Cryopreservation: Methods, Equipment and Critical Concerns

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Abstract

The technique of cryopreservation aims to maintain the biological samples in a metabolic suspension state, by which all chemical, physical and biological changes are diminished. The biology principles behind cryopreservation are widely investigated in the literature. However, little scientific interest has been focused to the current equipment used as cryogenic storages and how they affect the viability and functionality of the cells during their preservation. This review paper attempts to bridge the gap between the biology and engineering aspects of cryopreservation. The basic principles of cryobiology, the cryoinjury mechanisms, the role of cryoprotective agents, the state of the art cryopreservation methods and equipment, and concerns regarding the use of liquid nitrogen as cryogenic refrigerant are covered. Further to our knowledge there is no complete review in the literature describing the connection between the current cryogenic equipment and the physical events taking place during cryopreservation. The paper aims to provide researchers with the key knowledge of cryopreservation, not only from a biological point of view, but from a technical aspect, too, in order to contribute to the development of the cryobiology field.

Introduction

There has been a steady and continuous growth of the cryobiology field. Scientists try to gain better understanding of the effects of the effects of low temperatures and of freezing of living cells. Significant development of robust protocols and devices aim to overcome the freezing injury, in order to achieve viable storage conditions and to ensure the maximum rate of cells' post-recovery. Long term preservation of cells for scientific, industrial and medical applications is achieved when cells are stored at cryogenic temperatures. At this temperature range all biological, physical and chemical processes of any aqueous system (e.g. cells) are diminished, as there is insufficient thermal energy for chemical reactions and water does not exist in liquid phase below -130°C. Consequently, several medical and biological applications have benefited from storing biological materials at cryogenic temperatures. Red blood cells and platelets can be transfused after years of storage, artificial insemination, banking of cells and tissues, bone marrow transplantation, in-vitro fertilization, facilitated transport of cells and tissues, storage of seeds for reproduction purposes and many more applications are nowadays possible with cryopreservation. However, one of the challenges in the field of cryopreservation is the development of effective cryogenic storage systems, which guarantee the cells' viability and functionality after their recovery.

Cryobiology principles

There are four major steps in cryopreservation and the recovery process of cells. First, cryoprotective agents (CPAs) are added to the cells, in order to protect them from the freezing process. Then, the cells are cooled, in a controlled manner, until they reach a temperature below -130°C, at which all the metabolic processes of the cell stop. The recovery of the cells follows the reverse procedure; firstly the cells are thawed following a defrost protocol and then the CPAs are removed. The viability of the cells can be impacted by each of these steps. Many experimental findings indicate that cryopreserved living cells have been damaged or even destroyed by the freezing process; a type of damage called cryoinjury. Although, the cryoinjury of the cells is not caused by the cell's inability to endure low-cryogenic storage temperatures, cells are damaged due to the effects of an intermediate zone of temperatures in the range of -15 to -60°C, which they must traverse twice; once during cooling and once during thawing [1]. The cause of damage in these temperatures is the phase change of water in the extracellular and intracellular solution. Water is the main substance of all living cells. When all water is converted into ice, the cellular metabolism stops. Thus, it is important for the refrigeration system to provide slow cooling, in order to freeze the external of the cell without developing

intracellular ice. As ice forms at the extracellular environment of the cell, an osmotic imbalance occurs between the intracellular and extracellular mediums, which cause the discard of water out of the cell. As a result, an increase of solute concentrations outside and inside the cell occurs, which often means the death of the cell. On the other hand, when the cell maintains too much water on its intracellular medium, ice crystals begin to form, which will recrystallize during thawing and damage the cell.

Mechanisms of Cryoinjury

As the cell is cooled down to approximately -5°C, both its intracellular and extracellular mediums remain unfrozen and supercooled. Supercooling is a phase during which the temperature of the sample is below its freezing point, but its cellular infrastructure does not form ice crystals. Between -5°C and approximately -10°C, the chemical potential of the supercooled water inside the cytoplasm is increasing and causes the water to flow out the cell. With further reduction of the temperature, ice begins to form in the extracellular solution, but the intracellular solution remains supercooled and unfrozen, as the plasma membrane restrains the ice crystals growth inside the cytoplasm. The physical events that occur below -10°C, depend on the cooling rate to which the cell is being exposed. These mechanisms have been extensively described by Peter Mazur in his 'two-factor hypothesis' [2]. Mazur's theory suggests that if an optimal cooling rate is not applied, the cells are damaged due to the effects of water's transport rates.

