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Section 1 Chapter 1

Cryobiology

Cryobiology: an overview

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Introduction

Cryobiology deals with life at low temperature [1,2]. The word cryobiology is relatively new. Literature search indicates that cryobiology was first used in the early 1950s to describe the newly developing field of low temperature biology [3–9]. Living things must be able to adapt to the changing surface environment of the earth in order to preserve the existence of life itself. The principal effects of cold on living tissue are destruction of life and preservation of life at a reduced level of activity. Both of these effects are demonstrated in nature. Death by freezing is a relatively common occurrence in severe winter storms. Among cold-blooded animals, winter weather usually results in a coma-like sleep that may last for a considerable length of time. Therefore, the definition of cryobiology is to study living organisms at low temperature. In other words, cryobiology is the branch of biology involving the study of the effects of low temperatures on organisms (most often for the purpose of achieving cryopreservation).

In cryobiological applications, much lower temperatures are used are present in natural environments. Liquid nitrogen (at −196°C or −320°F) can either destroy living tissue in a matter of seconds or it can preserve it for years, and possibly for centuries, with essentially no detectable biochemical activity. The end result when heat is withdrawn from living tissue depends on processes occurring in the individual cells. Basic knowledge of the causes of cell death, especially during the process of freezing, and the discovery of methods which prevented these causes, have led to practical applications for long-term storage of both living cells and living tissues. In the industrial food area, the microorganisms used in cheese production can be frozen, stored, and transported without loss of lactic acid-producing activity. In the medical field, it is commonly known that whole blood or separated blood cells can be cryopreserved and stored for their valuable applications.

Water is the fundamental molecule of life. The biochemical constituents of a cell are either dissolved or suspended in water. Water is essential for the survival of all known forms of life; without an environment of water, life would not exist. Water has many distinct properties that are critical for the proliferation of life and these set it apart from other substances. It enables the proliferation of life by allowing organic compounds to react in ways that ultimately allow replication. Water is vital both as a solvent for many of the body's solutes and as an essential part of many metabolic processes within the body. Water is essential and central to these metabolic processes. Metabolic processes are affected by temperature. When the temperature falls, cells may slow down or stop all metabolic processes, and extremely low temperature may cause cell death.

During the physical process of freezing, water tends to crystallize in pure form, while the dissolved or suspended materials concentrate in the remaining liquid. In the living cell, this process is quite destructive. In a relatively slow-freezing process, ice first begins to form in the fluid surrounding the cells, and the concentration of dissolved materials in the remaining liquid increases. A concentration gradient is established across the cell wall, and water moves out of the cell in response to the osmotic force. As freezing continues, the cell becomes relatively dehydrated. Salts may concentrate to extremely high levels. In a similar manner, the acid–base ratio of the solution may be altered during the concentration process.

Dehydration can affect the gross organization of the cell and also molecular relationships, some of which depend on the presence of water at particular sites. Cellular collapse resulting from loss of water may bring into contact intracellular components that are normally separated to prevent any destructive interaction. Finally, as the ice crystals grow in size, the cell walls may be ruptured by the crystals themselves or

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by the high concentration gradients that are imposed upon the walls. To prevent dehydration, steps must be taken to stop the separation of water in the form of pure ice so that all of the cell fluids can solidify together.

Cryobiology is the core of fertility cryopreservation. The earliest application of fertility cryopreservation was in the storage of animal sperm cells for use in artificial insemination. The principal application for human fertility cryopreservation was also begun with sperm freezing, and then with embryo and oocyte as well as gonadal cryopreservation. Knowledge and medical achievement have steadily advanced in the field of fertility cryopreservation, especially with recent oocyte and ovarian tissue cryopreservation. These historic accomplishments in the application of the scientific method can provide overwhelming support for continuing on this path. This chapter will try to set out briefly the scientific background and our current basic knowledge of cryobiology.

