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CRYOPRESERVATION: BASIC KNOWLEDGE AND BIOPHYSICAL EFFECTS

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SUMMARY

It had been a great dream of humankind to be able to pause the time or stay young forever. Not the whole body yet; but some cells and even tissues can be conserved by the help of cryotechnology now. In practise, mostly, embryos are being frozen for the situations occur in assisted reproductive techniques, by means of slow cooling or vitrification. In this review the biophysical effects, that take place on cells during cryopreservation are discussed. Especially, the importance of cooling rates and the fate of water content of a frozen cell are emphasized. A historical backround as well as thawing methods and cryoprotectant solutions are also mentioned.

Key Words: *Cell, Cryopreservation, Physics, Vitrification*

ÖZET

Soğukta Koruma: Temel Bilgiler ve Biyofiziksel Etkiler

Zamanı durdurmak ya da sonsuza kadar genç kalmak eskiden beri insanlığın büyük bir hayali olmuştur. Bugün, kriyoprezervasyon teknolojisi ile henüz tüm vücut olmasa da, hücreler hatta dokular korunabilmektedir. Günümüzde en sık olarak uygulanan, yardımcı üreme tekniklerinde gametlerin, gonadların ve özellikle de embriyonların yavaş soğutma ve vitrifikasyon kullanılarak dondurulmasıdır. Bu derlemede soğukta koruma uygulanan hücrelerin maruz kaldığı biyofiziksel etkiler ele alındı. Özellikle soğuma hızlarının önemi ve donmuş hücrelerdeki su içeriğinin akıbeti üzerinde duruldu. Tarihsel zemin ve çözme metodlarının yanı sıra soğukta koruyan çözeltiler de özetlendi.

Anahtar Kelimeler: *Fizik, Hücre, Kriyoprezervasyon, Vitrifikasyon*

From the moment of fertilization onwards, life processes run by the rhythm of a biological clock. The technology of cryopreservation gives us the possibility of interfering with the clockwork by stopping biological time. Cryopreservation of a cell therefore involves the cooling of a cell and storage at a temperature where all metabolic processes are arrested. In practice, frozen cells are stored at the temperature of -196°C in liquid nitrogen. The freezing of single cells is considered cryobiologically the simplest, since only the physicochemical characteristics of one specific type of cell must be taken into account in predictions of the response to freezing.

Paradoxically, sperm cells and oocytes are single cells but seem rather sensitive to cryopreservation stress. This may be related to their highly specialized structure and function of reconstituting an entire organism from the fusion of two single cells. Today, the most common procedures are cryopreservation of the embryos, spermatozoa and some somatic cells and tissues.

Cryopreservation methods can be described under a few titles:

Cryopreservation at -196°C

The viability of biological material stored at -196°C (liquid nitrogen temperature) is essentially independent of the period of storage. The oldest sample available, bovine spermatozoa that has been stored for over 50 years, shows no reduction in viability. The stresses associated

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with cryopreservation are not mutagenic: millions of cattle have been produced from frozen sperm and the incidence of abnormalities is identical to that observed with non-frozen sperm.

Frozen storage at temperatures above -196°C

Storing biological material in a conventional freezer is more convenient than using liquid nitrogen and a number of freezers are available which maintain temperatures down to -130°C.

However, unless biological material is stored at temperatures below -135°C the viability decreases during long-term storage. Whilst, this may be appropriate with microbial suspensions or with mammalian tissue cultures where large numbers of cells are frozen and some loss of viability may not cause practical problems, storage at these temperatures would not be acceptable with embryos, oocytes etc.

Freeze drying

It would undoubtedly be very convenient if mammalian cells could be freeze dried to retain viability because all the costs and difficulties associated with frozen storage and the maintenance of cold chains would be avoided. Recently it has been demonstrated that freeze dried mouse sperm, when injected directly into mouse ova could support normal development. However, the evidence from yeasts and bacteria is that the freeze drying process is highly mutagenic and that viability would be expected to decrease with storage time: these two factors would preclude the use of freeze dried spermatozoa in IVF.

Vitrification

An alternative approach to cryopreservation is vitrification. Conventional methods of cryopreservation have been developed to accomodate the consequences of ice formation.