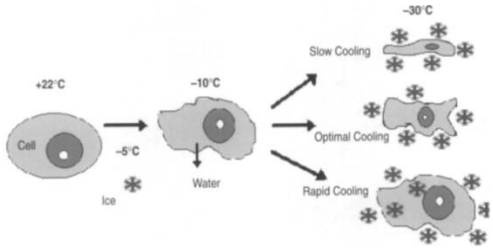

Figure 1: Schematic of physical events in cells during freezing [3]

Figure 1 represents the physical events in cells during freezing. In the case of rapid cooling, the water inside the cell does not have enough time to be transferred in the extracellular medium, as the chemical potential of the extracellular water reduces faster than the rate by which the intracellular water can flow out of the cell. As a result, the cytoplasm becomes increasingly supercooled and the only way to reach an osmotic equilibrium with the extracellular solution is to freeze internally [4]. This mechanism of the cell is known as *Intracellular Ice Formation* (IIF) and has lethal effects on cell viability [5,6]. Figure 2 shows photomicrographs of IIF in mouse oocytes cooled at 100°C/min. On the left of the figure 2 (A) the intact oocytes prior freezing are shown. A well-defined boundary between the extracellular and intracellular mediums is clearly shown. The middle photo (B) shows the frozen oocytes, as ice has propagate from the external solution to the intracellular solution; the blackening of the oocytes indicates the IIF and the darken background is due to the ice of the CPA solution. Finally, the right photo (C) shows the recovery of oocytes, after they have been thawed. It is apparent that the cell membrane which separated the extracellular and intracellular medium does no longer exist, indicating the damage of cell's morphology [7].

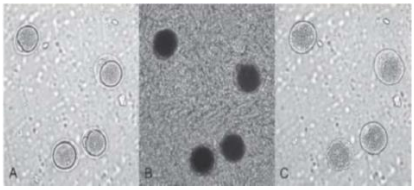

Figure 2: Rapid cooling injury of mouse oocytes [7]

In the case of slow cooling, the rate of water transport from the cytoplasm towards the extracellular medium is fast enough to eliminate excessive levels of supercooling, to dehydrate the cell and to eventually preclude IIF. However, several other events take place, causing the damage of the cell, which are known as *solution effects*. This general damage classification includes extreme dehydration of lipid vesicles and cell's membrane, high solute concentrations mainly electrolytes [8], which cause the denaturation of lipoproteins of cell's membrane, mechanical damage due to formation of 'cell channels' between adjacent ice crystals [9–

12], and finally, severe volume shrinkage, which applies physical compression forces in the cell's membrane and causes loss of cell's membrane area [13–16]. Mazur and Cole studied the survival rate of red blood cells, after they have been slowly frozen, and they found that the cause of damage changes according to the unfrozen fraction of water in the cell [10]; when the unfrozen water fraction of the cell is greater than 20% then, cell damage is caused by osmotic dehydration and the cell's survival depends on the electrolyte concentration. On the other hand, if the unfrozen water fraction of the cell is between 5 to 15% then, cell damage is caused by mechanical effects of cell-to-cell interaction, as they aggregate into small channels as they are excluded from the ice [17]. Figure 3 shows photomicrographs of frog red blood cells, for different value of unfrozen fraction of water, during slow freezing. One can notice that cells are confined to channels of unfrozen solution between the formed ice crystals and, as the unfrozen fraction of water reduces, the channels' diameter and cells' volume decrease, as well [5].

