THE TECHNOLOGY IN CRYOTECHNOLOGY

Janina Hagedorn[1*], Ghiath Alkurdi[1], Sven-Alexander Barker[1], Ricarda Brunotte[1], Tarek Deeb[1], Oleksandra Hubenia[1,2], Diaa Khayyat[1], Sara Leal-Marin[1], Tim Rittinghaus[1] and Birgit Glasmacher[1]

[1]Institute for Multiphase Processes, Leibniz University Hannover, Garbsen,30823, Germany
[2]Institute for Problems of Cryobiology and Cryomedicine, National Academy of Sciences of Ukraine, Kharkiv, 61016, Ukraine
*Corresponding author's E-mail: hagedorn@imp.uni-hannover.de
All authors contributed equally to this paper.

Abstract

The process of freezing biological material at extremely low temperatures is known as cryopreservation. To ensure the preservation of cells and tissues over an extended period of time, low temperatures are applied since biological processes, including the biochemical ones, come to a halt under cryogenic conditions and thus it is possible to maintain their structural and functional integrity. The field of cryopreservation gained more prominence in the 20th century and emerged as an unavoidable technology for different applications such as cell therapy, tissue engineering, or assisted fertilization. In this work we provide an overview of various technologies in the field of cryotechnology with regard to the freezing, storage and thawing of living cells. The first part covers the freezing process, starting with cryoprotective agents regarding their protection mechanisms and compositions, passing by cryoimaging, micro-fluidic systems, and the currently available freezing and biobanking equipment. The second part focusses on the thawing process as well as the hypothermic preservation for the short-term storage of biological materials and constructs.

Keywords: biobanking; CPA; freezing; hypothermal storage; nanowarming; microfluidic.

INTRODUCTION

Kryos" is the Greek word for (ice) cold and introduces the subject area of a research field that deals with the long-term preservation of different cell types or natural and artificially produced three-dimensional tissues at cryogenic temperatures (1). Cryogenic temperatures cover a range from -120°C to absolute zero (-273.15°C) (2). Peter Mazur pioneered the research field, by discovering that the result of cryopreservation depends on the cell type, as well as that the main parameters for freezing and thawing have to be optimised for each cell type and tissue (3). To achieve the best possible cell viability after thawing, existing techniques, e.g. for precise control of ice nucleation, are constantly being improved and new aspects related to cell-specific physical and biological parameters are being explored (1, 2). In the following, we provide an overview of possible technologies in cryotechnology with regard to the freezing,

thawing and storage of living cells. Thereby different methods and containers for the freezing of living cells as well as the necessary antifreeze agents with their respective advantages and disadvantages are presented. Finally, the topic of thawing with different methods and hypothermal storage is discussed.

FREEZING

Cryoprotective agents

As a critical component of cryogenic storage, cryoprotective agents (CPAs) are necessary to freeze and recover the cells with high levels of integrity and functionality (4). CPAs are water-soluble molecules that are utilized in cryopreservation for their protective effect to greatly increase the survival rate of frozen cells and tissue. This occurs by inhibiting the growth of ice and reducing the dehydration of cells during the freezing and thawing process (5).

CPAs are classified into two main categories, namely: penetrating CPAs (pCPAs) and non-penetrating CPAs (npCPAs). The pCPAs are non-ionized molecules with a relatively low molecular weight and small size, which enables them to cross the cell membrane and also diffuse into the cell interior. Because of these properties, they have been the most commonly used CPAs to date. The best known pCPAs are glycerol and dimethyl sulfoxide (DMSO) (6). In contrast, npCPAs are too large or too ionized to cross the cell membrane. Therefore, they are present only in the extracellular space. npCPAs (e.g., sugars) are usually less toxic than pCPAs and are often applied in addition to reduce the concentration of pCPAs used. However, the exact mechanisms of CPAs to protect cells during freezing and thawing have not been fully explored (7).

When ice forms as temperatures drop below 0°C, the solutes are excluded from the ice and concentrate in the remaining unfrozen solution. The cells are also located in the remaining spaces between the ice crystals. As the ice grows, these spaces become increasingly smaller. When the spaces become insufficiently small for the cells, the ice crystals also cause mechanical damage to the cells. CPAs inhibit ice growth and lower the melting point of water, resulting in less ice at any given temperature and larger areas for the cells to reside. Thus, larger areas enable reduced damage to cells from both types of freezing injury (8).

Due to the concentration of solutes, osmotic processes take place, which are lethal to the cell. By adding CPAs, the concentration of electrolytes and salts decreases, mitigating the osmotic effects (6, 9). Additionally, formed ice concentrates the CPAs in the remaining solution and the increased concentration will increase this effect, but also the side effects of the CPAs. This increase in concentration must be considered in advance so that the cells are not exposed to toxic amounts of CPAs. In some cases, a mixture of several different CPAs is also applied to keep the toxicity of a single CPA lower (10, 11).

In principle, npCPAs act in the same way as pCPAs, but they are only present in the extracellular space. As a result, they have the additional effect of promoting earlier water efflux out of the cells via osmosis, and the reduced amount of intracellular water decreases the likelihood of intracellular ice (8). There are two theories as to how sugars protect cells from death. The first is the water replacement hypothesis. According to this hypothesis, biological membranes and proteins are stabilized by disaccharides forming hydrogen bonds to the structures at risk. This direct interaction results in proteins and membranes retaining their physical state and not being deformed or destroyed. The second theory is that the disaccharides form a glass when dry, and thus have a protective effect. When the cell loses water by osmosis during freezing, this leads to a high molecular density and a reduction of molecular mobility. As a result, the cell is protected until water re-enters the cell interior (12, 13, 14) . The two theories are not mutually exclusive and Crowe et al. (14) assume that both described processes are necessary for the survival of organisms. However, for a protective effect of sucrose, the sugar must also be present in the cytoplasm of the cell.

Today the gold standard in cryopreservation is the application of DMSO as a CPA with a concentration between 5% and 10% (4, 15). Using DMSO works perfectly in many cryopreservation applications but also presents a huge drawback. DMSO is highly cytotoxic at room temperature which demands one or several post-thawing washing steps to eliminate residual DMSO (16). Besides that, there were some reports about DMSO causing genetic and epigenetic alterations. This largely intensifies the search for alternatives in cryotechnology (17, 18, 19).