Vitrification is a process which, by combining the use of concentrated solutions with rapid cooling, avoids the formation of ice. Samples reach low temperatures in a glassy state, which has the molecular structure of a viscous liquid and is not crystalline. This method has the potential advantages of being rapid to carry out and does not require controlled rate cooling apparatus. Good survival of mammalian embryos has been demonstrated by vitrification in a number of laboratories, however this approach is still experimental and the high levels of additives that are necessary to achieve vitrification are potentially cytotoxic. A further practical problem arises from the tendency of vitrified solutions to devitrify, i.e. crystallise into ice, during storage or thawing leading to loss of viability. (1)

Historical Background of Cryopreservation

More than two centuries ago, in 1776, Spallanzani was the first to report the maintenance of motility of human spermatozoa after exposure to low temperatures.

Mantagazza in 1866 suggested sperm banks for frozen human sperm. In 1949 Polge and colleagues discovered the effectiveness of glycerol as a cryoprotective agent for fowl spermatozoa. Freezing of human spermatozoa was reported shortly afterwards by Sherman (1953), observing that human spermatozoa, after freezing on dry ice, were able when thawed to fertilize and to produce normal embryonic development and offspring. The first birth after freezing human spermatozoa with glycerol in liquid nitrogen vapor was described by the same group.

The development of human sperm cryobanks in the 1970s gave rise to a growing need for standardization. Especially in the United States commercial and university sperm banks were established to permit later fertilization after vasectomy. The first association of human sperm banks was set up in France, the Center d'Etude et de Conservation du Sperme, in 1973, followed by the creation of the American Association of Tissue Banks in 1976, which also covered cryopreservation of gametes. The growing use of cryopreserved human semen led to the First International Meeting on Human Semen Cryopreservation, held in Paris in 1978.

The pioneers of oocyte cryoconservation set out their goals almost 50 years ago. It is therefore

surprising that now, in the turn of the twenty-first century, there is still no successful method for the cryoconservation of the human oocyte. In 1957 Lin, Sherman, and Willet demonstrated that the mouse oocyte can survive cooling to -5°C in a 5% glycerol-containing medium. In 1958 Sherman and Lin reported the birth of live young following in vitro fertilization (IVF) of mouse oocytes that had been "frozen" at -10°C in a 5% glycerol-containing medium. However, no oocytes survived exposure to a temperature of -20°C. The question with regard to these experiments is whether the oocytes were really frozen at -10°C, or whether they were reduced to a "supercooled" state (see below).

It was not the human oocyte but the human embryo that was the first to be successfully cryopreserved. Indeed, over a period of approximately 20 years (1960-1980) cryobiology had been raised from the level of pragmatism to science by pioneers of mathematical cryobiology such as Mazur (1970) and Leibo (1977, 1978, 1980). These authors had setup mathematical models for the freezing of biological systems based on the physicochemical characteristics of cells, such as membrane permeability and surface-to-volume ratio. In 1972 Whittingham and coworkers applied mathematically based cryopreservation protocols to mouse embryos, so leading to the first report of the birth of live young after storage of mouse embryos at -196°C in liquid nitrogen. The protocols that had been shown to work well with mouse embryos were largely copied for the human embryo, and in 1983 the first paper on a pregnancy from a cryopreserved human embryo was published by Trounson and Mohr (1983). Unfortunately a live birth did not ensue, and it was the Dutch group of Zeilmaker and colleagues (1984) who reported the first recorded live birth after cryopreservation of human embryos in 1983. It was again Wittingham (1977) who reported on the first successful cryoconservation of the mouse oocyte at -196°C in liquid nitrogen followed by the birth of live young. Despite the early successes oocyte cryopreservation did not find widespread clinical application. The reasons for this were that the efficiency of oocyte cryopreservation was low due to low survival rates, and that alarming reports questioning the genetic safety appeared in the literature. It was found that the degree of polyploidy increased significantly after cryopreservation of the mouse oocyte and of human oocytes. Almost all clinical teams stopped oocyte cryopreservation around 1987; and only a few research teams continued to investigate the effects of cryopreservation on the oocyte before considering moving oocyte cryopreservation back to the clinic again. (2)