Basic science of cryobiology

Nature of water

Water appears in nature in all three common states of matter: vapor, liquid, and solid. Water is a tasteless, odorless liquid at standard temperature and pressure. The color of water and ice is, intrinsically, a very light blue, although water appears colorless in small quantities. Ice also appears colorless, and water vapor is essentially invisible as a gas. The maximum density of water occurs at 3.98°C (39.16°F). Water becomes even less dense upon freezing, expanding 9%. This causes an unusual phenomenon: ice floats upon water, and so organisms can live inside a partly frozen pond because the water on the bottom has a temperature of around 4°C (39°F). The boiling point of water is 100°C (212°F) at sea level and one atmosphere pressure. The freezing point of water is very close to 0°C (32°F) in the presence of nucleating substances, but in their absence it can be supercooled to −42°C (−43.6°F) before freezing. For most substances, freezing and melting points are approximately equal. Therefore, the melting point of ice at one atmosphere pressure is very close to 0°C (32°F). The melting point of water is relatively insensitive to change in pressure because the solid–liquid transition represents only a small change in volume.

The transition between liquid water and solid ice is one of the most commonly observed events in nature. As mentioned above, when water is cooled, it often is taken substantially below the freezing point before ice begins to form. This is because of the need for nucleation to occur before an ice crystal can begin to grow. Nucleation refers to the process by which a minimum crystal is formed, which can then expand. The continued expansion of the crystal is a process known as growth. When an ice nucleus begins to grow, any solutes that are present in the liquid will be excluded from this growing ice front. If the rate of crystal growth is faster than the rate at which diffusion of the particular solutes can carry them away from the ice front, then a concentration gradient will very quickly form in the liquid that surrounds the ice crystal. The concentrated solute will then lower the freezing point of the solution. When a certain amount of ice has formed, the solution at the interface will have a freezing point equal to the temperature of the interface. At this point, ice growth will be limited by diffusion of the solute away from the crystal. If the temperature is reduced to far below the melting point with supercooling speed, the solution may be prevented from reaching this situation of ice crystal nucleation and growth. If water is cooled sufficiently fast enough so that nucleation cannot occur, it is possible to avoid ice crystal formation [10]; this process is known as vitrification.

Temperature measurement

Thermometers measure temperature by using materials that change in some way when they are heated or cooled. In a mercury or alcohol thermometer, the liquid expands as it is heated and contracts when it is cooled, so the length of the liquid column is longer or shorter depending on the temperature. Modern thermometers are calibrated in standard temperature units such as Fahrenheit (F), Celsius (C), or kelvin (K).

Celsius is converted to Fahrenheit by multiplying by 1.8 (or 9/5) and add 32, and to Kelvin by adding 273 (e.g. 37°C is equivalent to 98.6°F and 310K).

Glass transition temperature

The glass transition temperature (T_g) is the temperature at which an amorphous solid becomes brittle on cooling or soft on heating. Glass transition is a pseudo-second phase transition in which a supercooling melt yields on cooling a glassy structure with properties similar to those of crystalline materials. Below T_g, amorphous solids in a glassy state, and most of their joining bonds are intact. It is important to note that T_g is a kinetic parameter and, therefore, parametrically depends on the melt cooling rate. Consequently, the slower the melt cooling rate, the

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lower the value of T_g. In addition, T_g depends on the measurement conditions, which are not universally defined.

At a certain temperature, the average kinetic energy of molecules no longer exceeds the binding energy between neighboring molecules, and growth of an organized solid crystal begins. Formation of an ordered system takes a certain amount of time since the molecules must move from their current location to energetically preferred points at crystal nodes. As the temperature falls, molecular motion slows down further and if the cooling rate is fast enough, molecules never reach their destination: the substance enters into dynamic arrest and a disordered glassy solid form. A full discussion of T_g requires an understanding of mechanical loss mechanisms of specific functional groups and molecular arrangements. The value of T_g is somewhat dependent on the time-scale of the imposed change in contrast to the melting point temperatures of crystalline materials. Time and temperature are interchangeable quantities when dealing with glasses, a fact often expressed in the time–temperature superposition principle. An alternative way to discuss the same issue is to say that a T_g is only a point on the temperature scale if the change is imposed at one particular frequency. Since T_g is dependent on the cooling rate as the glass is formed, the glass transition is not considered a true thermodynamic phase transition by many in the field.

