LP (Chart, USA, Planer Biomed, UK) vessel. The containers are used for storage of bags with haematopoietic progenitor cell concentrates (Měřička et al., 1999, Bláha et al., 2003, Měřička et al., 2003). One smaller container XLC 230 is used for storage of control ampoules serving for assessment of freeze-thaw recovery of colony forming progenitor cells, the second one for storage of samples of tumour tissue for special diagnostic and research purposes. These containers are filled manually (Fig. 2). One container (Cryosystem 2000) is used for storage of ampoules with gametes of oncology patients, two additional containers (KL 32, Cryometal Říčany, Czech Republic) (Fig. 3) for storage of liquid nitrogen itself. One container Eurocyl 230 LP serves as back-up cooling of

two mechanical freezers used for storage of deep-frozen solid human tissues for transplantation (Měřička et al., 2002). The whole system is connected with an external vessel with the pure capacity of 4,690 kg of liquid nitrogen (VT 6 Ferox Děčín, Czech Republic).

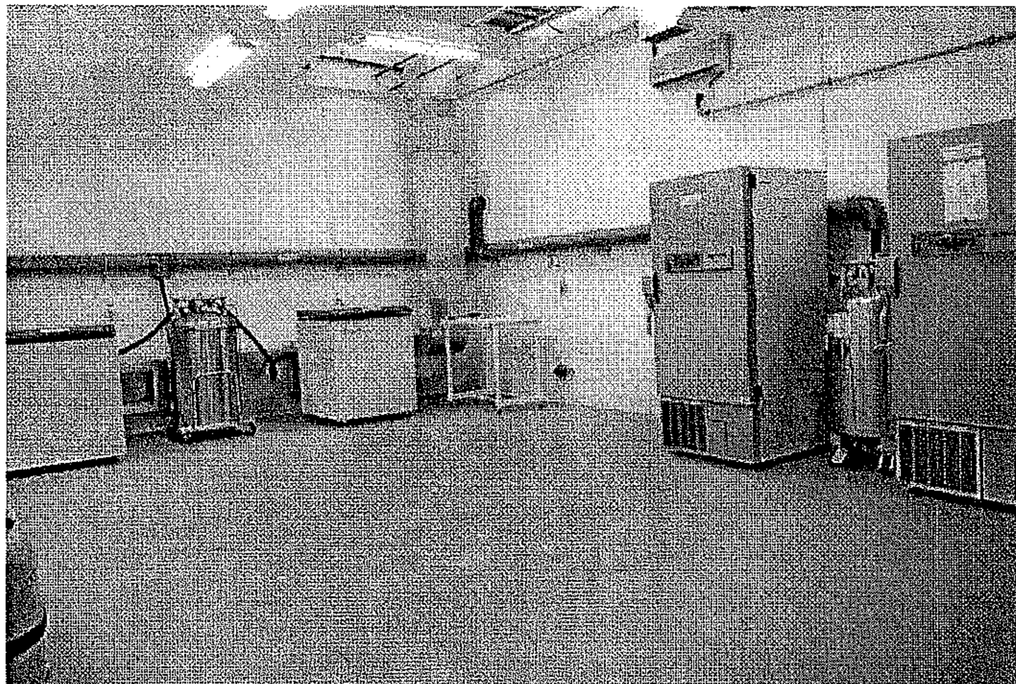

Figure 1: General view of the cryostorage facility of the cell and tissue bank of the University Hospital Hradec Králové. The fixed monitor with display and acoustic alarm is mounted on the right wall in the height of 35 cm.