Physics in Cryopreservation

Since liquid water is considered essential to the structure and function of living cells, it is not surprising that the solidification of water by freezing is usually lethal. Yet paradoxically freezing can also preserve cells for long periods of time in a viable state. It can slow or stop some biochemical reactions, but it accelerates others. Contrary to the usual impression, the challenge to cells during freezing is not their ability to endure storage at very low temperatures; rather it is the lethality of an intermediate zone of temperature (~–15 to –60˚C) that a cell must traverse twice –once during cooling and once during warming. No thermally driven reactions occur in aqueous systems at liquid N_2 temperatures (–196˚C), the refrigerant commonly used for low temperature storage. One reason is that liquid water does not exist below –130˚C. The only physical states that do exist are crystalline or glassy, and in both states the viscosity is so high (>10^{13} poises) that diffusion is insignificant. Moreover, at –196˚C, there is insufficent thermal energy for chemical reactions.

The only reactions that can occur in frozen aqueous systems at –196˚C are photophysical events such as the formation of free radicals and the production of breaks in macromolecules as a direct result of "hits" by background ionizing radiation or cosmic rays. Over a sufficiently long period of time, these direct ionizations can produce enough breaks or other damage in DNA to become deleterious after rewarming to

physiological temperatures, especially since no enzymatic repair can occur at these very low temperatures. The dose of ionizing radiation that kills 63% of representative cultured mamlian cells at room tempreature is 200-400 rads. Because terresterial background radiation is some 0.1 rad/year, it ought to require some 2,000-4,000 years at –196˚C to kill that fraction of a population of typical mammalian cells. Besides this proportional calculation, there is no evidence that storage at –196˚C results in the accumulation of chromosomal or genetic changes. (3)

One of the most important things is the fate of intracellular water during freezing. At this stage it may be useful to go over some terms. It is necessary to know about the term "supercooling", to understand the freezing mechanisms of cells better.

Supercooling is the ability of an aqueous solution, to cool down it's normal freezing point, without changing it's state; from liquid to solid (ice). At this situation, if the aqueous solutions or water are touched by a crystal or a metal object, the crystallization will occur and temperature will immediately return to the freezing point. This process is called "seeding" and is used to obtain a manually controlled freezing. Crystallization can be induced by the particules in the medium, mechanical vibration or the rough surfaces. Supercooling is a situation, that takes place in the atmosphere frequently.

Water and aqueous solutions have a strong tendency to cool below their melting point before nucleation of ice occurs. For example, whilst 0°C is the melting point of ice, the temperature of water may be reduced significantly below 0°C before ice formation occurs, and in carefully controlled conditions water may be cooled to approximately –40°C before ice nucleation becomes inevitable.

Following ice nucleation and initial crystal growth the temperature rises to its melting point and remains relatively constant at that temperature during the subsequent phase change to ice ('latent heat plateau'), when the temperature then changes more rapidly to the environment temperature (fig. 1). The tendency of a system to supercool is related to a number of factors including temperature, rate of cooling, volume, exclusion of atmospheric ice nuclei and purity of particulates. In cryopreservation of cells and tissues in IVF systems, there is thus a strong tendency for supercooling to occur. To avoid the damaging effects of supercooling on cells and in particular embryos ice formation is initiated in a controlled manner. This is commonly referred to as 'seeding' -although, strictly speaking, this term refers to the introduction of a crystal to an under

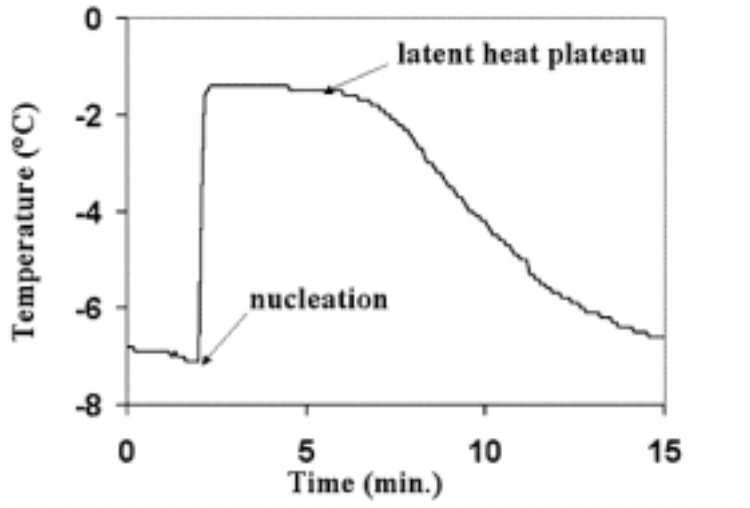

Figure 1: Temperature changes during the freezing of an aqueous solution of glycerol