The viscosity at T_g depends on the sample preparation (especially the cooling curve), the heating or cooling curve during measurement, and the chemical composition. Proteins possess a T_g value below which both anharmonic motions and long-range correlated motion within a single molecule are quenched. The origin of this transition is primarily a consequence of caging by glassy water, but it can also be modeled in the absence of explicit water molecules, suggesting that part of the transition reflects internal protein dynamics. Glass formation of water below the melting point can occur, usually through very rapid cooling or the introduction of agents that suppress the formation of ice crystals.

Vitrification

Vitrification is defined as the process of glass solidification of a liquid. The liquid is in a metastable state until it gets below a characteristic temperature, T_g, which is indicated by a sharp exothermic event. This heat loss occurs because of the loss of metastable clusters. Those clusters that have more energy than can be held by the bonds which they are able to form will oscillate for a short time and then disintegrate. Upon reaching T_g, the excess energy will be lost, thereby stabilizing the clusters. Once below T_g, the system is not merely a viscous liquid but is also a solid that is in a stable thermodynamic state. Achieving vitrification with pure water requires very small amounts and incredibly fast cooling. However, it is important to mention that vitrification can also occur in aqueous solution during slow freezing.

Aqueous solutions

A solution is a mixture containing at least two kinds of pure substance. In most solutions, one material predominates and this is called the solvent, with the other compounds being called solutes. When the components of a solution are in different states of matter, the solvent is considered to be the one that does not undergo a change of state upon mixing. Aqueous solutions are important for cryobiology since the freezing of biological systems always involves solutions containing substances such as electrolytes, non-electrolytes, polymers, and so on. During the phase change that occurs with vitrification, the concentration and distribution of the solutions are altered, sometimes accompanied by irreversible chemical reactions.

Molarity and molality

The composition of a solution is described by the concentration of its constitutions. There are two primary ways of expressing concentration: molarity and molality. Molarity is another term for concentration (M) and is the number of moles of solute in 1 liter of solution. Molality (m) is the number of moles of solute associated with 1000 g of solvent. Therefore, molarity is based on the volume of solution whereas molality is based on the weight of solvent. The difference becomes most noticeable when temperature effects are considered. Because the volume of liquids can expand or contract with changes in temperature, molarity can change with change of temperature. By comparison, the weight of solvent is constant with temperature, so molality gives a measure of concentration that is independent of temperature.

Solubility

A property of any particular combination of solute and solvent is the solubility of the solute in the solvent. This is the amount of solute that can be associated with a given amount of solvent in the context of

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a solution. Most solids show a well-defined saturation point in liquid when no more solid can be dissolved. The ratio of solute to solvent at this point defines the solubility. In general, solubility in liquids increases with temperature. Therefore, the solubility is a temperature-dependent property, although there are some exceptions. If a saturated solution is made at a certain temperature and then the temperature of the solution decreases, the solubility of the solution will be exceeded. The solution becomes supersaturated and exists in a metastable state. The solute will precipitate out of solution, usually forming crystals in the liquid. Even in crystalline form, solute molecules continually leave and join the crystal surface, going back and forth from solution to the solid phase. This has the effect of increasing the average crystal size, since small crystals have a high surface energy while large crystals have a small surface energy. The constant movement between crystal and solution tends to minimize the total surface energy of all the crystals present in the solution. The solubility is important for cryobiology because the solubility of solutes in extracellular and intracellular water will be changed following the change of temperature. Therefore, changes in solubility during freezing may induce ice crystal formation and cause cell death.

