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Fundamental Aspects of Gamete Cryobiology

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Fundamental Aspects of Gamete Cryobiology

A. M. Petrunkina

The storage of reproductive cells and tissues in cryobanks and their use in reproductive medicine and biotechnology have become an indispensable part of modern science and clinical service. However, current methods have severe limitations, in particular resulting in sub-optimal maintenance of viability after freezing-thawing of gametes. The improvement of gamete cryopreservation systems is therefore of great importance for genetic resource banking, agriculture and human reproduction. So far, advances have been made largely via empirical rather than analytical approaches. When an analytical biophysical approach had been applied, severe discrepancies between theory and practice often have been revealed. A basic understanding of the cellular processes which accompany gamete cryopreservation is therefore necessary for successful development of the relevant technology. In particular, the concepts of biological variability between cell types, species and individuals, as well as of heterogeneity between cells of the same type must be considered.

This review focuses essentially on the fundamental physical and chemical principles of gamete cryobiology and cryoinjury, as well as on the biological properties of gametes in different species that are mainly responsible for the heterogeneous outcome and poor rates of improvement in their cryotechnology. J Reproduktionsmed Endokrinol 2007; 4 (2): 78–91.

Key words: gametes, osmotic properties, plasma membrane, permeability, cryoinjury, cryoprotectant, biological heterogeneity

The era of reproductive cryobiology began in 1949 with the discovery of glycerol as a potent cryoprotectant. In that year, Polge and colleagues first reported the successful use of glycerol for sperm cryopreservation [1]. Since this initial fundamental technological step, much progress has been made in the cryopreservation of reproductive cells and tissues. New technologies of slow freezing, freeze-drying and vitrification have been developed, and nowadays not only can all types of reproductive germ cells be frozen, but cryopreservation of ovarian and testicular tissues has also found a wide application [2–4]. Considerable advancements have thereby been achieved in the biotechnology and storage of important murine lines, in genome resource banking for endangered species, in agriculture by germplasm transfer of genetically superior animals, and in human reproductive medicine [3].

In human medicine, especially, the storage of reproductive cells and tissues in special cryobanks and their use in reproductive medicine and assisted biotechnology for artificial insemination and in vitro fertilization, intracytoplasmic sperm injection and embryo transfer have become an indispensable part of modern therapy: these have introduced not only new concepts and opportunities but also new ethical considerations in the life of individuals and the entire society [5–7].

However, despite this crusade of reproductive cryobiology, most attempts to improve cryopreservation systems, even when successful, have been made in empirical ways. Although the use of the protocols developed thus far for slow freezing and vitrification result in adequate therapeutic outcomes in human assisted reproduction, further improvement of cryobiological techniques would be desirable, especially in the cryopreservation of oocytes and embryos [6, 7]. Even more critical is the situation in many animal systems, where only small improvements in basic techniques have been made since the early 1950s [3, 8]. Whereas in the dairy cattle breeding industry considerable successes have been achieved with

respect to freezing technology, insemination and embryo transfer, successful applications of these to the breeding of pigs, wild animals or even beef cattle have been rather restricted [9, 10]. It has become clear that protocols which are optimal for one cell type are not transferable to another cell type, and even not applicable within this cell type from one species to another. Although differences between species in sperm physiology and biochemistry and in physiology and anatomy of the female genital tract may be major sources of this wide biological variability [11], a relatively poor understanding of the fundamentals of the cryobiological processes themselves may also play a large part in limiting progress.

Historically, research in the field of cryobiology has proceeded almost independently along two parallel modes: 1) via a biophysical approach which is obviously limited in its applicability to living cellular systems; 2) via a cellular approach which is to a high degree empirical and often lacks detailed quantitative analysis. The cultural gap between these two has been narrowed somewhat in recent years as cryobiology has developed as an autonomous science. However, especially in reproductive biology and medicine, these two different research approaches continue to run in parallel on their Euclidean path. A primary example of this latter problem is the evident discrepancy between theoretically predicted optimal cooling rates and the empirically obtained cooling rates that result in maximal cell survival [12].

This review will therefore focus on basic aspects of cryobiology and cryoinjury from the point of view of fundamental physical principles, and on the phenomena and biological cell properties which appear to be mainly responsible for the heterogeneous outcomes and poor rates of improvement in conventional technologies (e. g. slow freezing) of gamete cryopreservation.

Basic Principles of Cryobiology

Cryobiology as a science describes the effects of sub-zero temperatures on live organisms. The complexity of cryobiology is deeply rooted in the complex behavior of living cellular systems and their responses to massive temperature changes. Thus understanding of the effect of cryobiological processes at the cellular and molecular level requires interaction between many fields of biological and physical science: cell physiology, pathology and

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biochemistry, biophysics and physical chemistry, thermodynamics and mathematics.