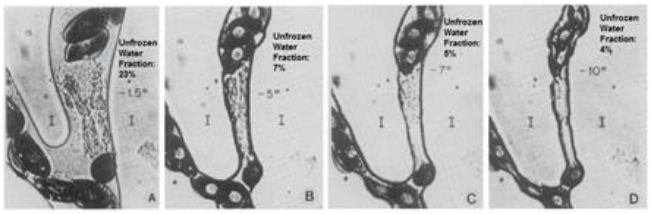

Figure 3: Slow cooling injury – Cells' aggregation into channels [5]

However, if the cooling rate complies with the optimal cooling rate of the cell, they can be recovered without showing any significant changes to their morphology, function and viability. At the intermediate zone of temperatures (-15 to -60°C) and while ice starts to form in the extracellular medium and the intracellular medium remains unfrozen and supercooled, an increase of the osmolality of the extracellular medium occurs, as very little solute can be concentrated into the ice [3]. As long as the intracellular and extracellular mediums maintain their osmotic equilibrium as the temperature continues to drop, more ice is progressively formed and the solution concentrations of both mediums continue to increase, until they reach either a eutectic temperature, in which the remaining solution solidifies or a glass transition temperature, in which the remaining solution vitrifies. At this point, the cells can be stored at cryogenic temperatures for long-term periods, without being cryoinjured.

The 'two-factor hypothesis' and the cell's survival rate can be graphically presented as a function of the cooling rate. Figure 4 illustrates the trend of cells' survival according to optimal, rapid or slow cooling rate. The survival curve follows a characteristic inverted U-shape, which its peak corresponds to the optimal cooling rate. This ideal cooling rate allows the optimal survival of the cell, as it is slow enough that precludes IIF and rapid enough to protect the cell form the solution effects.

The optimal cooling rate differs for each cell type, as it depends on the water permeability of cell's membrane, which has unique value for every cell type. The following graph shows the relationship between percentage of survival and cooling rate for three types of cells; mouse bone marrow stem cells, hamster oocytes and human red blood cells. All of the curves have an inverted U-shape, only the values of the optimal cooling rate changes, as it can be seen in Figure 5.

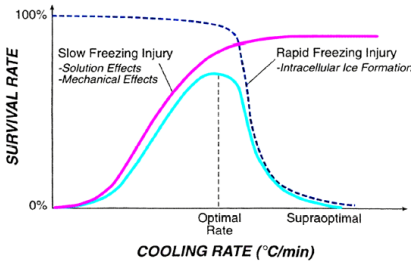

Figure 4: Relationship between the cooling and survival rates of hypothetical cells [18]

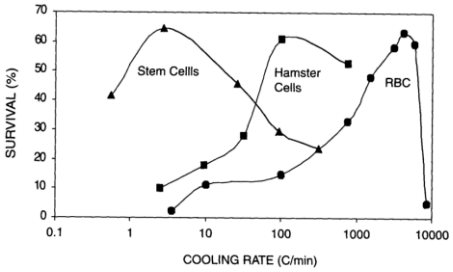

Figure 5: Relationship between percentage of survival and cooling rate for three types of cells [3]

Cryoprotective Agents

The role of cryoprotective agents (CPAs) is to diminish the effects of cryoinjury during freezing or vitrification. CPAs are chemicals added to the cellular solution and alter the physical behaviour of the cell. There are numerous CPAs of different types, which can create hundreds combinations of CPA solutions. The most widespread CPAs are dimethyl sulfoxide (Me_2SO), glycerol, sucrose and trehalose. CPAs are generally categorised into two types, according to their ability to penetrate through the cellular membrane; permeating CPAs, such as Me_2SO, glycerol, 1,2-propanediol, and non-permeating CPAs, mainly polymers (polyvinyl pyrrolidone, hydroxyethyl starch) and sugars. Low concentrations of CPAs are capable of protecting the cells against the solution effects, rather than the IIF, however their mechanism of protection depends on their type [19].

Non-permeating CPAs act by increasing the osmolality of the extracellular solution, which forces the cell to shrink before freeze. In this case, the initial freezing of the cell may require more time, but the intracellular composition, solute concentration and size will not be altered, and they will remain a function of the extracellular osmolality, as if the CPA were never added. Permeating CPAs have the ability to decrease the concentration levels of electrolytes and the amount of extracellular ice formation in the cell. However, they cause the increase of cells' final size, as they penetrate the intracellular membrane, adding more components in cell's volume.