The alternatives to DMSO vary between syntactic materials, such as polymers, and natural substances like osmolytes. Sugars are often studied as alternatives to DMSO. But as previously mentioned, they lack the ability of

penetrating the cell membrane due to their large molecular size. This motivated investigations of enhancing the intra-cellular permeability of sugars. For example, some chemical approaches have been undertaken, such as conjugating trehalose with 2, 4 or 6 acetyl groups to improve its lipophilicity and thus its membrane permeability. Using six groups, it was possible to significantly enhance the intracellular trehalose delivery and no irreversible cellular damage was observed when examined on rat hepatocytes after 14 days of incubation (20). Another work in this field investigated the delivery of esterified trehalose analogues, whether acelytated or propionylated (an easter with a longer chain), in a wider range of human cells. The results showed consistency with the last-mentioned study. The only drawback of this approach is the long incubation time required to deliver the sugars (21). Zhang et al. tried a different approach. They employed nanoparticles (NP) as sugar carriers into the cells. The utilized NPs were cold responsive, meaning that they disassembled after being exposed to cold temperature (0°C) (22). An alternative approach has applied electrical fields to reversibly or irreversibly create pores in the cell membrane, enabling an intracellular delivery of sugar. There have been many promising reports about electroporation being a successful substitute for DMSO (13, 23, 24).

Pollock et al. investigated the use of multi-component solutions including osmolytes to substitute cytotoxic CPAs. After computationally optimizing the composition of these solutions, with regard to the freezing rate and components, they used differential evolution (DE) algorithms (25). The group experimented with different solutions and incubation times on mesenchymal stem cells (MSCs). These solutions contained sugars (sucrose and glucose), sugar alcohols (mannitol and glycerol), and small molecule additives (creatine) in multiple concentrations. This work showed that the incubation time for the suggested DMSO- alternatives is high compared to DMSO or glyc- erol and might reach 90 min. Besides that, it could be shown that the osmolyte mixtures did not show a colligative effect as a protection mechanism but rather supposedly a biological one. This is based on the fact that there was no detected physical change in ice formation between these solutions but a variance in recovery rates. The authors hypothesized that there could be a favourable composition at which an optimal recovery can be reached. An explanation might be that the use of osmolytes offers protection to the membrane as well as to internal proteins (26). In the search of this composition Pi et al. tried to characterize the mechanisms of protection, offered by osmolyte mixtures as CPAs (diverse concentrations of sucrose glycerol and isoleucine), and applied them to Jukart cells. The results were consistent with the findings of Pollock et al. (27). The gathered data in this work was used to evaluate the effect of the control parameters as well as the model of the differential evolution algorithms on the prediction ability of a computational model to optimize the formula of multi-component DMSO-free CPAs (28).

Parallelly, Gertrudes et al. reported the role of osmolytes as CPAs and suggested that a group of deep eutectic solvents (DES), called nat- ural DES or (NADES), might be a novel group of DMSO-free CPAs. This is based on the fact that NADES were detected in animals that survive extreme low temperature (29). In 2021 the group reported the potential of NADES as CPAs and their thermal behaviour in combination with water by preparing two mixtures (glu- cose:urea:proline) at a molar ratio of 1:1:1 and (proline:glucose) with a 5:3 ratio. They showed that a higher NADES concentration might lead to a total suppression of water crystallization. Additionally, reducing the concentration can alter the crystallization temperature as well as the number and shape of these crystals. Furthermore their results suggest a lower cytotoxicity of NADES compared to DMSO (30). The next step was to employ NADES as CPAs and there were multiple trials which showed promising results (31, 32, 33).

Imaging

Cryomicroscopy is one of the most important techniques for visualizing freezing and thawing processes. It enables an effective screen ing and identification of novel cryoprotectants (CPAs) for cells and tissue biobanking. Additionally, it allows the investigation of a variety of freezing processes under controlled thermal conditions. It is possible to assess the CPAs' ice crystal growth velocity, ice recrystallization inhibition (IRI) activity, intra- and extracellular ice formation and devitrification during slow freezing and vitrification (34, 35).

Over the past two decades, cryomicroscopic methods have been improved. Developments have included the mounting of a cold stage on a microscope instead of cooling the entire microscope, the utilization of a digital control system instead of the less accurate analogue

control system, and the application of video recordings to record the freezing events. Cryomicroscopy also helps in characterizing intracellular ice formation (IIF) through the visual inspection of the blackening of cells due to light scattering by opaque ice inside the nucleated cells (36, 37).

The study of crystallization is very important and complex. Prykhodko et. al have analysed microscopic images during freezing and thawing. Therefore, they used a semi-automated method (38). Here, the pre-filtering stage is not necessary and the application of threshold segmentation is not always possible. Thus, they applied the Otsu method in their study. Finally, the resulting image is morphologically processed using erosion and proliferation operations. The advantages of this method are: (a) it allows analysis of recrystallization during thawing and isothermal annealing, (b) it is possible to determine the efficiency of inhibition of ice recrystallization of novel and low-toxic cryoprotectants, (c) the tracking of certain ice crystals is possible. For complex crystal formation Tymkovych et. al have developed specialised software for the segmentation and analysis of microscopic ice images with active crystal contours (39). The following steps are required for segmentation of microscopic ice crystals: region of interest selection, preliminary segmentation, morphologic processing, seeding, active contour process, active contour simplification, contours intersection, contours combining, and seeds updating. The choice of the size of the initial contour depends on the scale of the image and the size of the recorded structures. Due to the absence of unified software for the automated analysis of ice recrystallization, Tymkovych et al. used a U-Net deep neural network for the segmentation of ice crystals on cryomicroscopic images (40). On the basis of 100 images, as training set, the resulting accuracy was approximately 74% of the test sample (30 images). This result showed the possibility of segmentation considering the overlapping of intensity levels of an object and background.

Cryomicroscopy methods have become widespread and effective tools for life scientists, pharmaceutical researchers, and more, to examine biological structures close to their native state. One of these techniques is the cryo-correlative light and electron microscopy (cryo-CLEM) (41). Cryo-EM uses extremely low, cryogenic temperatures to overcome the challenge of measuring biological specimens with high water content by utilizing electron beams in vacuum conditions. Cryo-CLEM brings the advantages of low temperature fluorescence together with cryo-EM, to increase the sensitivity for the detection of biological, chemical, and genetic processes inside living cells. Cryo-CLEM enables direct fluorescent labelling and targeting of molecules or molecular assemblies.