supercooled solution. Controlled ice formation during freezing is recognized to be a key factor in determining the viability of embryos following freezing and thawing. In a carefully controlled series of experiments, samples which were nucleated below -9°C had a low viability, whilst nucleation (seeding) at higher subzero temperatures of -5°C to -7.5°C resulted in much higher viability (fig. 2). Normal practice is to cool straws to a temperature of approximately -7°C, hold at this temperature for thermal equilibration, and then initiate ice formation in the straw by touching the outside of the straw with cold forceps etc. The temperature of the straw rises to the melting point of the solution, and then following ice formation the temperature returns at a rate of 2.5°C/min to -7°C. Cellular dehydration then occurs during subsequent slow cooling. (1)

Down to –5˚C, the cells and their surrounding medium remain unfrozen both because of supercooling and because of the depression of the freezing point by the protective solutes that are frequently present. Between –5 and –15˚C, ice forms in the external medium (either spontaneously or as a result of seeding), but the cell contents remain unfrozen and supercooled, presumably because the plasma membrane blocks the growth of ice crystals into the cytoplasm. The supercooled water in the cells has a higher chemical potential than that of water in the partly frozen solution outside the cell, and in response to this difference in potential, water flows out of the cell and freezes externally.

The subsequent physical events in the cell depend on cooling velocity. If cooling is sufficiently slow (fig. 3, upper right), the cell is able to lose water rapidly enough by exosmosis to concentrate the intracellular solutes sufficiently to eliminate supercooling and maintain the chemical potential of intracellular water in equilibrium with that of extracellular water. The result is that the cell dehydrates and does not freeze intracellularly. But if the cell is cooled too rapidly (fig. 3, bottom and center right) it is not able to lose water fast enough to maintain equilibrium; it becomes increasingly supercooled and eventually attains equilibrium by freezing intracellularly. Intracellular ice formation leads to cell death.

In the course of time it is needed to formulate these physical events and to see what kind of factors are effective on cryopreservation. More specifically, the proposal was that the rate of exosmosis of water during freezing can be described by four simultaneous equations. The first relates the loss of cytoplasmic water to the chemical potential gradient expressed as a vapor pressure ratio; second expresses the change in this vapor pressure ratio with temperature

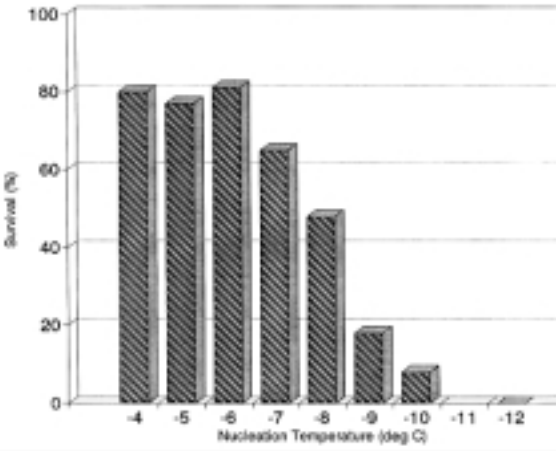

Figure 2: The survival of mouse cell embryos after seeding at various subrero temperatures

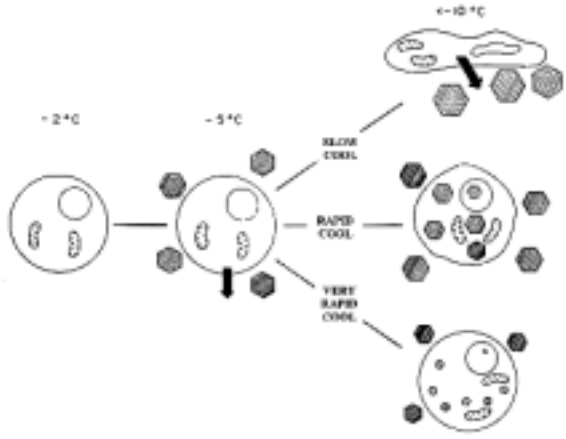

Figure 3: Schematic of physical events in cells during freezing. *Cross-hatched hexagons, ice crystals.*

(Clausius-Clapeyron equation and Raoult's law). Third equation is about time and temperature which are related by the cooling rate (B). And finally the fourth one is related to the activation energy and temperature (3).