In a biophysical approach to cryobiology, the behavior of cells is predicted based on models of mass solute transfer at various temperatures. Most calculations used for the estimation of optimal cooling protocols are based on the so-called "ideal solution" theory. In this section, the basic terms and concepts of biophysics relevant to cryobiology will be re-iterated: solution theory, osmosis, water and solute transport. Because of space limitations, the literature referring to the basics of biophysics has not been extensively cited, and only a few crucial final quantitative biophysical equations have been addressed. The biophysical and thermodynamic principles of cryobiology have been summarized and reviewed many times, and these books or publications may be consulted for further details [13–19]. An excellent text book in cryobiology is available online [20]. Further information about the relevant basics of physical chemistry, cell biology and biophysics as concerned in this paper are also obtainable from books [17–20].

Cellular Water

Water plays a crucial role in the life of the cell: not only does the living state of the cell depend entirely on intracellular water as a vehicle for the intracellular contents (e.g. hydrophilic structures such as proteins, metabolites and ions), but the extracellular environment also consists, in a generic sense, of aqueous solutions. These solutions (intracellular and extracellular) can exist in different states, and phase changes will occur in them during freezing and thawing, containing as they do electrolytes, osmolytes, macromolecules and gases. The concentration and distribution of these solutes in the extracellular and intracellular environments undergo changes during cryopreservation.

Water Transport and Osmotic Properties

To describe water transport in cells, usually two major assumptions are made: 1) cells are surrounded by a membrane which is impermeable for solute but permeable for water and 2) cells are normally in a state of osmotic equilibrium.

Water can be transported across these semi-permeable membranes between compartments containing different concentrations of solutes. Changes in osmotic pressure (internally as well as externally) will produce changes in cellular volume and shape, phenomena which have been seen in a variety of cell types [21]. According to osmotic theory, as osmotic conditions change, water movement will take place until there is no osmotic gradient across the plasma membrane and the osmotic pressure of the cytoplasm matches the external osmotic pressure:

$$\pi_i = \pi_e$$

Then the cell volume V at these conditions will fit the equation:

$$\pi_e(V - V_b) = \pi_o(V_o - V_b) \quad (1)$$

where V_o is the isotonic volume and V_b the osmotically inactive volume (non-solvent component of the cytoplasm).

This equation in its re-arranged form is widely used in cryobiology to determine the osmotic parameters at a

given temperature, and is known as the Boyle Van't Hoff Equation:

$$V = \pi_o/\pi_e(V - V_b) + V_b \quad (2)$$

It implies that at a constant temperature cell volume changes reciprocally with osmolality. Typical Boyle Van't Hoff plots show the relative volume changes of cells in environments of differing osmolality (Fig. 1). The cell volume decreases with increasing osmolality. However, only part of the cellular volume is osmotically active: the point at which the line crosses the Y-axis (15.1–16.2 fl) corresponds to the osmotically inactive cell volume.

However, the Boyle Van't Hoff equation describes only the equilibrium cell volume without giving any information about how rapidly this equilibrium will be achieved. The kinetics of cell volume as a function of time are determined by the physical structure of the membrane and depend on the hydraulic conductivity of the membrane (L_p). The flux of water across the bilayer obeys Fick's law; so, assuming membrane semi-permeability to solute and solvent, the osmotic swelling and shrinkage of cells is given by the equation:

$$\frac{dV}{dt} = L_p A R T (\pi_i - \pi_e) \quad (3)$$

where L_p is the hydraulic conductivity (water permeability), A is the surface area of the membrane, R is the universal gas constant, and T the temperature.

The hydraulic conductivity is a property that is unique to a particular cell type. For example, there is more than one order-of-magnitude difference in water permeability between human red blood cells ($L_p \sim 9 \mu\text{m}/\text{min}/\text{atm}$) and lymphocytes ($L_p \sim 0.5 \mu\text{m}/\text{min}/\text{atm}$) [23, 24]. Cell separation technologies are often based on differential physical properties like density, osmotic water loss, or swelling and lysis [25]. However, even within one cell type, the physical and chemical cell properties may vary between

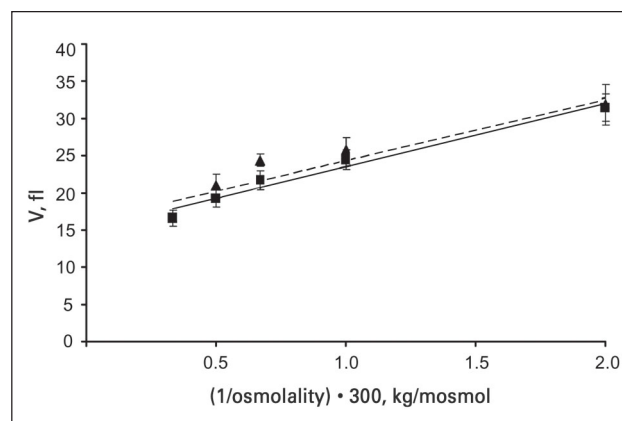


Figure 1: Boyle Van't Hoff Plot of boar spermatozoa (Drawn on the basis of data presented in [22]).