Microfluidic systems

From the initial application area of producing microfluidic systems (MFS) for the manufacture of microsensors and microactuators to manipulate liquid or gaseous samples, a novel and widely used microtechnology has been developed. This technology allows the performance of precise laboratory work on only one system, lab-on-a-chip (42, 43). Consequently, material, time and laboratory costs can be minimized (44). MFS enable sensitive technical analyses, such as drug screening or material synthesis, and are often used for blood analysis, blood cell separation or characterisation (45, 46). They are produced in a size range of 100 nm to 100 μm (47). More recently in microfluidic manufacturing, modular MFS have been established. These are individual modules which are connected via plug-in or clamp connections. They are also highly customizable and easy to use (42). In the field of cryotechnology, MFS can be used to automate the manual process of loading and unloading with the required concentration of CPAs followed by controlled freezing. With regards to cells, potential osmotic shock or dangerous ice crystal formation can be minimised. Controlled freezing also offers the advantage of enhanced regulation of the vitrification process and a significant increase in cell survival rate. The formation of cell-damaging intracellular ice is completely avoided (48, 49).

In the early 1990s, the first microfluidic systems were produced out of silicon. Silicon is easy to handle and very precise structures can be created, but it is quite expensive to use disposable systems (43). Today, microfluidic systems are made from a variety of materials, depending on the requirements of the application. These include polymers such as polydimethylsiloxane (PDMS), plastics such as polystyrene or polypropylene, and glass or ceramics (43, 44, 50). Probably the most widely used polymer is PDMS. It belongs to the siloxanes and is based on silicon atoms that are alternately connected with oxygen atoms. Advantages, apart from low cost, include high

transparency and stability to moisture and temperature stresses. Good gas permeability enables cell cultivation or cell-adhesive or -repellent coatings (43, 50, 51). Since 2000, hydrogels have been increasingly used in microfluidic technology to optimise their respective advantages. Beebe et al. were the first to introduce a hydrogel microvalve that responds to stimuli to autonomously regulate flow in microfluidic channels (44).

MFS can be produced very easily and cost-effectively. In current applications, manufacturing processes such as three-dimensional (3D) printing, lithographic techniques or casting processes are used. MFS production can generally be divided into two categories: additive and subtractive manufacturing processes. In additive manufacturing, MFSs are built through layer-by-layer application of the selected material. Some of the applied methods are: (a) fused deposition modelling (FDM) - in this process, a thermoplastic filament is melted and pushed through a nozzle to build up the object layer by layer, (b) stereolithography (SLA) - a laser is used to cure a liquid that photopolymerises to build up the object, (c) the electron beam melting (EBM) - this process uses an electron beam to melt and weld a powder material to create the object (44, 43, 51). Unlike additive processes in which material is added to create the desired shape, subtractive processes remove material from an initial piece using various techniques. These techniques stand out for their good structural accuracy and complexity using removable templates (52). Some widely used methods are laser ablation, microcutting including drilling and milling processes with a drill bit < 0.01 mm or ultrasonic machining. Ultrasonic machining (USM) uses ultrasonic waves in the kHz range to transfer patterns or templates to the surface of the workpiece. Abrasive particles are used to create the desired structure. The particles consist of tough substances, including boron carbide, aluminium oxide, as well as silicon carbide and enable a high level of hardness (51, 52). For the production of very complex microfluidic systems, both processes can be combined (53).

Slow freezing and vitrification

Designing a cryopreservation protocol begins with the CPAs selection. Additionally, other parameters such as loading time, freezing method, and storage temperature have to be selected appropriately. The most common freezing methods are slow freezing and vitrification, both aiming to avoid the ice crystal formation that causes cell damage and tissue rupture. A major requirement in the case of slow freezing is to find an optimal cooling rate related to the specific cell type (2). Additionally, the technology plays an important role in slow freezing, as controlled freezers are required for cooling rates between 0.2 and 50 K/min. The slow cooling associated with the technique of replacing water with pCPAs facilitates the intracellular water efflux from cells which allows the elimination of supercooling (2, 54, 55). Previously, this was the preferable method for long-term storage of germinal cells, but nowadays, it is being replaced by vitrification (56).

By applying vitrification, ice crystal formation is avoided due to the rapid cooling of samples in direct contact with liquid nitrogen (LN2) and an amorphous state is formed. This technique requires an increased viscosity in the cryoprotective solution. However, an increase in the CPA concentration can affect cell viability (57). Also, maintaining an extremely high cooling/warming rate is essential to avoid ice recrystallization and devitrification. Thus, the critical warming rate is usually several orders of magnitude higher than the critical cooling rate and therefore represent huge challenges (58). A current focus of research is the mixture of magnetic or metallic nanoparticles with CPAs to improve the heat transfer during cooling and reducing the CPA concentration. Further this approach can improve the distribution of heating during thawing by utilizing warming techniques such as nanowarming (59) or joule induction (60).

Commercial freezing devices

Commercial freezing devices can roughly be divided into the two groups: passive freezing devices (insulated freezing containers), and controlled rate freezers (CRF). The first group is limited to one cooling rate close to 1 K/min while the CRFs provide a range of cooling rates from 0.2 K/min up to 50 K/min.

Freezing containers such as Biocision®Cool Cell®, FreezerCell™ and Nalgene® Mr.Frosty™ are comparatively cheap and can be placed into a -80°C freezer for the cryopreservation of around 12 to 30 samples in 2 mL cryo vials. Containers for bigger vials are also available. One concern is that the cooling rate of 1 K/min is defined differently between manufacturers, for example

by Mr.Frosty™ and CoolCell® (61, 62). When changing from one system to the other an adjustment of the freezing protocol might be necessary.

Controlled rate freezers use active cooling by injection of nitrogen steam (Planer Cryo Series, Thermo Scientific™ CryoMed™, SY-LAB IceCube, CustomBioGenic) or by utilization of the Stirling process with helium as the working gas (Cytiva, STREX, Grant Instruments). LN2-based systems provide cooling rates up to 50 K/min whereas the Stirling based systems usually provide cooling rates up to 2 K/min (in some cases up to 10 K/min). Another system is a combination of a cryo workbench for sample handling in a -130°C environment. It is combined with an integrated freezing system using a metal basket above a pool of LN2 (Askion C-Line® Workbench). During the cooling process the basket moves downwards to the LN2 pool to reduce the sample temperature. It should be noted that the highest cooling rates specified by the manufacturer can often only be achieved with very small sample volumes and thin-walled sample containers. Modern controlled rate freezers often provide a function for induced nucleation, i.e., latent heat compensation by an increase of sample cooling performance (63, 64).