High concentrations of CPAs increase the viscosity of the extracellular and intracellular medium, which reduces dramatically the ice formation process and the crystal growth. As a result, the rate of ice formation is decreasing along with the temperature. Usually, in glass-transition temperatures the viscosity is high enough to stop all phase transitions inside the cell. Ice may forms at any temperature below the melting point of the cell, but when the temperature is above the glass-transition temperature, the ice formation rate can be controlled, by controlling the freezing time between the cooling down from the melting point temperature to the glass-transition temperature [20].

However, high CPA concentrations correspond to high toxicity levels, which damage the cells instead of protecting them [21]. The toxicity of CPAs can be reduced either by using lower concentrations of CPAs or by reducing the period of time of cell's exposure to the CPA solution. Moreover, studies have shown that CPAs increase the retention of intracellular water and thus, enhance the probability of IIF [22,23]. Finally, cells can be damaged by excessive osmotic forces developed during the addition and removal of the CPAs, before and after their cryopreservation [24].

Cryopreservation Methods

The viability and extend of cryoinjury in a cell depend on the amount of water in it and the ability of that water to crystallize during the freezing process. The variables that influence the survival rate of a cell are the concentration of CPAs, the cooling rate, the storage temperature and the rewarming rate used. The effects of cooling rates, the use of cryoprotective agents and their mechanisms of action have been extensively described in the previous section. In this part the significance of the storage temperature and the current equipment system technologies are outlined.

Initial Freezing

All cryopreservation processes, despite the long-term storage method used afterwards, begin with the initial freezing of the sample, during which the sample is transferred from its physiological environment to a cryoprotective solution, to minimise the cryoinjury effects. This phase is also known as *hypothermic phase* and the way it works is by diminishing the cell's metabolism and the chemical toxicity of the CPAs. Biological materials can be introduced into the hypothermic phase either by being directly packaged into the precooled (0 to 4°C) cryoprotective solution and then transferred to a cryopreservation chamber of the same temperature range, or by being placed into a control rate freezer. However, the later method has been proven to result in better post-cryopreservation survival rates, as it provides robust temperature compensation for the release of the latent heat produced by the cells. This heat release is continuous and caused by the freezing process, as ice formation is an exothermic process. [25]

Equilibrium Freezing

Two are the main methodologies currently used for the initial freezing of cells; equilibrium freezing and vitrification. During equilibrium freezing, cells are exposed to relatively low CPA concentrations and then slowly cooled at a controlled cooling rate, in order to gradually freeze the extracellular solution and in this way to reduce the probability of IIF.

During equilibrium freezing, the cells are exposed to low concentrations of CPAs, equilibrated at room temperature for a short period (10 to 25 min), placed into containers (straws or vials), sealed and stored into a controlled-rate freezer. Thus, the cells are exposed to gradual increase of CPAs concentrations, as more extracellular ice is formed.

To avoid the lethal consequences of IIF and solute concentrations, the controlled freezing systems must facilitate the equilibration of the sample and its surrounding medium, at a temperature marginally below the sample's freezing point. To do that, the sample is forced to freeze in specific regions, by introducing several nucleation points in the cell. This process is called *seeding* and is induced extracellularly either by contacting the cell with a cold instrument or by a short-lived introduction of liquid nitrogen (LN_2) into the preservation chamber. As a result a solute gradient is created between the intracellular and extracellular medium, which forces the freezing water to flow out of the cell, minimizing that way the chances of IIF. [26]

Equilibrium freezing has three major disadvantages. First of all, the ice crystals formed in the extracellular solution may cause direct mechanical damage to the cell membrane or other fine structures (e.g. sperm tails) and lead to the loss of cell's biophysical function and viability. Secondly, in order to achieve the highest survival rates of the preserved cells, a very tightly controlled optimal cooling rate is required, which many existing cryogenic systems find difficulties to obtain. Finally, the cooling requirements differ from one cell type to another, as the optimal cooling rate depends on individual cell characteristics. As a result, different cell types require different cooling devices, a fact that limits the application of the equilibrium freezing method [27].