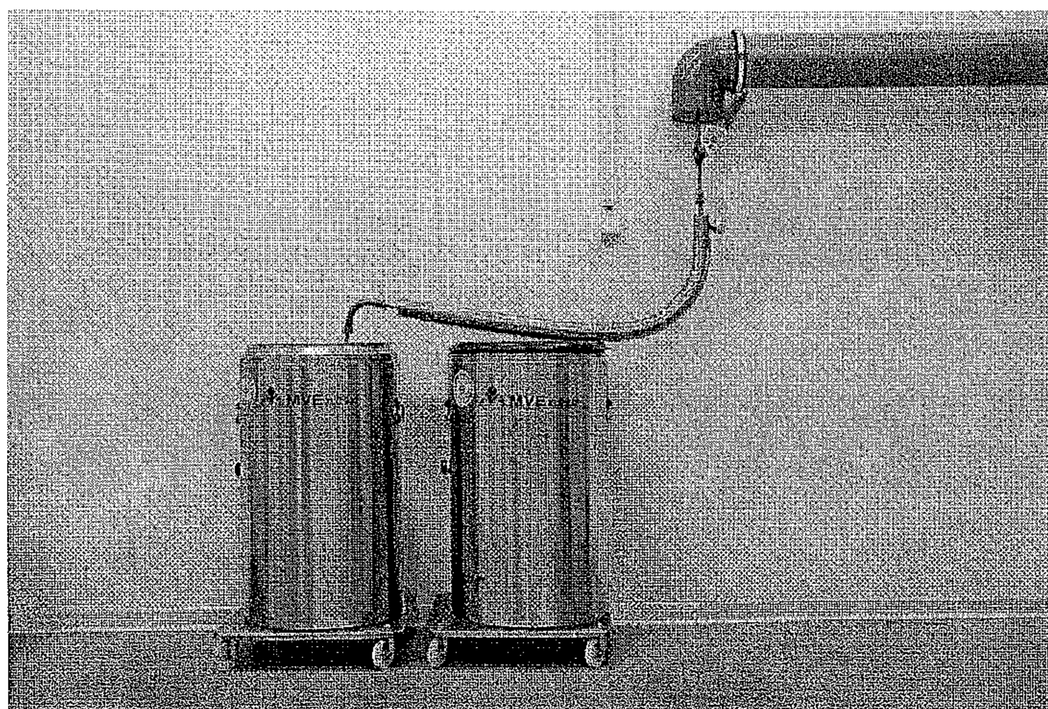

Figure 2: Containers used for storage of ampoules

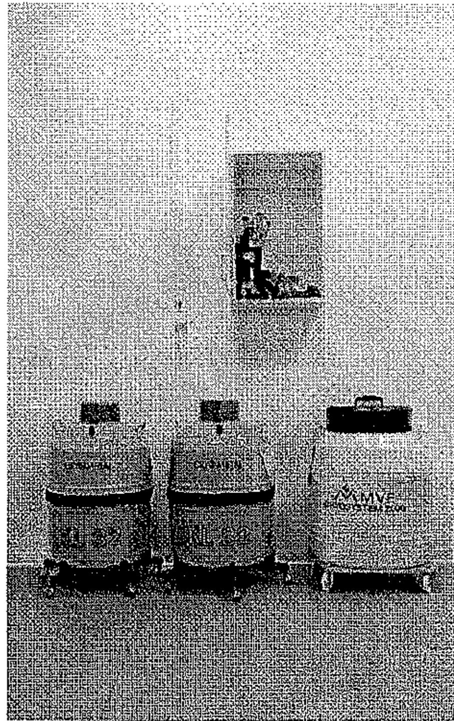

Figure 3: The container used for storage of gametes of oncology patients (right). Containers used for storage of liquid nitrogen. An opened box with a breathing apparatus .