Heat transfer

Cooling or thawing rates and the related heat transfer play a critical role in the freezing and thawing of cells and tissues. Knowing and controlling the cooling rate is essential for cell survival. The freezing container used further influences the cooling rate. Small volumes can be frozen in cryovials. The geometry of the vial is not optimal for heat transfer. Whilst small sample volumes remain unproblematic, the cylindrical geometry is not optimal for larger sample volumes (64). Due to the low surface-to-volume ratio, temperature layers can form as a result of heat conduction, leading to different local cooling rates. The latent heat released in the outermost layer impedes freezing in interior layers. This obstruction causes a delayed temperature drop, deviating the cooling rate (65, 66).

Various sample holding devices are available for freezing multiple sample vessels simultaneously. The CoolCell® and Mr Frosty™ products are cylindrical with cut-outs for the vials. The temperature profile of the vials within these passive cooling devices (PCDs) depends on their positioning (Figure 1). According to Hunt (67), the temperature profile within the CoolCell® is only associated with a small deviation between the vials due to the uniform distribution of the vials. In Mr Frosty™'s PCD, the distribution is given by an inner and an outer ring in which the vials can be positioned. Hunt undertook temperature measurements in two different locations. However, compared to the temperature trace from the Cool-Cell® these two traces have a larger deviation from each other of about 5 K. The cooling rate for both was 1 K/min. In comparison, the CoolCell FTS30 was studied with a cooling rate of 0.64 K/min. The deviation is negligible here due to the position of the vials. The mean cooling rates were determined between -10°C and -40°C (67).
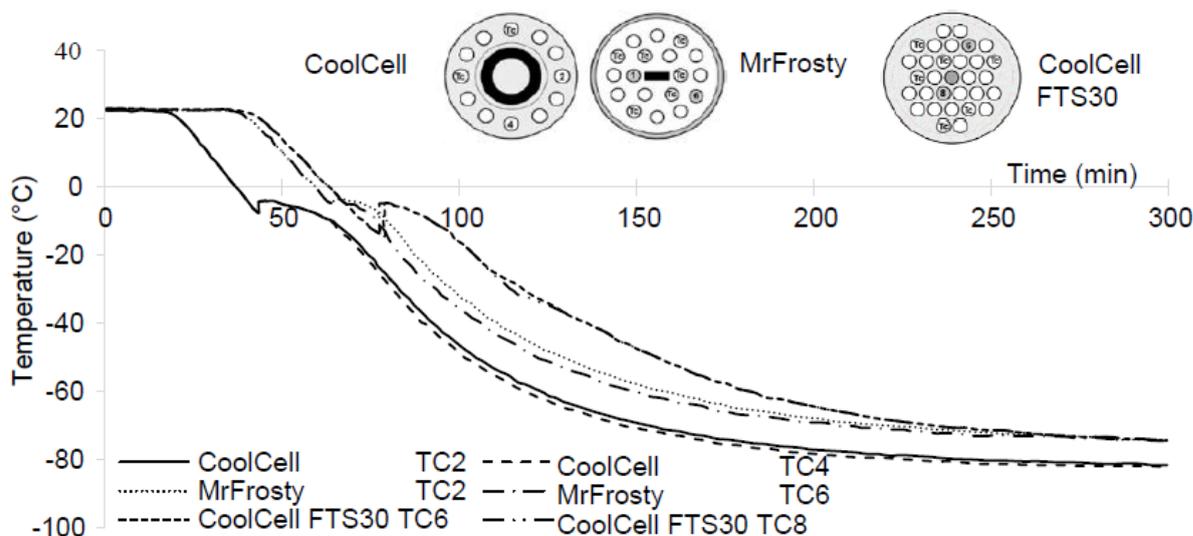

Figure 1. Comparison of cooling curves for the passive cooling devices CoolCell®, Mr Frosty™ and CoolCell FTS30. Examination of different positions within the device (67).

Biobanking devices

Storage temperatures below the glass transition temperature of -135°C are recommended for the long-term storage of living biological material to prevent devitrification and recrystallisation within the valuable samples. Mechanical -150°C freezers are advantageous, since they can be installed in every room with power sockets as long as the heat released by the device can be dissipated. However, a failure of the freezer or the power supply requires immediate action to save the samples from reaching normothermal temperatures. Sample storage in LN2 cooled devices is more fail-safe since the LN2 usually lasts for many days up to several weeks. Besides storing directly in LN2, storage in the gas phase above a pool of LN2 is commonly applied. This reduces the probability of contamination of the samples by LN2 penetrating the sample container. In some devices dry storage is used. Here the LN2 is located in the sidewalls and the bottom wall of the device. The samples are not in direct contact with the LN2 or nitrogen vapor, decreasing the chance of sample contamination even more. Automated storage systems like Azenta Life Sciences Biostore™ II, Hamilton BiOS and Askion C-Line® HS are used to handle and manage a large number of samples efficiently. They can be equipped with automated scanning and sorting systems and a sample data logging. Depending on the system, storage temperatures between -80°C down to below -150°C can be provided. The downside of some devices is that certain areas are kept at temperatures up to -20°C to assure sufficient function of moving parts. This may result in a potentially critical warming of the samples (67, 68, 69).

THAWING

The simplest way to thaw samples is to use a water bath, usually at body temperature. However, here the thawing rates cannot be precisely adjusted and controlled. Some of the freezing devices mentioned before, such as the Planer Cryo Series and the SY-LAB IceCube Series, provide a heating function with programmable heating rates up to 15 K/min depending on the device specification. Much higher heating rates can be achieved by the following methods described (69).

Nanowarming

In the case of vitrification, it is necessary to exceed the critical warming rate to prevent devitrification (70). This can be obtained by mixing magnetic nanoparticles with CPAs for nanowarming and to improve the heat transfer. During thawing, the magnetic nanoparticles are excited with radiofrequency waves. Due to the uniformity of the generated field, small organs can be thawed without the challenges of heat distribution (71) and thawing rates of > 300 K/min can be reached (72). From a technological point of view this technique requires high power radiofrequency generators, a minimum power of 15 kW and a frequency of 100 kHz (73, 74, 71). Similar parameters are commonly used in other fields for melting materials.

Joule heating

Joule heating is the conversion of electric energy into heat energy through the incorporated resistance in an electric circuit (75). This is methodologically easier to implement in comparison to nanowarming and increases the thawing rates from 5000 K/min to 60000 K/min. It is a novel way of thawing biological materials, proving to be effective for adherent cells to organ slices (60).