1. $dV/Dt = (L_p ART \ln P_e/P_i)/V_i°$
2. $d\ln(P_e/P_i)/dT = L_f/RT^2 - [N_2V_1°/(V+N_2V_1°)V]dV/dT$
3. $dT/dt = B$
4. $L_p = L_p^g \exp [-E/R`(1/T-1/T_g)]$

According to these equations, the "optimum" rate of cooling may be considered to be the fastest rate of cooling at which intracellular ice formation does not occur. Whilst the existence of an optimum rate of cooling has been reported for a wide range of cell-types, a clear optimum does not seem to exist with some cells of importance to IVF. The response of cells to the hypertonic conditions encountered during freezing is determined by a number of biophysical factors:

1. Cell volume (V) and surface area (A)
2. Cellular permeability to water (Lp)
3. Arrhenius activation energy (E)
4. Type and concentration of cryoprotective additives
5. Cooling rate (B)

Thus to avoid the probability of intracellular ice damage to embryos, which have low surface area to volume ratio and low water permeability, slow rates of cooling are required. Sperm, with a large surface area to volume ratio and a higher value for water permeability may be cooled faster before the intracellular compartment becomes significantly supercooled (4).

An increase in diameter (D) proportionally reduces the cooling rate required to produce a given probability of intracellula freezing because the fractional water loss during cooling is proportional to the ratio of the cell surface (D^2) to cell volume (D^3). The development of cryomicroscopes has permitted experimental tests of the presumed relation between cooling rate and the occurence of intracellular freezing. It has been seen that the ovum cooled at 1.2˚C/min dehydrates without evidence of internal freezing, but the ovum cooled at 32˚C/min undergoes no discernable shrinkage and freezes intracellularly at –40˚C as evidenced by its sudden opacity.

To avoid intracellular freezing, the ova must be cooled slowly enough to permit their water content to approach the equilibrium value before they have reached their ice-nucleation temperature. The available evidence indicates that when ~90% of cell water is removed, the residual ~10% cannot freeze at any temperature. (3)

Effects of Freezing on Cells

Cells are exposed to a continuously increasing hypertonic solution medium during freezing. It is essential to remove a particular amount of water from cell osmotically, to avoid intracellular freezing, before and –partially- after the ice nucleation. This is something related to the cooling rate. At slow rates of cooling, cells try to remain in equilibrium with the external solution. The external solution freezes before the intracellular medium because of the protective effects of cell membranes. Thus, outside of the cell becomes more hypertonic and a water-flow from inside to outside (exosmosis) occurs. This is like a knife that cuts both ways for the cell. Removal of too much water may lead to increase in the solute load that means the disturbance of equilibrium. As the cooling rate is increased there is less time for water to move from the cell, which becomes increasingly supercooled and eventually intracellular ice formation occurs that is inevitably lethal.

So, an optimum rate of cooling results from the balance of these two phenomena. At rates of cooling slower than the optimum, cell death is due to long periods of exposure to hypertonic conditions. At rates of cooling faster than the optimum, cell death is associated with intracellular ice formation.