Vitrification

An alternative approach is vitrification, by which any signs of ice formation are prevented. This method maintains the cell as a whole in a vitreous state and precludes both intracellular and extracellular ice formation. In order to achieve that, high concentration of CPAs and a relatively fast cooling rate in excess of the critical cooling rates (the minimum cooling rate to vitrify a solution) are used. During the process, the liquid phase of the cell eventually becomes so viscous that the water molecules do not have enough energy to form crystalline structures, and as a result they start to become amorphous solid or glass. The temperature at which this phenomenon takes place is known as *glass transition temperature* and below this temperature the cell considers to be biologically inert [18].

Vitrification with no or low concentrations of CPAs would be suitable for almost all cell types, but it would require the freezing of the samples at ultra-fast cooling rates, on the order of tens of thousands of degrees Celsius per minute. The only method that approaches ultra-fast freezing is the drop of a small cell suspension volume directly into LN_2, but the cooling rate cannot be controlled. A thin vapour coat film forms around the surface of the sample when plunged in LN_2, insulates the sample and prevents a more rapid temperature loss (Leidenfrost effect). Vitrification protocols that require the direct contact of the sample to the LN_2 raise concerns of potential viral contamination, while the whole process of vitrification is very challenging, due to the limited amount of time of cell handling and loading. New techniques of vitrification, though, show very promising results, which overcome the current shortcomings of rapid freezing. For examples, S^3 vitrification exposes the sample to a series of CPA solutions, loaded into sterile straws (0.25 ml), heat sealed them at both ends and vitrifies them. In this way, there is sufficient time for exposure in the CPA solution, loading and vitrification, while the relatively large sized container and the significant lower cooling rates, simplifies the vitrification process and diminishes the amount of LN_2 used. [28]

Laser Pulse Vitrification

In 1998 *Fowler and Toner* developed a protocol for successful recovery of rapidly frozen red blood cells by vitrification, without the use of CPAs. There technique included the use of a laser pulse prior thawing, which selectively targeted the intracellular ice, so that it was melted and resolidified into a glass phase. The erythrocytes were loaded into straws (1mm x 1μm), plunged into methanol slurry (-96°C) and transferred

into LN_2. The average cooling rate of the samples was approximately 10,000 °C/min. Then, the samples were removed from the LN_2, placed on a cryostage (-170°C) and a laser pulse (7 ns width, 532 nm wavelength) irradiated the haemoglobin, which exists in the intracellular solution. The short laser pulse was able to be absorbed only by the intracellular medium, which to the increase of its temperature above 0°C, while the extracellular solution remained at -170°C. Up to 80% of cells treated that way remained viable after thawing. [29,30]

Liquidus Tracking (LT)
The principle of LT is the dynamic control of CPA concentrations, throughout the cooling process, in order to maintain the cell just above its freezing point at all times, without the formation of ice. As a result, vitrification can occur without the extremely high CPA concentrations and without the need of ultra-rapid cooling rates. LT systems adjust the CPA concentrations, temperature and time program to the characteristics of the stored cells at all stages of the process. LT systems consist of a conventional controlled rate freezer, with a cooling rate of 0.1 to 0.3 °C/min, while the solution composition is controlled by standard peristaltic pumps. [31]

Isochoric and Hyperbaric Cryopreservation
All the above cryopreservation methods are performed under constant pressure of 1atm of the chamber (isobaric process). A new approach is the freezing under pressure, i.e. the freezing under constant volume of the chamber. Two techniques are currently used, the isochoric and hyperbaric cryopreservation, and both of them use none or few amounts of CPA solutions. The isochoric method has proved to minimise significantly the increase of solute concentration during the freezing process, by almost an order of magnitude, compared to the isobaric cryopreservation. Isochoric and hyperbaric methods follow the same theoretical principles (changes in pressure), but they are different processes. The isochoric cryopreservation is a two-phase equilibrium process, in which ice and liquid exist simultaneously at equilibrium under constant temperature and volume, while hyperbaric cryopreservation the solution is maintained in a single phase, as liquid. In hyperbaric cryopreservation, the elevating pressure changes and rapid cooling rates prohibit the ice formation in the solution, thus the biological sample maintains its original composition. However, cells and organs cryopreserved by the hyperbaric method do not show very high survival rates [32–34]. Isochoric cryopreservation offers a more robust method of freezing, by which the system adjusts itself to the minimal pressure for a particular temperature, instead of continuously increasing the pressure of the chamber [35]. Erythrocytes in 5% v/v Me_2SO or 8% v/v glycerol solutions, under 120 MPa pressure and cooling rate of 35°C/min or 160°C/min showed survival rates of 92% or more [36].