HYPOTHERMAL STORAGE

Hypothermic preservation

Cell and gene therapies have grown significantly over the past decades due to their unrivalled potential to improve the treatment landscape for a wide variety of diseases (76). The applied processes for storage and transportation must ensure that the cell product is consistently GMP (good manufacturing practice)-compliant and safe to maintain cell viability as well as potency during the time window that separates the release of the product from a GMP facility to the clinical trial centre or therapeutic facility (77). Cell suspensions are usually slowly frozen in the presence of DMSO in concentrations of 1.5 M (78). Recent studies have demonstrated the successful cryopreservation of mesenchymal stem cells (MSCs) within a 3D medium, made up of alginate microspheres (AMS) with DMSO in the same concentration range (79). However, due to the toxic nature of DMSO and the requirement for its removal prior to use post- thaw, the development of alternative cryopreservation protocols has become a pertinent research topic.

Hypothermic preservation is practical and adaptable method for storing viable cells at refrigerated temperatures, which is particularly valuable for cell diagnosis and transportation. On the other hand under hypothermic conditions, cellular swelling occurs due to a disruption in metabolic and ionic homeostasis. This can result in the accumulation of reactive oxygen species that can lead to the activation of apoptosis and cause structural damage to the cells (80). Specialized solutions have been developed to prevent cell swelling, and effectively reduce cellular damage. Petrenko et al. (81) presents a clinically relevant solution for the preservation of human multipotent mesenchymal stromal cells (hMSCs) for transportation and storage. The use of cryoprotective agents, such as trehalose, was shown to enhance the survival and functional properties of hMSCs during hypothermic storage. Additionally, careful temperature and oxygen control during hypothermic storage was crucial for preventing cellular damage and maintaining cell viability (81).

Correia et al. (82) presents an innovative method for hypothermic storage of human pluripotent stem cell-derived cardiomyocytes. The study was conducted to assess the viability and functionality of 2D monolayers and 3D aggregates after being stored at low temperatures for long periods. The research involved the utilization of cardiomyocytes, which were stored in a hypothermic solution at 4°C for up to 7 days. The cells were then analysed to determine their viability, proliferation, and functionality. The results showed that the cells remained viable and functional after hypothermic storage, with no significant differences observed compared to cells that were not stored (82).

A well-known method for preserving and transporting cells safely using hypothermic storage involves the use of various natural and synthetic polymers for cell encapsulation (79). Currently, alginate appears to be the most suitable material for producing hydrogel microspheres that can effectively contain viable cells. This is due to the fact that alginate has no adverse effects on cells (83), and its physicochemical properties are similar to those of the extracellular matrix (84). Furthermore, the process of gel formation can occur under physiological conditions. When cells are enclosed within AMS, the hydrogel serves as a barrier against molecules with a mass greater than 100 kDa, but still allows diffusion of oxygen and nutrients (85).

Chen et al. (86) successfully stored human MSCs and mouse embryonic stem cells (ESCs) in alginate hydrogel for 5 days at room temperature (18 - 22°C) in sealed conditions. After release from the hydrogel, the viability of human MSCs and mouse ESCs was 74% and 80% respectively. What is important is that the level of proliferation and the main markers of stem cells in both human MSCs and mouse ESCs released from alginate hydrogel were equivalent to those in cryopreserved cells. Encapsulation of cells in alginate was found to be effective in preserving cell viability during 72 hours of hypothermic storage, with the optimal temperature being 15°C. This approach helped in maintaining the adhesive characteristics of cells even after they were returned to conditions of monolayer cultivation. Furthermore, the cells exhibited a normal phenotype, characteristic growth kinetics, and retained their ability to induce differentiation after attachment. Swioklo et al. (87) conducted a study on hypothermic storage of MSCs obtained from adipose tissue of an adult. The study involved storing the cells at temperatures ranging from 4 - 23°C, provided they are encapsulated in 1.2% alginate. This approach gave good results during 72 hours of storage, while the preservation of cells largely depended on the optimum temperature being 15°C. At this temperature, encapsulation in alginate contributed to the preservation viability of cells and their adhesive characteristics after returning to conditions of monolayer cultivation. After attachment, the cells remained normal in phenotype, demonstrated characteristic growth kinetics and capacity to induce differentiation. It was shown that after encapsulation of MSCs of adipose tissue origin and their storage for 5 and 12 days, after recovery, the cells demonstrated a strong differentiation potential, expression of immunomodulatory molecules and maintained viability at the level of 77 ± 6% (77).

Hypothermic preservation solutions

The hypothermic preservation of cells, tissues and organs as well as tissue engineered constructs takes place at temperatures above 0°C.

Unlike during cryopreservation where the metabolic activity almost comes to a halt, it is reduced at hypothermic temperatures. Hence, cells still require nutrients to stay metabolically active at hypothermic temperatures and produce waste products (88, 89, 90, 91, 92).

According to Arrhenius, the metabolic activity is coupled to the temperature. Thus, a decreased temperature influences cellular transport processes. In the case of hypothermic preservation, this effects the ionic transport via Na^+/K^+ pumps, causing alterations in Na^+/K^+ ATPase and leading to cell oedema, the depletion of ATP reserves combined with rising ADP levels, and reactive oxygen species (ROS) (81, 88, 89, 90, 91, 93, 94, 95, 96, 97). Kerkweg et al. have also reported the impact of ROS on temperature- induced apoptosis (91, 98). Additionally, cell swelling also effects intracellular pH (90, 99).

Therefore, special preservation solutions are required in order to protect the cells against cell injury during and post-preservation. Belzer et al. (100) have previously defined five properties that hypothermic preservation solutions have to fulfil, these are:

1. minimize cell swelling
2. inhibit intracellular acidosis
3. preserve interstitial space
4. prevent ROS-related injury
5. provide energy-compounds for reperfusion.

One of the first solutions, dating back to the 1960s, was the Collins solution (Table 1), later modified to todays Euro-Collins solution (91, 93, 94). Develped in 1988, the University of Wisconsin (UW) solution is the current 'gold standard' (93, 94, 81, 97) and is used for various organ preservations such as heart, lung, liver, kidney or pancreas (101–104).

Over time, this solution has been modified (99, 101, 105, 106) and other solutions have been developed, aiming to enhance post-preservation outcomes. Known and widely applied solutions are Celsior, Histidine-Tryptophan-Ketoglutarate (HTK), and HypoThermosol (HTS).

Generally, in order to protect cells during hypothermic storage, the preservation solutions include different substances, supporting specific parameters during preservation.

Key components of all solutions are Na^+ and K^+, since these ions are of upmost importance for cellular metabolic activity and need to compensate reduced ionic transport upon preservation (92, 96).