Cells in suspension are not punctured by ice crystals, nor are they mechanically damaged by ice. (1)

Stress Encountered	Potential Cellular Response
Reduction in temperature	Membrane lipid phase changes Depolymerisation of the cytoskeleton
Increase in solute concentration	Osmotic shrinkage
Increase in ionic concentration	Direct effects on membranes, including solubilisation of membrane proteins
Dehydration	Destabilisation of the lipid bilayers
Precipitation of salts and eutectic formation	Not known
Gas bubble formation	Mechanical damage to membranes and the cytoskeleton
Solution becomes extremely viscous	Diffusion processes, including osmosis may become limited
Changes in pH	Denaturation of proteins etc.
Cells become closely packed	Membrane damage

Thawing & Post Thaw Handling

During thawing of cryopreserved samples the physical processes which occur during freezing will be reversed. The solid system will partially melt and cells will become again suspended into hypertonic solutions which will become more dilute during thawing. Cryoprotective additives and water may be transported across cell membranes and any intracellular ice may grow before it finally melts. In most cases examined, rapid rates of thawing are generally better than slow rates of warming.

The measured rates of warming of the samples are relatively high between -196°C and approximately -10°C, in this temperature range the amount of ice to be melted is relatively small. The warming curve is then observed to flatten off between -10°C and the melting point of the solution, within this temperature zone a large amount of ice needs to be melted -this is the reverse of the latent heat plateau.

Conventionally, material cryopreserved in straws is held in air for 40 seconds, during which time the temperature will rise rapidly to approximately -50°C, before the straw is transferred to a water bath held at 30°C for 1 minute. The step of holding the straw in air was originally employed to allow the boiling off of any liquid nitrogen trapped within the straw because immersion of straws containing entrapped liquid nitrogen into warm water would lead to rapid boiling of the liquid nitrogen with possible fracture of the straw or violent expulsion of the plug. Development in plugs for straws now make it unlikely that nitrogen can leak into straws.

Upon thawing the cryoprotective additives are diluted out either in a single step or in two steps. Following freezing and thawing cells will contain cryoprotective additive and upon exposure to normal growth medium will tend to expand. To prevent swelling of cells, shrinkage is induced by using wash out solutions which contain hypertonic sucrose (0.2 M), this sucrose is then diluted away by washing with growth medium.

Cryoprotectants

In dilute aqueous solutions such as growth media the increase in ionic composition following ice formation is dramatic and by -10°C the ionic concentration reaches approximately 3 molar which is, not surprisingly, lethal to cells.

A number of compounds, so called cryoprotective additives, are employed to reduce cellular damage following freezing and thawing. The general properties of cryoprotective compounds are that they have low molecular weight, are non toxic and can permeate cells. Cryoprotective additives achieve their protective effects by increasing the unfrozen fraction at a

given temperature and thereby reducing the ionic composition. This effect is illustrated here with glycerol but the other commonly employed additives (propanediol, dimethlysulphoxide etc.) act in the same manner. Cells are exposed to a high concentration of the cryoprotective additive during freezing rather than a high ionic concentration, which is less damaging. Cells can be permeated by all of the commonly employed cryoprotective additives used in IVF, and it is standard practice to "incubate" cells in the cryoprotective additive before freezing commences to allow them to attain an equilibrium intracellular concentration.

When compared at the same concentration (molar), all cryoprotective additives have a very similar effect to that described above. However the protective efficiency of these compounds may vary from cell-type to cell-type: for example it has been found that human embryos are best frozen with propanediol whilst human blastocysts are optimally frozen with glycerol. This may be related to the relative cellular toxicity or the differing permeability of these additives to differing cell-types, but because experiments to compare them have been mostly carried out at a single freezing protocol, the explanation may be more complex than these experiments suggest.

Sucrose is employed in the standard protocols for embryo and oocyte cryopreservation with a low level (0.1 M) used in addition to the cryoprotectant. To prevent swelling of cells on thawing, shrinkage is induced by using wash out solutions which contain hypertonic sucrose (0.2 to 0.3M). This sucrose is then diluted away by washing with growth medium. (4)

Conclusion

Today, by the help of progressing biotechnology, it is more often that we apply cryopreservation. While, there are still insufficient researches especially for the oocytes, studies on the cryopreservation of immunological memory lymphoid cells, aortic root allografts and osteoblasts for bone banking, are going on. (5-8). An updated, but discursive subject is postmortem human reproduction; and it currently has many ethical and legal problems awaiting to be solved. (9). Recently, it can be seen that vitrification is more preferable than slow cooling as it is cheaper and easier to handle. (10). Cryopreservation of cornea, umbilical cord and hematopoietic cells and sperm banking procedures are performed routinely. (11)

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