Colligative properties

The properties that solutions exhibit that arise from the behavior of the collection rather than from the behavior of individual components are called colligative properties. Vapor pressure, boiling point elevation, and freezing point depression are three such properties, depending upon the concentration of the solutions rather than the chemical properties of the constituents.

Osmosis

Water can be transported across semipermeable membranes separating compartments containing different concentrations of solutes. The membrane must be impermeable to the solute but permeable for water. This process is called osmosis and has enormous significance for living organisms. The most important and most widely occurring process for water transfer in and out of living cells is osmosis.

Cell permeability

Changes in the extracellular osmotic pressure will create a situation in which a cell will attempt to attain equilibration by either gaining or losing water until there is no osmotic gradient across the plasma membrane. If the cell volume is measured as a function of time, then it can be seen that equilibrium is only achieved after a certain amount of time has elapsed. The kinetics of water movement out of the cell is determined by the physical structure of the membrane. With biological membranes, the phenomenological permeability is complicated. Since freezing and thawing introduce opportunities for osmotic swelling and shrinkage of cells, it may be important to know the tolerance of each type of cell for the osmotic pressure during a given cell type's response to exposure to low temperatures.

Cryoinjury

Some of the classic papers in the field of cryobiology describe the theories and the mechanisms of cryoinjury during cell freezing and thawing [11–14]. These theories have made great contributions to developments and understanding in cryobiology [15–19]. Cryoinjury has been successfully simulated by changing the concentration of solutes surrounding cells in suspension so as to simulate the changes in concentration that take place upon freezing and thawing when water is subtracted or added back to yield the original pre-freezing concentrations [20–29]. Many theories and mechanisms have been proposed for cryoinjury, but none may exactly explain the nature of the phenomenon. For example, it is not surprising that survival rates can vary from cell type to cell type for the same cooling rate and freezing solution. Although there are mathematical models describing how to calculate appropriate cooling rates for avoiding intracellular ice formation [30–41], the theoretical predictions may not apply for all types of cell, particularly for aqueous solutions supplemented with cryoprotective additives. Some cryoprotectants reduce the injury of cells during freezing and thawing. Cryoprotectants are usually divided into two broad classes based on their ability to diffuse across cell membranes. Penetrating cryoprotectants are able to move across cell membranes whereas non-penetrating agents cannot. Since Chapter 4 specifically deals with cryoprotectants, this chapter will describe the penetrating cryoprotectants and will touch briefly on the potential mechanisms involved in cell cryopreservation.

Cryoprotectants

Discovery of cryoprotectant properties

Although a good survival rate of deep-frozen cells has occasionally been observed without a protective

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agent, a suitable cryoprotectant usually increases the survival rate. Usually the literature indicates that Polge *et al.* [3] first reported that glycerol has cryoprotective function to improve survival rate of frozen–thawed cells (chicken spermatozoa). However, the cryoprotective effect of glycerol was discovered much earlier than is usually stated [42–45]. Although survived cells have been occasionally without a cryoprotectant, the presence of a suitable cryoprotectant usually increases the cell survival rates considerably. The discovery that glycerol, first, and later dimethyl sulfoxide protect eukaryotic cells against freezing damage marked the beginning of modern cryobiology [9].