Buffers, such as phosphates, bicarbonate, histidine or HEPES are applied to stabilize pH

Table 1. Composition of different hypothermic preservation solutions. The abbreviations of the solutions are University of Wisconsin solution (UW), Euro-Collins (EC), Celsior® (CS), Histidine-Tryptophan-Ketoglutarate (HTK), HypoThermosol (HTS), Institut Georges Lopez-1 (IGL-1), BES-gluconate-sucrose (BGS), sucrose-based solution (SBS), Marshall's Solution (MS). BGS was developed by Mandolino et al. The abbreviations of the components are phosphate (Phos), bicarbonate (HCO_3), histidine (His), N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), lactobionate (Lac), hydroxyethyl starch (HES), raffinose (Raf), mannitol (Man), glucose (Glc), dextran (Dex), sucrose (Suc), polyethylene glycol (PEG), adenosine (ADN), glutathione (GSH), allopurinol (AP), dexamethasone (DXM), gluconate (Glu), tryptophan (TRP), ketoglutarate (KG) and bovine serum albumin (BSA). (73–99).

Components		UW	EC	CS	HTK	HTS	IGL-1	BGS	SBS	MS
Electrolytes	Na^+	30	10	100	15	100	125	-	<15	<95
	K^+	125	115	15	<93	42.5	30	25	<30	<84
Buffers		Phos	Phos HCO3	His	His	HEPES	Phos	Phos BES	Phos	-
Impermeants / Colloids		Lac HES Raf	Man Glc	Lac Man	Man (Sugars)	Lac Man Dex Suc Glc	Lac Raf PEG	Glu PEG (Sugars)	Suc PEG	Man
Pharmalogical agents		ADN GSH AP DXM Insulin	-	GSH Glu	TRP KG	ADN GSH	ADN GSH	ADN GSH AP	BSA	-
Osmolality*		320	340	360	310	350	320	335	310	300
pH		7.4	7.3	7.3	7.2	7.6	7.4	7.4	7.4	7.1

All units in mmol/L unless indicated otherwise.

* Osmolarity in mOsm/kg.

values and thus to supress metabolic acidosis (90, 92, 93, 96, 107).

The addition of impermeant but osmotically active substances or colloids, shall counteract osmotic stress and therefore prevent cellular oedema. Widely utilized impermeants are lactobionate, mannitol, sugars (such as sucrose and raffinose), and polyethylene glycol (PEG). Colloids, added as oncotic support also include hydroxyethyl starch (HES) or dextran (81, 90, 92, 93, 94, 95, 96, 108).

Pharmalogical agents are widely added to hypothermic preservation solutions. For example, supplementation with the ATP-precursor adenosine functions as an energy resource upon rewarming. Antioxidants, such as allopurinol, glutathione (GSH), tryptophan or mannitol help prevent the cells from harmful ROS-related effects (92, 93, 94, 96, 105, 109).

According to Southard et al. not all components of the preservation solutions are known to be vitally essential and to induce the required preservative effect (94, 105).

Comparably new solutions are sucrose-based solutions (SBS). SBS show a low ionic strength. Somov et al. demonstrated the successful preservation of cellular bioenergetics (97). Also, Kravchenko et al. (110) as well as Tarusin et al. (111) reported the alternative utilization of SBS with hepatocytes and MSCs, respectively, compared to UW. Additionally, the protective application of other sugars, such as trehalose has been reported (112).

Studies comparing the effectiveness of different hypothermic preservation solutions have shown different outcomes. This is majorly governed by the set parameters, such as storage temperature and duration. For example, Mathew et al. (113) have analysed different cell types with UW and HTS. They showed that the cells react differently to the applied solutions. Thus, there is no universal hypothermic preservation solution but individual, specialised solutions with correlating preservation parameters. In general, the preservation of MSCs (81, 111, 112, 114, 115, 116, 117), HUVEC (118) and hepatocytes (98, 99, 106, 110, 119, 120, 121, 122) is possible with the respective storage solution.

REFERENCES

1. Chian R-C & Quinn P (2010) (eds) *Fertility Cryopreservation*, Cambridge University Press 271 pp.
2. Buriak IA, Elliott G, Fleck RA et al. (2022) in *Cell Engineering and Regeneration. Reference Series in Biomedical Engineering*, (eds) Gimble JM et al., Springer, Cham., pp 1–60.
3. Mazur P (1984) *The American Journal of Physiology* **247,** C125-42.
4. Elliott GD, Wang S & Fuller BJ (2017) *Cryobiology* **76,** 74–91.
5. Wowk B (2007) *Cryonics* **Third Quarter,** 3–7.
6. Hubel A (1997) *Transfusion Medicine Reviews* **11,** 224–233.
7. Raju R, Bryant SJ, Wilkinson BL et al. (2021) *Biochimica et Biophysica Acta. General Subjects* **1865,** 129749.
8. Elliott JAW & Toner M (2019). *Langmuir : the ACS Journal of Surfaces and Colloids* **35,** 7335–7336.
9. Mazur P (1970) *Science (New York, N.Y.),* **168,** 939–949.
10. Pancrazio JJ, Wang F & Kelley CA (2007) *Biosensors & Bioelectronics* **22,** 2803–2811.
11. Pegg DE (2002) *Seminars in Reproductive Medicine* **20,** 5–13.
12. Stewart S & He X (2019) *Langmuir : the ACS Journal of Surfaces and Colloids* **35,** 7414–7422.
13. Dovgan B, Barlič A, Knežević M et al. (2017). *The Journal of Membrane Biology* **250,** 1–9.
14. Crowe JH, Carpenter JF & Crowe LM (1998) *Annual Review of Physiology* **60,** 73–103.
15. Whaley D, Damyar K, Witek RP et al. (2021) *Cell Transplantation* **30,** 963689721999617.
16. Awan M, Buriak I, Fleck R et al. (2020) *Regenerative Medicine* **15,** 1463–1491.
17. Verheijen M, Lienhard M, Schrooders Y et al. (2019) *Science Reports* **9,** 4641.
18. Tunçer S, Gurbanov R, Sheraj I et al. (2018) *Science Reports* **8,** 14828.
19. Chatterjee A, Saha D, Niemann H et al. (2017) *Cryobiology* **74,** 1–7.
20. Abazari A, Meimetis LG, Budin G et al. (2015) *PloS One* **10,** e0130323.
21. Bragg JT, D'Ambrosio HK, Smith TJ et al. (2017) *Chembiochem : a European Journal of Chemical Biology* **18,** 1863–1870.
22. Zhang Y, Wang H, Stewart S et al. (2019) *Nano Letters* **19,** 9051–9061.
23. Campbell LH & Brockbank KGM (2011) *In*

Vitro Cellular & Developmental Biology. Animal **47,** 195–199.