Forced ventilation is controlled by the Honeywell unit that is operated continuously during day and night. Breakdowns are reffered automatically to the central management of the hospital air conditioning system. The air is introduced in the vertical stream from the ceiling (Figure 1 on top) and exhausted from the bottom of the room (behind the large XLC 1200 vessels). The mean daily consumption of liquid nitrogen in the XLC 1200 containers is calculated automatically and indicated on the display as difference of liquid levels in mm. This value is about 27-30 mm per day. The difference of levels in XLC 230 containers is measured manually by an approved measuring stick (Chart Europe). The data on evaporation of other vessels were taken from data declared by the manufacturer. The total of 52.4 l of liquid nitrogen is transformed daily to the gas phase in the cryostorage room. Wall mounted oxygen monitors with displays calibrated annually with the calibration gas are used for continuous monitoring of atmospheric oxygen in two rooms, where sink of oxygen level can be encountered. The data are recorded in 5-minute intervals and can be stored in the memory box for maximum of 3 months. Downloading of data into the personal computer is performed weekly. Two alarm levels which are identified by acoustic signals are adjusted. The first level of 20% is valid for women according to the Czech legislation (Ministry of Health of the Czech Republic, 1998), the second level of 18% is the recommendation of the manufacturer of oxygen monitors. Personal and portable monitors are in the disposal of the staff for case of the breakdown of wall mounted monitors. The entries into the oxygen defficiency hazard area are equipped with light signals: green - oxygen level above 20 %, orange - oxygen level between 18.1 till 20 %, red - oxygen level 18.0 % or below. If the red light is switched on, entry without breathing apparatus is not allowed.

2 RESULTS

Operation of the system under normal ventilation conditions is demonstrated on the Figure 4. The oxygen level is maintained between 21.0 and 21.1%. In case of automatic filling the XLC 1200 containers the oxygen level sinks, the values below 20% do not occur, however. In case of filling the back-up vessels Cryocyl or Eurocyl or manual filling of smaller containers deep sinks below the

level of 20% are encountered, the alarm level of 18% is not achieved, however. Operation of the system under suboptimal ventilation conditions (exhausting of the air from the top of the room only) is demonstrated on the Figure 5. Automatic filling of the XLC 1200 vessels leads to sinking of the oxygen level below 20%. The sinks below 18% are encountered in cases of filling the back-up vessels. The lowest recorded level was 17.5 %.