Today, the most commonly used cryoprotectants in the field are glycerol, dimethyl sulfoxide, ethylene glycol, and propylene glycol. The cryoprotective action of each type of cryoprotective agent must be similar, but although many hypotheses have been proposed to explain their mechanism of action, it is still unclear what role they do actually play in the freezing or vitrification solutions. For example, Lovelock [4] proposed that glycerol acted colligatively (altering the phase diagram of the solution) to reduce the high salt concentration that occurs during freezing. Later, phase diagrams were produced that described the mechanism of action of cryoprotectants, especially dimethyl sulfoxide [15]. Phase diagrams are used to describe equilibrium situations in which two or more phases of matter exist together as pure substances or in solutions. In the freezing system, the primary component is water, and the entire system is a collection of compartments filled with an aqueous solution. As aqueous solutions are cooled, the water forms a crystalline solid that has almost no solubility for the solutes that were in the aqueous solution. As ice forms, the solutes will be confined to the remaining liquid phase, becoming more concentrated. Because this lowers the freezing point of the aqueous phase, the system can remain in equilibrium with a substantial unfrozen fraction. As cooling continues, the solubility limit of the solution will also be reached, leading to the precipitation of solutes. The ternary systems of glycerol–NaCl–water and dimethylsulfoxide–NaCl–water have been described [15,26,27]. From these diagrams, it is clear that the solubility and eutectic behavior of a single solute can be altered significantly by the amount and type of additional solutes introduced into the system. It is also clear that the equilibrium between solids and liquid becomes increasingly complex as the number of components is increased. Therefore, the action of cryoprotectants can be described as lowering the freezing point and reducing/preventing ice crystal formation of aqueous (freezing) solutions.

The following compounds are commonly used cryoprotectants in field of cryobiology. Since Chapter 4 will deal with more details about the cryoprotectants, here we just briefly introduce these classic permeable cryoprotectants.

Glycerol

Glycerol, also known as glycerin or glycerine, is a sugar alcohol. It is a colorless, odorless, viscous, sweet-tasting liquid that is soluble in water and low in toxicity. Each glycerol molecule has a three-carbon chain, with a hydroxyl group (OH) attached to each carbon atom (Figure 1.1a). The hydroxyl groups are responsible for making the substance highly soluble in water and hygroscopic. A hygroscopic substance is one that attracts water molecules from the surrounding environment. It has only slight solubility in organic solvents such as ethyl acetate and diethyl ether, and it does not dissolve in hydrocarbons. Its melting point is 18°C (64.4°F), and its boiling point is 290°C (554°F). Its surface tension is 64.00 mN/m at 20°C, and it has a temperature coefficient of −0.0598 mN/(m K). The glycerol substructure is a central component of many lipids. Glycerol is useful for numerous applications. It is a common component of solvents for enzymatic reagents stored at temperatures below 0°C as the presence of glycerol depresses the freezing temperature of the solution. Glycerol is compatible with other biochemical materials in living cells and is frequently used in cell preservation to reduce damage caused by ice crystal formation.

Dimethyl sulfoxide

Dimethyl sulfoxide, also known as methyl sulfoxide or methylsulfinylmethane, is a clear and colorless liquid. The sulfur center in dimethyl sulfoxide is nucleophilic toward soft electrophiles and the oxygen is nucleophilic toward hard electrophiles (Figure 1.1b). The methyl groups are somewhat acidic in character ($pK_a = 35$) because of the stabilization of the resultant carbanion by the S(O)R group, and so are deprotonated with strong bases such as lithium diisopropylamide and sodium hydride. Dimethyl sulfoxide is an important polar aprotic solvent that dissolves both polar and non-polar compounds and it is miscible in a wide range of organic solvents as well as water.