24. Mutsenko V, Barlič A, Pezić T et al. (2019) *Cryobiology* **91,** 104–114.

25. Pollock K, Budenske JW, McKenna DH et al. (2017) *Journal of Tissue Engineering and Regenerative Medicine* **11,** 2806–2815.

26. Pollock K, Yu G, Moller-Trane R et al. (2016) *Tissue Engineering, Part C, Methods* **22,** 999–1008.

27. Pi C-H, Yu G, Petersen A et al. (2018) *Scientific Reports* **8,** 16223.

28. Pi C-H, Dosa PI & Hubel A (2020) *Journal of Biomechanical Engineering* **142**(7), 071006.

29. Gertrudes A, Craveiro R, Eltayari Z et al. (2017) *ACS Sustainable Chem. Eng.* **5,** 9542–9553.

30. Craveiro R, Castro V, Viciosa MT et al. (2021) *Journal of Molecular Liquids* **329,** 115533.

31. Hornberger K, Li R, Duarte ARC et al. (2021) *AIChE J,* **67(2)**, e17085.

32. Jesus AR, Duarte ARC & Paiva A (2022) *Scientific Reports* **12,** 8095.

33. Jesus AR, Meneses L, Duarte ARC et al. (2021) *Cryobiology* **101,** 95–104.

34. Spindler R & Glasmacher B (2009) *KI - Kälte, Luft, Klimatechnik* **45(4),** 28–31.

35. Acharaya T & Devireddy RV (2010) *The Open Biotechnology Journa,* **4,** 26–35.

36. Liu K, XuY & Yu H (2019) *Chem. Res. Chin. Univ.* **35,** 542–548.

37. William N & Acker JP (2020) *Cryobiology* **97,** 217-221.

38. Prykhodko MV, Tymkovych MY , Avrunin OG et al (2018) in *Proceedings of the 11th International Conference on Bioelectromagnetism, Aachen, Germany, ICBEM*, 4 pp.

39. Tymkovych M, Avrunin O, Gryshkov O, Semenets V & Glasmacher B (2019) in *2019 IEEE 39th International Conference on Electronics and Nanotechnology (ELNANO).*

40. Tymkovych M, Gryshkov O, Se-livanova K, Mutsenko V, Avrunin O & Glasmacher B (2021) *Process for Cryo-preservation. EMBEC 2020,* 102–111.

41. Kühlbrandt W (2022) *Faraday Discussions,* **240,** 323–335.

42. Lai X, Yang M, Wu H et al. (2022) *Micromachines* **13(8)**, 1363. https://doi.org/10.3390/mi13081363.

43. Nguyen N-T (2004) in *Mikrofluidik,* Vieweg+Teubner Verlag. https://doi.org/10.1007/978-3-322-80069-

44. Nie J, Fu J & He Y (2020) *Small,* **16(46),** e2003797.

45. Kim H, Zhbanov A & Yang S (2022) *Biosensors* **13(1)**, 13. https://doi.org/10.3390/bios13010013

46. Szafraniec HM, Valdez JM, Iffrig E et al. (2022) *Lab on a Chip* **22,** 1565–1575.

47. Convery N & Gadegaard N (2019) *Micro and Nano Engineering* **2,** 76–91.

48. Isachenko V, Isachenko E, Katkov II et al. (2004) *Biology of Reproduction* **71,** 1167–1173.

49. Tirgar P, Sarmadi F, Najafi M et al. (2021). *Biomicrofluidics* **15,** 34104.

50. Zhao X-M, Xia Y & Whitesides GM (1997) *J. Mater. Chem.* **7,** 1069–1074.

51. Scott SM & Ali Z (2021) *Micromachines* **12(3)**, 313.

52. Salieb–Beugelaar GB, Liu K & Hunziker P (2018) *Adv. Eng. Mater.* **20,** 1800243.

53. Levine LM & Campbell T (2007) *NSTI Nanotechnology Conference and Trade Show* 2007, **3**, 385.

54. Leal-Marin S, Kern T, Hofmann N et al. (2021) *Journal of Biomedical Materials Research Part B: Applied Biomaterials* **109,** 1198–1215.

55. Marques LS, Fossati AAN, Rodrigues RB et al. (2019) *Sci Rep* **9,** 15353.

56. Vanderzwalmen P, Ectors F, Panagiotidis Y et al. (2020) *Reprod. Med.* **1,** 142–157.

57. Arav A (2022) *Cells* **11,** 1072.

58. Lin M, Cao H & Li J (2023) *Acta Bio-materialia* **155,** 35–56.

59. Gangwar L, Phatak SS, Etheridge M et al. (2022) *CryoLetters* **43,** 303–315.

60. Zhan L, Han Z, Shao Q et al. (2022) *Nature Communications* **13,** 6017.

61. Foussat A, Rietze R, Pottier E, Schryver B, Thompson ML & Ehrhardt RO (2014) *BioProcess International* **March 2014, Supplement**, (online). https://bioprocessintl.com/category/2014/march-2014-supplement/.

62. Simione FP & Thermo Fisher Scientific (2012), *A Guide for Proper Cryogenic Preservation*, (online) https://tools.thermofisher.cn/content/sfs/manuals/D21111.pdf

63. Hunt CJ (2019) *Transfusion medicine and hemotherapy* **46,** 134–150.

64. Rittinghaus T (2021) *Float-Verfahren zur Kryokonservierung von Erythrozytenkon-zentrat*, Doctor Thesis, Hannover.

65. Solanki PK, Bischof JC & Rabin Y (2017) *Cryobiology* **76,** 129–139.

66. Spindler R (2012) *Einfluss der Nukleationstemperatur und der Konzentration an Frierschutzmitteln auf die Kryokonservierung humaner Zellen*, Doctoral Thesis, Gottfried Wilhelm Leibniz Universität Hannover, Diss., 2012, XIV, 152 S.