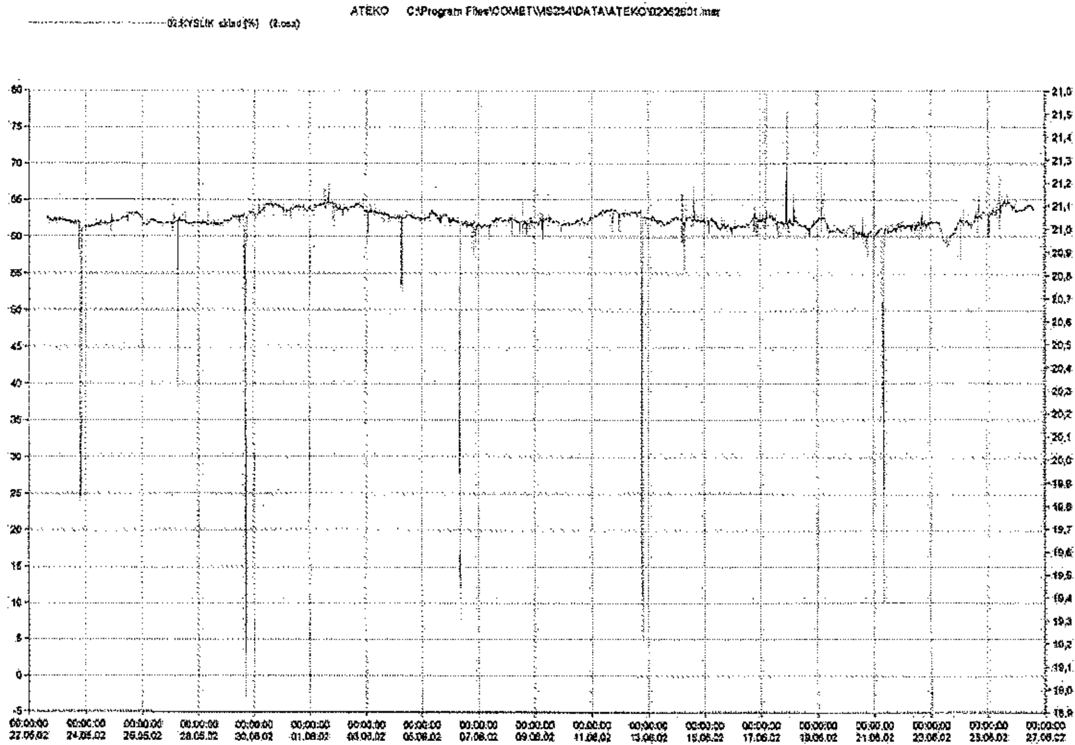

Figure 4: Results of oxygen monitoring under normal ventilation conditions

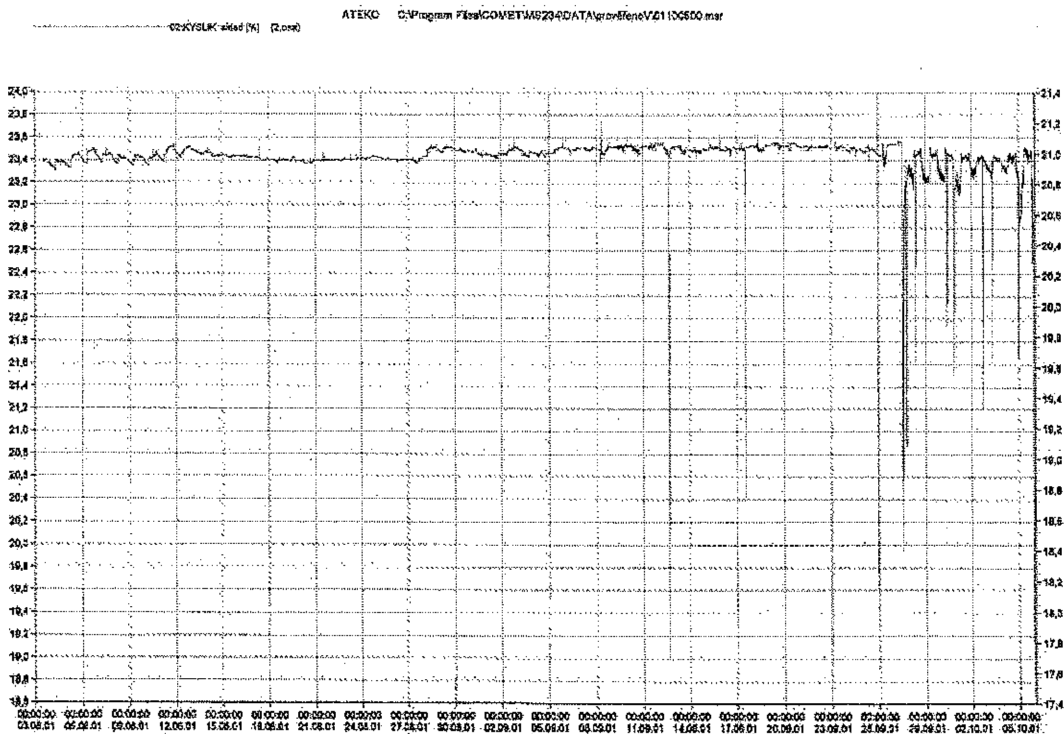

Figure 5: Results of oxygen monitoring under suboptimal ventilation conditions

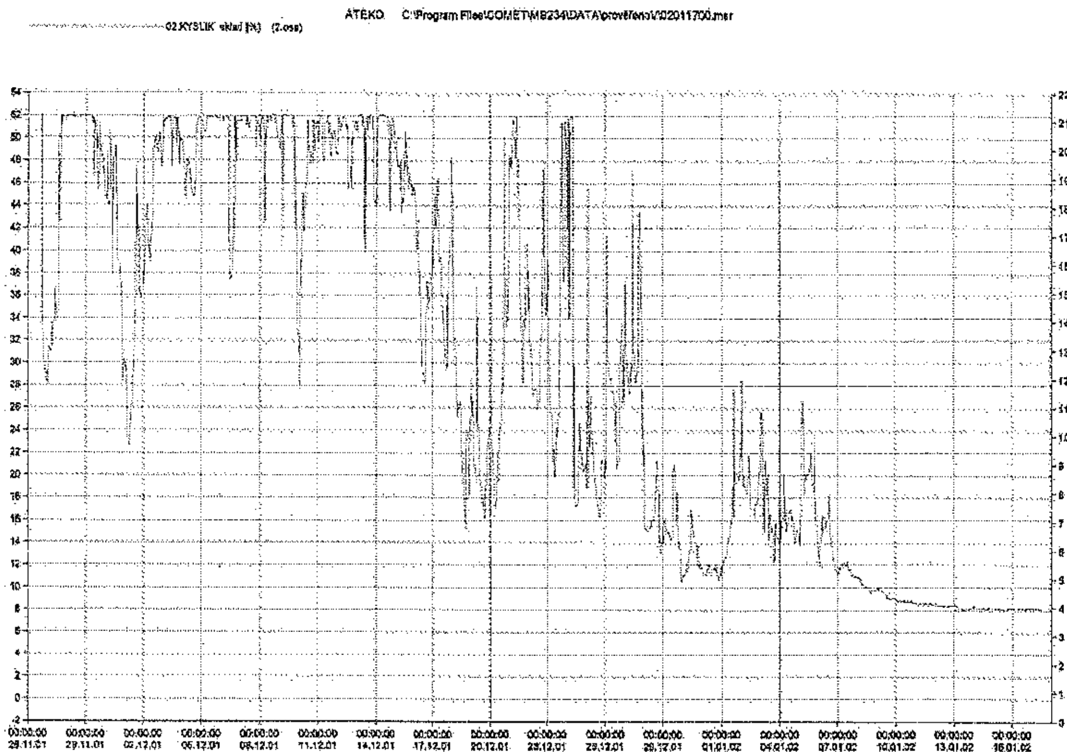

Figure 6: Record of oxygen level at the end of the service life of the monitor

The oxygen level recorded at the end of the service life of the oxygen monitor is demonstrated on the Figure 6. The considerable fluctuations of the levels (in the wide range between 10 and 21% (left part of the record) are followed by continuous and irreversible decrease to the level of 4% (right part of the record).

3 DISCUSSION

The two-year operation of our system showed that the forced ventilation with exhausting the air from the bottom of the room assures acceptable conditions for safety of the personel of the cell and tissue bank. At normal ventilation conditions the oxygen level only rarely sinks below 20% with the exception of manual filling of vessels. The system assures safety conditions for work activities of women even under very strict criteria given by the Decree of the Czech Ministry of Health No. 185/1998. The most difficult situation can occur at the end of the service life of the wall mounted monitor, that can indicate false low oxygen levels. The use of portable monitor checked at normal oxygen level conditions (open air) before entering the potentially dangerous area is necessary for distinguishing such situations from the real ventilation breakdown. The breathing apparatus is at the disposal of the staff for managing such situations as well as the cases of real ventilation breakdown or excessive evaporation of the liquified gas.

CONCLUSIONS

Our experience shows that in routine operation of the system the oxygen level may exceptionally sink below 20% in case of automatic filling of high capacity containers and regularly during filling of back-up vessels (Cryocyl or Eurocyl 230). Sinking of the oxygen level below 18% does not occur if the forced ventilation works well. The ventilation breakdown can lead to lowering the oxygen level below 18%. Portable monitors are necessary to identify false low oxygen levels that can be indicated at the time of the end of the service life of the monitor.

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