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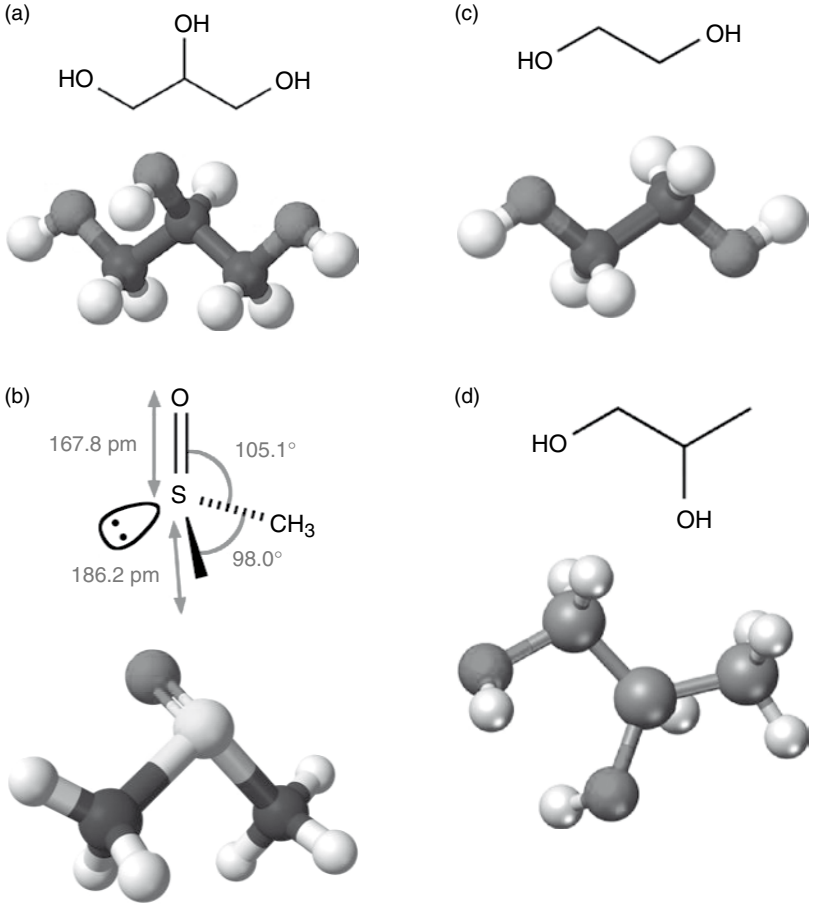

Figure 1.1. The structure of cryoprotectants commonly used in cryobiology: (a) glycerol; (b), dimethyl sulfoxide; (c) ethylene glycol; (d) propylene glycol (1,2-propanediol). (Adapted from Wikipedia.)

It dissolves a variety of organic substances, including carbohydrates, polymers, and peptides, as well as many inorganic salts and gases. Its melting point is 18.5°C (65.3°F), and its boiling point is 189°C (372.2°F). It has a distinctive property of penetrating the skin very readily. Its taste has been described as oyster- or garlic-like. Other reported side effects include stomach upset, sensitivity to light, visual disturbances, and headache. Skin irritation can develop at the site where dimethyl sulfoxide is applied topically. Loading levels of 50–60 wt% are often observed compared with 10–20 wt% with typical solvents. For this reason, dimethyl sulfoxide plays a role in sample management and high-throughput screening operations in drug design [46]. In cryobiology, dimethyl sulfoxide has been used as a cryoprotectant and it is still an important cryoprotectant for vitrification used to preserve organs, tissues, and cell suspensions.

Ethylene glycol

Ethylene glycol, also known as monoethylene glycol or 1,2-ethanediol, is an alcohol with two hydroxyl groups (a diol) (Figure 1.1c). It may also be used as a protecting group for carbonyl groups in organic synthesis and it is widely used as an antifreeze in vehicles. In its pure form, it is an odorless, colorless, syrupy, sweet-tasting, toxic liquid. Its melting point is –12.9°C (8.8°F), and its boiling point is 197.3°C (387.1°F). The major use of ethylene glycol is as a medium for convective heat transfer in, for example, automobiles and liquid-cooled computers. Because of its low freezing point, it is used as a de-icing fluid for windshields and aircraft. It is also commonly used in chilled water air conditioning systems that place either the chiller or the air handler outside, or systems that must cool below the freezing temperature of water. Ethylene glycol is also used in the manufacture of some vaccines, but it

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is not itself present in the vaccines. The major toxicity from ethylene glycol is through ingestion, where it is oxidized to glycolic acid and then oxalic acid, which is toxic. Ethylene glycol and its toxic byproducts first affect the central nervous system, then the heart, and finally the kidneys. Ingestion of sufficient amounts can be fatal. Ethylene glycol is used widely for vitrification, especially oocyte and embryo vitrification.