67. Hunt CJ (2019) *Transfusion Medicine and Hemotherapy* **46,** 134–150.

68. Schiewe MC, Freeman M, Whitney JB et al. (2019) *Journal of Assisted Reproduction and Genetics* **36,** 5–14.

69. Rittinghaus T, Keller JN, Nussbeck SY & Glasmacher B (2021) *KI-Kälte Luft Klimatechnik* **08-09-2021,** 52–55.

70. Zhou X, Liu Z, Liang XM et al. (2013) *International Journal of Heat and Mass Transfer* **65,** 381–388.

71. Sharma A, Lee CY, Namsrai B-E et al. (2023) *Ann Biomed Eng* **51,** 566–577.

72. Chiu-Lam A, Staples E, Pepine CJ et al. (2021) *Science Advances* **7(2),** eabe3005, 8 January 2021.

73. Bischof JC & Diller KR (2018) *Annual Review of Biomedical Engineering* **20,** 301–327.

74. Etheridge ML, Xu Y, Rott L et al. (2014) *Technology* **02,** 229–242.

75. Xuan X (2022) *Electrophoresis* **43,** 167–189.

76. Aijaz A, Li M, Smith D et al. (2018) *Nature Biomedical Engineering* **2,** 362–376.

77. Branco A, Tiago AL, Laranjeira P et al. (2022) *Bioengineering (Basel, Switzerland),* **9(12),** 805.

78. Janowska-Wieczorek A, McGann LE et al. (2015) *Cryobiology,* **71,** 181–197.

79. Gryshkov O, Hofmann N, Lauterboeck L et al. (2015) *Cryobiology* **71,** 103–111.

80. Fuller BJ, Petrenko AY, Rodriguez JV et al. (2013) *CryoLetters* **34,** 432–452.

81. Petrenko Y, Chudickova M, Vackova I et al. (2019) *Stem Cells International* **2019,** 5909524.

82. Correia C, Koshkin A, Carido M et al. (2016) *Stem Cells Translational Medicine* **5,** 658–669.

83. Paredes Juárez GA, Spasojevic M, Faas MM et al. (2014) *Frontiers in Bioengineering and Biotechnology* **2,** 26.

84. Maia FR, Fonseca KB, Rodrigues G et al. (2014) *Acta Biomaterialia* **10,** 3197–3208.

85. Nicodemus GD & Bryant SJ (2008) *Tissue Engineering. Part B, Review,* **14,** 149–165.

86. Chen B, Wright B, Sahoo R et al. (2013) *Tissue Engineering, Part C, Methods* **19,** 568–576.

87. Swioklo S & Connon CJ (2016) *Expert Opinion on Biological Therapy* **16,** 1181–1183.

88. Freitas-Ribeiro S, Reis RL & Pirraco RP (2022) *PNAS Nexus,* **1(4),** pgac212.

89. Boutilier RG (2001) *The Journal of Experimental Biology* **204,** 3171–3181.

90. Fuller BJ, Petrenko AY, Rodriguez JV *et al.* (2013) *CryoLetters* **34,** 432–452.

91. Hawkins BJ, Abazari A & Mathew AJ (2017) *Cell Gene Therapy Insights* **3,** 345–358.

92. 'tHart NA, Leuvenink HG & Ploeg RJ (2002) *Transplantation Reviews* **16,** 131–141.

93. Latchana N, Peck JR, Whitson B et al. (2014). *Journal of Thoracic Disease* **6,** 1143–1149.

94. Guibert EE, Petrenko AY, Balaban CL et al. (2011) *Transfusion Medicine and Hemotherapy* **38,** 125–142.

95. Mandolino C, Dolores Pizarro M, Quintana AB et al. (2011) *Annals of Hepatology* **10,** 196–206.

96. Baust JM (2005) *BioProcess International* **3,** 46–56.

97. Yu Somov A, Semenchenko OA, Green CJ et al. (2009) *CryoLetters* **30,** 1–12.

98. Kerkweg U, Li T, Groot H de et al. (2002) *Hepatology (Baltimore, Md.)* **35,** 560–567.

99. Mamprin ME, Petrocelli S, Guibert E et al. (2008) *CryoLetters* **29,** 121–133.

100. Belzer FO & Southard JH (1988) *Transplantation* **45,** 673–676.

101. Hall SM, Komai H, Reader J et al. (1994) *The American Journal of Physiology* **267,** L508-17.

102. Steffen R, Krom RA, Ferguson D et al. (1990) *Transpl Int* **3,** 133–136.

103. Gu K, Kin S, Saitoh Y et al. (1996) *Transplant Proc* **28,** 1906–1907.

104. Paushter DH, Qi M, Danielson KK et al. (2013) *Cell Transplantation* **22,** 1113–1121.

105. Southard JH & Belzer FO (1993) *Transplantation Reviews* **7,** 176–190.

106. Shanina IV, Kravchenko LP, Fuller BJ et al. (2000) *Cryobiology* **41,** 315–318.

107. Baicu SC & Taylor MJ (2002) *Cryobiology* **45,** 33–48.

108. Petrenko A, Carnevale M, Somov A et al (2019) *Transfusion Medicine and Hemotherapy* **46,** 151–172.

109. Southard JH, van Gulik TM & Ametani MS et al. (1990) *Transplantation* **49,** 251–

257.

110. Kravchenko LP, Petrenko A, Somov A et al. (2001) *Cryobiology* **42,** 218–221.

111. Tarusin DN, Petrenko YA, Semenchenko OA et al. (2015). *Probl Cryobiol Cryomed* **25(4),** 329–339.

112. Di G, Wang J, Liu M et al. (2012) *Journal of Cellular Physiology* **227,** 879– 884.

113. Mathew AJ, Baust JM, van Buskirk RG et al. (2004) *Tissue Engineering* **10,** 1662–1671.

114. Corwin WL, Baust JM, Baust JG et al. (2014) *Cryobiology* **68,** 215–226.

115. Ginis I, Grinblat B & Shirvan MH (2012) *Tissue engineering. Part C, Methods* **18,** 453– 463.

116. Chen Y, Yu B, Xue G et al. (2013) *Cell Transplantation* **22,** 1075–1086.

117. Heng BC, Cowan CM & Basu S (2008) *Cytotechnology* **58,** 69–75.

118. Post ICJH, de Boon WMI, Heger M et al. (2013) *Experimental Cell Research* **319,** 2501–2513.

119. Gramignoli R, Dorko K, Tahan V et al. (2014) *Cell Transplantation* **23,** 1143–1151.

120. Pless G, Sauer IM & Rauen U (2012) *Cell Transplantation* **21,** 23–37.

121. Duret C, Moreno D, Balasiddaiah A et al. (2015) *Cell Transplantation* **24,** 2541–2555.

122. Ostrowska A, Gu K, Bode DC et al. (2009) *Archives of Toxicology* **83,** 493–502.