Cryopreservation Equipment

LN_2 - Direct Temperature Feedback Method
These systems monitor and control the temperature of the chamber. More advanced systems are additionally monitoring the samples' temperatures and they adjust the chamber's operational temperature accordingly. T-type thermocouples are continuously monitoring and controlling the chamber's and samples' temperatures, compare them to the programmed optimal temperatures and the appropriate amount of LN_2 is determined and injected into the chamber. The delivery of the LN_2 in the chamber is provided usually by dual injection valves for more precise temperature control, faster cooling rates and as a back-up system in case of failure of one of the valves. Solenoids can also be used, but they tend to wear over time [37].

LN_2 - Timed Pulse Method
Similarly to direct temperature feedback systems, timed pulse systems control the chamber temperature. They usually consist of a microprocessor control system, a pump, a heater and one or more solenoid valves with openings, which meter a timed pulse of LN_2 into the chamber. The appropriate amount of LN_2 is determined and injected into the chamber by several variables, such as the valves' sizes, the tank's pressure and the number of solenoid openings. The heater heats the LN_2 to its boiling point, in order to provide constant tank pressure and to ensure that the appropriate amount of LN_2 is provided to the chamber. The disadvantage of timed pulse systems is their low reliability, as the same cooling program can produce different cooling curves of the cells over time [37].

LN_2 - Plunge Freezing Method
This is the simplest method of controlled rate freezing with the use of LN_2 and it is usually used for small numbers and low volume straws and vials. A heat block is loaded with the biological samples and then is submerged into a tank of LN_2. The heater increases or decreases its power in order to achieve a controlled freezing rate of the chamber [37].

Step Down Method - Mechanical Freezers
This is a non-automated freezing method, but is still in practise. The samples are initially cooled down by being placed in a refrigerator for several hours, then transferred to a mechanical freezer and finally stored either into a LN_2 freezer or continue their storage into an Ultra-Low Temperature (ULT) freezer.

ULT freezers follow the principles of any mechanical refrigeration system and consist of the same basic components; a compressor, a preservation area, evaporator, condenser and refrigerant. The refrigeration of the system is provided by the basic vapour-compression cycle, in which the refrigerant circulates through the system and passes through a number of processes. The temperature range of an ULT freezer is typically between -56 to -86°C, but it is important to be able to operate within the range of -70 to -80°C [38]. There is no conventional refrigerant that can cover the temperature gap between ambient room and operational temperatures; for that reason ULT freezers are equipped with two refrigeration circuits, each one with its individual compressor and refrigerants, using a cascade technique. Based on the cascade technique, the first refrigeration cycle is used to cool down the system from ambient to an intermediate temperature, while the second refrigeration circuit is able to further reduce the temperature of the preservation area to the required operational conditions [39].

The disadvantages of step down method are that is a long time process, is difficult to repeat and cannot provide controlled cooling rates. Moreover, the post-thaw recovery rates of cells maintained into ULT are very low, even for robust cells, due to power outages, mechanical failures or human caused mishaps, such as frequent door openings or improper closing of the freezer's door. As far as energy consumption is concerned, ULT systems account for more than 50% of the total energy used in a laboratory space [40].