Propylene glycol

Propylene glycol, known also by its systematic name 1,2-propanediol and as 1,2-dihydroxypropane, methylethyl glycol, methylethylene glycol, Sirlene or Dowfrost, is an organic compound (a diol alcohol) (Figure 1.1d). Propylene glycol is usually a faintly sweet and colorless, clear viscous liquid that is hygroscopic and miscible with water, acetone, and chloroform. It contains an asymmetrical carbon atom, so it exists in two stereoisomers. Propylene glycol has properties similar to those of ethylene glycol. Pure optical isomers can be obtained by hydration of optically pure propylene oxide. Its melting point is −59°C (−74.2°F), and its boiling point is 188.2°C (370.8°F). Propylene glycol usually is used in antifreeze solutions, in hydraulic fluids, and as a solvent. It has numerous applications, for example as a solvent in many pharmaceuticals and as a less-toxic antifreeze, especially for human embryo cryopreservation.

Toxicity of cryoprotectants

The toxicity of cryoprotectants refers to at least two effects. The first is the chemical reacting with cells before cryopreservation, and the second is the chemical causing the change of osmosis of freezing solutions. Relatively low concentrations of cryoprotectants are usually used in cryobiology and, therefore, the chemicals themselves may not be a major concern for toxicity, although the concentration of cryoprotectants used in rapid cooling is relatively high. For assessment of the toxicity of cryoprotectants, it seems necessary to consider the colligative property of the aqueous (freezing) solution, which may be related directly to the cell permeability of each cryoprotectant and may cause osmotic stress in the cells before freezing and thawing procedures. The permeating speed of cryoprotectants is related directly to temperature. Consequently, major factors to be considered in assessing the toxicity of cryoprotectants are their concentration, the exposure temperature, and the time in aqueous (freezing) solution.

Cryoprotectants can interact with each other in a mixture, or with crucial cell molecules, thereby producing effects other than those that would occur with an individual cryoprotectant [47]. It has been observed that the protective effect of combinations of cryoprotectants can be greater than would be expected if the action of each agent were simply additive [48]. Whether the toxicity of cryoprotectants can be reduced, or not, by mixing two or more cryoprotectants (in a system where there is a reduction in the concentration of each cryoprotectant) in the aqueous (freezing) solution needs to be further investigated.

Equilibration

It is common practice to suspend cells in aqueous (freezing) solution containing permeable cryoprotectants for the time that is required to equilibrate intracellular solutes before freezing [49]. Many cells, especially eukaryotic cells, are sensitive to osmotic stress. Therefore, the permeating cryoprotectants are added gradually to the freezing solution in order to minimize osmotic stress as well as to allow removal of the cryoprotectants from the suspensions gradually. It means that the cells need time to balance and to adapt to osmotic shock. Different cell types require different equilibration times. Based on the permeating speed of the cryoprotectants and the cell types, different equilibration times will be required. Normally, lower temperature requires a longer period of equilibration, and higher temperature needs a shorter period.

The success of cryopreservation is determined by whether or not the cell undergoes intracellular ice formation during freezing. As mentioned above, vitrification can occur by either a slow-freezing or a rapid-cooling procedure. In the slow freezing procedure, intracellular ice formation is avoided by sufficiently slow cooling that osmotic dehydration results in the water remaining in near chemical potential equilibrium with the outside solution and ice. In the rapid cooling procedure, the cooling rate needed for vitrification is approaching T_g. Different freezing solutions with different concentrations of cryoprotectants need different cooling rates for vitrification. These rates are derived primarily from calculations using mathematical models of ice crystallization in very dilute solutions [20,50–53]. However, the actual situation may differ from the theoretical rate in the different cell types.

Conclusions

Cryobiology is the branch of biology involving the study of the effects of low temperatures on organisms and is most often used for the purpose of achieving

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cryopreservation. Cryobiology is the core of fertility cryopreservation. The principal application for human fertility cryopreservation began with sperm freezing, and then developed to include embryo and oocyte as well as gonadal cryopreservation. Although knowledge and medical achievements have advanced in fertility cryopreservation, especially with the recent development of oocyte and ovarian tissue cryopreservation, cryobiology can still be considered as a relatively new branch of biology.