Data are presented in volume equivalents for ejaculated (unbroken line) and epididymal (broken line) boar spermatozoa at 39°C as measured by electronic cell counter. Boar spermatozoa behave as "perfect osmometers" in the range between 150–900 mOsmol/kg. The changes in aniso-osmotic volume V are given by the regression equation where x is the reciprocal of relative osmolality:

- for epididymal spermatozoa, $V = 8.2 \cdot x + 16.2$
where $x = 1/300 \text{ mOsmol/kg}$ $R^2 = 0.94$
- for ejaculated spermatozoa, $V = 8.5 \cdot x + 15.1$
where $x = 1/300 \text{ mOsmol/kg}$ $R^2 = 0.99$

The osmotically inactive fraction of cell volume corresponds to $0.645 \cdot V_{\text{iso}}$ in epididymal spermatozoa and to $0.626 \cdot V_{\text{iso}}$ in ejaculated spermatozoa.

species: in particular, the diffusional water permeability of red blood cells varies by as much as a factor of 4 across 21 mammalian species [26]. Cells with higher L_p will reach equilibrium faster: for example, in the case of bovine, canine or human erythrocytes, osmotic equilibrium in an anisotonic solution will be achieved within less than 1 s ($L_p \sim 9\text{--}12 \mu\text{m}/\text{min}/\text{atm}$ [27, 28]), whereas equilibration will take more than 10 s in bovine or human spermatozoa ($L_p \sim 0.7\text{--}1.8 \mu\text{m}/\text{min}/\text{atm}$ [29, 30]). The property of hydraulic conductivity is associated with the ability of cells to survive cryopreservation stress, because it characterizes how quickly the cells can respond to a changing osmotic environment [23].

Water and Solute Transport

In most cases, the cellular environment cannot be considered as a simple aqueous solution of impermeable solute. Especially in cryopreservation, additional substances are included in the conservation media which can permeate the membrane. In this case, not only will water flow take place but also the flow of the permeable solute.

The water flow will result from the water transport through the membrane due to the osmotic gradient. This osmotic gradient will be changing because the osmotic pressure of the intracellular solution in particular will depend on transport of the permeable solute across the membrane pore:

$$\frac{dV}{dt} = -L_p A [\Delta C_i + \sigma \Delta C_p] \quad (4)$$

where ΔC_p and ΔC_i are the gradients for permeable and impermeable solutes across the membrane and σ is the fraction of molecules of permeable solute allowed through the pore (the so-called reflection coefficient).

For a system with the solute volume V_s and the volume of osmotically active water V_w

$$V_s = N_s \bar{V}_s \quad (5)$$

$$V_w = (V - V_b - V_s) \quad (5a)$$

(N_s is the mole amount of the solute, $\bar{V}_s$ is partial molar volume of the solute), the overall solute flow

$$\frac{dN}{dt}$$

will result from the flow due to the solute concentration gradient (dependent on the permeability of solute P_s) and from the solute transport across the membrane through the pore due to the flow of water:

$$\frac{dN}{dt} = P_s A (\Delta C_p) + (1 - \sigma) \bar{C}_s \frac{dV}{dt} \quad (6)$$

where $\bar{C}_s$ is the mean solute concentration in the inner and outer compartments.

For example, if cells are more permeable to water than to a cryoprotectant, they will initially shrink because of water efflux. As the cryoprotectant subsequently enters the cell, the cell volume will increase.

In the generalized form (for k different solutes) these two thermodynamic equations are known as the coupled water-solute transport or Kedem-Katchalski model for vol-

ume flow (change in volume with time) J_v and solute flow (change in solute concentration with time) J_s [16].

$$J_v = \frac{1}{A_s} \frac{dV}{dt} = -L_p \sum_k \sigma_k (C_k^e - C_k^i) RT \quad (7)$$

$$J_s = \frac{1}{A_s} \frac{dN_k}{dt} = \bar{C}_k (1 - \sigma_k) J_v + P_s (C_k^e - C_k^i) \quad (7a)$$

where C_k is the concentration of the relevant solute.

Two boundary cases of the Kedem-Katchalski model are especially interesting. If the solution contains only impermeable solutes, the swelling rate is given by equation (3); if the solution contains only impermeable solutes plus one cryoprotectant, the simplified equations (4) and (6) can be used for model calculations.