Alcohol Bath Freezers
These units are commonly used in laboratories, for preservation of cells at temperatures above -80°C. Alcohol bath freezers are basically refrigerated circulators, in which an amount of alcohol is used as cooling medium to pass through a cooling system and exchange temperature, in order to reduce it. These systems require a relatively large volume of cooling medium, in order to provide stable temperatures and accurate controlled cooling rates, due to medium's high heat capacity. However, alcohol bath freezers are not capable of achieving rapid cooling rates. [31]

Vitrification Systems
These systems usually include the rapid transition of the sample to a glassy state, by direct submersion into LN_2. The cooling rate of the equipment can be increased either by the use of negative pressure systems, which depress the freezing point of LN_2 to -205°C, or by minimising the sample's volume. Other available systems freeze the cell by getting them into contact with an aluminium block cooled in LN_2, in order to avoid the direct contact of the sample and the LN_2, for contamination reasons and to eliminate the Leidenfrost effects. [31]

Stirling Engines Freezers
The original idea of Stirling engines is to convert thermal energy to mechanical work. However, their operation principle can be used in reverse and thus cryopreservation systems use Stirling engines to convert mechanical energy to thermal energy; in other words, Stirling engines operate as heat pumps. Most refrigeration systems operate on the Rankine cycle and their cooling capacity depends on the properties of their refrigerant. Triple stage Rankine refrigerators are currently the state of the art of mechanical freezers, but they can achieve temperatures of only -140°C. Sterling cycle is by definition less efficient than Rankine cycle, but is capable of providing lower temperatures than the Rankine cycle. Moreover, they can freeze small amounts of samples and their cooling capacity is much lower than the LN_2 systems. However, due to personal safety and cross-contamination concerns of LN_2 systems, Sterling engines freezers find great application in clean rooms and laboratories where the use of LN_2 is not possible. [31]

Liquidus Tracking Freezers
LT method is relatively a novel technique, thus there are not many types of equipment available. The typical configuration is a conventional slow cooling freezer coupled with a LT controller and two dynamic peristaltic pumps. The cooling is provided by the same way as in the conventional slow rate freezing process, but the LT controller monitors the temperature inside the chamber and adjusts the speed of the peristaltic pumps, in order to alter the concentration of CPA solution of the cells. The decrease of the temperature in the chamber, increases the CPA concentration of the cell, but as the temperature of the cell decreases, the toxicity of the CPA decreases, allowing the sample to tolerate the high CPA concentrations. The only requirements of the LT freezers are the large volumes of CPAs and their proper mixing around the sample. [31]

Isochoric Systems
Isochoric systems are quite simple and inexpensive, however they are not commercially available. One of the systems described in the literature was used by *Rubinsky et al.* and it consists of a constant volume chamber that is hermetically sealed and in which the pressure is monitored with a pressure gauge. The chamber is filled with fluid and is cooled by immersion in a constant temperature bath. The major advantage of isochoric cryopreservation is that the solution concentrations during the freezing are lower at each temperature by almost an order of magnitude, compared to the isobaric cryopreservation [35]; a fact that may increase the post recovery success rates of the cells.

Long-Term Storage in LN_2 - Contamination and Safety Issues
The temperatures at which biological materials are stored have great influences on their shelf life. Generally, the lower the storage temperature, the longer the length of time after which the biological material can be recovered. Ultimate cell viability and stability is achieved, when samples are preserved below -130°C the glass transition temperature of water. It is believed that at this temperature all metabolic processes are retarded and biological stability is obtained, which makes possible the intact storage of samples even for 2,000 years [5,41,42]. The only consideration is the slow accumulation of background ionizing radiation, but the effects on the cells will appear after centuries of storage.

Storage at temperatures of -150°C is typically recommended, because it provides a 20°C safety margin between the storage and glass transition temperature of cells. Storage in LN_2, with a boiling point of -196°C, is a convenient method to maintain low operating temperatures and to provide even greater safety margin for better sample security. The reasons of why LN_2 finds such great application in cryopreservation systems are due to the extremely low cryogenic temperatures that offers, the fact that it is chemically inert, relatively inexpensive, readily available, non-flammable and does not require electrical power to provide freezing; power supply is only used for the electronic features of the systems, such as auto-filling, monitoring and alarms.