Many factors affect successful cryopreservation of cells. First, it may depend on the cell type, cell size, cell growth phase, cell water content, cell lipid content, and the composition of the cells, as well as cell density. Second, it may depend on the composition of the freezing or vitrification medium, the cooling rate, the storage temperature, the duration of storage, and the warming rate and recovery medium. Third, it may be important to add cryoprotectants(s) into aqueous (freezing) solutions for freezing.

The mechanism of action of cryoprotectants can be considered as lowering the freezing point and preventing ice crystal formation in intracellular and extracellular solvents. It has been considered that there may be minor or severe cryoprotectant toxicity. This toxicity is related directly to the concentration used, and the cell exposure temperature and time. Although some mathematical theories have been developed in cryobiology [54], these theories may not be applicable to all types of cell. Further theoretical considerations are needed for developments in the field of cryobiology.

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Chapter 2

Suppression of ice in aqueous solutions and its application to vitrification in assisted reproductive technology

Patrick Quinn

Introduction

This chapter draws on several sources that have comprehensively reviewed this topic and here I want to especially acknowledge Dr. Helmy Selman, who organized a conference in Perugia, Italy on cryopreservation in 2006. Other sources are the classic work by Fahy and colleagues [1], which gives a thorough and in-depth look at the theoretical and biophysical aspects of this topic. This review is structured so that the more practical aspects are considered first followed by some of the more theoretical components involved. This approach will allow first an analysis of what works in a clinical setting and then a return to consider what is happening at the physical and molecular level. I believe this approach will allow a better understanding of the process.

Background

Slow cooling cryopreservation of mammalian oocytes and embryos has been extensively discussed and reviewed both in the chapters of this book and in other literature in the area for more than four decades and will not be further discussed in detail in this chapter. A concise description can be found in Wikipedia [2].

Although cryopreservation of human embryos and zygotes has become a well-established procedure in assisted reproductive technology (ART), oocyte freezing has proved to be technically challenging and until recently remained experimental. For several decades, attempts to cryopreserve human oocytes have been performed in many in vitro fertilization (IVF) centers worldwide, with variable results. When the slow freezing method traditionally used for embryos or zygotes was initially applied to oocytes, there were poor success rates. A major factor contributing to the poor results was that much of the cryobiology carried out by embryologists was empirical, based on "rules of thumb" rather than basic principles and knowledge.

Increasing knowledge of cryobiological mechanisms gave major insights to improve freezing–thawing protocols (Chapter 1). As one of the major components in any cell, including oocytes and embryos, is water, great care has to be taken that lethal ice crystal formation does not take place. Other critical factors during slow cooling include solution effects, osmotic stress, and intracellular dehydration. A summary of some of these factors and how they are handled with vitrification are given in Tables 2.1 and 2.2 [3].

Membrane permeability to water and solutes

The oocyte is the largest human cell (130 μm diameter). Large cells have a low surface-to-volume ratio and hence they are less efficient at taking up cryoprotectants (CPAs) and at losing water. The overall effect is that oocytes are more likely to retain water during cryopreservation and thus can be damaged by intracellular ice formation and growth. Also, oocytes can be seriously damaged even before freezing by excessive osmotic stress resulting from the addition or dilution of CPAs. The flow of water across each unit of the cell surface as a function of time is called the hydraulic conductivity or hydraulic coefficient (LP). Hydraulic conductivity is related to the volume of the cell. There is great variability between cell types and even species among mammals. In fact, membrane permeability changes with the developmental stages of oocyte, zygote, and embryo [4–6]. An example with human oocytes that caused some confusion initially is that freshly collected human oocytes are more prone to cryoinjuries than ones that are 1 day old, such as oocytes that fail to fertilize. Subsequently, it was found that methods established with "old" oocytes were often not suitable for the more sensitive freshly collected oocytes with full developmental potential [7], and it was suggested that this

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