However, besides the advantages of LN_2 systems, it is important to outline that the exposure to an environment with high levels of LN_2 in the atmosphere can cause dizziness, unconsciousness and even death, as the molecules of nitrogen displaces the molecules of oxygen. Thus the handling of LN_2 requires extra care, as it is colourless, odourless, and tasteless and it is breathed as if it were air, making its detection by the human senses impossible.

LN_2 storage systems can provide cooling in either liquid or vapour phase. Storage in liquid LN_2 offers uniform temperature profile across the chamber, while the vapour phase storage shows a temperature gradient and relatively higher temperatures than the liquid phase. However, storage in liquid phase LN_2 is contains the risk of contamination and explosion of the samples, as the vials used for packing the biological materials may leak and allow the interaction between the LN_2 and material. Thus, the rest of the samples stored in the chamber can become cross contaminated with microbes [43] and LN_2 can enter the leaking vial. As a result, when the leaking sample is retrieved, the trapped LN_2 will expand, transform into gas, change its volume and cause a small explosion, and the destruction of the sample. [44]

LN_2 usually has a very low microbial count but, during storage and distribution may become contaminated with microorganisms and potentially infect the stored material by transmitting viruses, bacteria, fungi and animal cells in the holding tank [45–47]. By opening the liquid nitrogen container, the water vapour in the air

above the container becomes cooled and forms small ice crystals with high electrostatic charge, which capture airborne microorganisms and fall into the container. However, the contamination can be controlled by the appropriate emptying and cleaning of the vessel. Other potential contaminations can occur from contaminated surfaces or leaking samples. [44]

In 1976, *Schafer et al.* suggested that LN_2 should be treated as biohazardous substance, as the screw cap of the plastic vials allowed contact between the contaminated LN_2 and the sample [48]. Another example of contamination is the Hepatitis B Virus (HBV) outbreak caused by LN_2 storage. In 1995, *Tedder et al.* reported how the bone marrow and stem cells harvested from one patient with HBV led to infect other five patients, who received transplants stored in the same tank [46]. Also, a survey of microbial contamination of hematopoietic stem cells stored are LN_2 freezers was conducted by *Fountain et al.* and showed that of the 583 cultures tested, 1.2% were found to be contaminated by microorganisms [45]. Four out of five freezers examined contained low level of microbial contamination, however the fifth freezer was heavily contaminated with Aspergillus, the same microbes found in the contaminated samples.

A more recent assay of *Grout and Morris* [49] showed that small particles, can be transmitted in the vapour used to cool programmable vapour LN_2 freezers, and as the immediate storage environment for bulk storage and transport of cryopreserved samples and remain suspended in the vapour phase of LN_2 for at least 24 hours. The longer a sample has been held in a storage tank then the greater the risk of contamination from the accumulating microbial load of the bulk cryogen.

Attempts to eliminate the danger of cross contamination include cooling in LN_2 passed through a 0.2µm pore-size filter and placing the carrier (vial, straw, etc.) into a container that partially or completely isolates it from the LN_2 during storage [50–52].

Storage in vapour LN_2 can address the contamination and the explosion problems, however cannot provide the uniform temperature distribution in the chamber, developing that way different temperature gradients along the storage area. However, new designs of vacuum insulated chambers, with low evaporation rates of LN_2 are showing promising results and vapour LN_2 temperatures of -190°C [31]. These vapour LN_2 systems restrict the amount of energy entering the preservation area, ensuring that the temperature of the vapour is maintained close to the liquid LN_2 temperature, and the heat exchange surface extends from the bottom to the top of the freezer.

Discussion

The paper reviewed the cryobiology principles, the cryoinjury mechanisms, the role of cryoprotective agents, the state of the art cryopreservation methods and equipment, and concerns regarding the use of LN_2 as long-term storage systems. Cryopreservation of cells and tissues is a slow developing field, due the complexity of biological samples. The development of more efficient freezing protocols and cryogenic storage systems, which will ensure the proper preservation of biological materials, is crucial. The goal of this review paper is to provide researchers with a better understanding of the physical events occurring during cryopreservation, in respect with the performance of the cryogenic storage equipment.

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