Theoretical Prediction of Osmotic Response

From the mass transport model of Kedem-Katchalski it is apparent that the theoretical osmotic response of a cell can be characterized completely if the following parameters are known:

- cell size (especially the surface area to volume ratio A/V)
- fractional osmotically inactive cell volume V_b
- water permeability L_p (hydraulic conductivity) of the plasma membrane
- permeability coefficients (P_s) of relevant solutes
- reflection coefficient σ of these solutes
- activation energy E_a (to allow cellular response to be extrapolated to different temperature conditions)
- initial concentration of solutes

The parameters L_p , P_s and σ can be determined by fitting experimental data to the transport equations. The isotonic cell volume can be measured directly using cell counter techniques, and osmotically inactive cell volume can be determined by using Boyle Van't Hoff plots. Activation energy can be calculated using the Arrhenius equation and an Arrhenius plot for data collected at different temperatures [19]. The kinetics of hydration of cells during freezing can be modelled mathematically [13, 23]. Differences in these parameters results in differing responses to freezing/thawing conditions, whence the high degree of cryobiological variability between different cell types can be explained.

Kleinhans [31] has shown that in the majority of cases a two-parameter model for volumetric changes due to water flux V and solute flux V_s (based on equations 4 and 6) is an adequate for predicting osmotic response. For calculation of the permeability to cryoprotective agents, the measured volumes are fitted to a simplified model defined by the following equations:

$$\frac{1}{A_s} \frac{dV}{dt} = -L_p (C^e - C^i) \quad (8)$$

$$\frac{1}{A_s} \frac{dV_s}{dt} = \bar{V}_s P_s (C_s^e - C_s^i) \quad (8a)$$

This model has been expanded to multi-solute systems with special attention to the presence of impermeable solutes [32, 33]. Applications of this model in cryobiology are very valuable because the maximal volume expansion or contraction ("excursion") can be calculated in a easy and practical way according to relatively simple mathematical formulae [33].

Cryoinjury and its Prevention

The process of cryopreservation leads to morphological and functional damage of cells because it is associated with considerable osmotic changes: during freezing, cells become dehydrated, and during thawing, as water re-equilibration takes place, they are subjected to hypotonic challenge. The phase transition from water to ice is the major critical event with respect to cell survival: there is a critical region of temperature within which the cells are irreversibly damaged if they remain within it longer than a few seconds [34]. Therefore, this section will discuss the concepts of ice nucleation and crystal growth, increasing salt concentrations, tolerable osmotic excursion of cell volume, post-hypertonic lysis, and mechanisms of cryoprotectant action.

Colligative Properties of Extracellular Solutions: Freezing Point Depression and Osmolality

The cellular state and water balance in cells during freezing and thawing is determined by the colligative properties of the external aqueous solution, i. e. by the properties which depend on concentrations of solutes rather than on behavior of individual chemical compounds. The freezing point of a solution is primarily dependent on the concentration of solute(s) which it contains. The freezing point depression is determined by the minimum radius of an ice crystal that is stable at a given temperature, and is given by the mathematical equation

$$\Delta T = cK \quad (9)$$

where $K = 1.86 \text{ }^{\circ}\text{C/mol}$ for aqueous solutions and C the molar concentration of the solute.

During freezing, the pure water precipitates out of solution in the form of ice, whence the extracellular salt concentration increases. In simple systems consisting only of two compounds, e. g. NaCl and water, binary phase diagrams can be used to describe the changes of osmolality in which the cell must exist. The osmolality of the unfrozen solution at any particular temperature is described by a simple quadratic function of temperature:

$$\pi = -0.52823T - 0.00492T^2 \quad (10)$$

Freezing injury will take place if the cell is exposed to a critical osmolality (i.e. that at which irreversible damage or lysis occurs) [35].

The eutectic temperature also plays an important role in cell response to cryopreservation. At the eutectic temperature, the system is in thermodynamic balance, at which point the unfrozen fraction (solution and solute) becomes solid after spontaneous crystallization ("eutectic formation").

Mechanisms of Cryoinjury

The milestones in understanding mechanisms of cryoinjury were set about a third of century ago as the "Two-factor Hypothesis of Freezing Injury" by Mazur, Leibo and Chu [36] has appeared. In this work, the kinetics of cellular water loss were investigated, and both too slow and too rapid freezing was found to be associated with lethal cryoinjury.

Rapid freezing will lead to intracellular ice formation because higher cooling rates lead to supercooling of cells

below their nucleation temperature. In this case water will be kept in the cell, whence eventually either large lethal, or small less damaging ice crystals will be formed within. Rapid freezing may also lead to large gradients in osmotic pressure across the plasma membrane, which will become damaged at a critical pressure gradient, and intracellular freezing will also occur as a result of this damage [37, 38]. The building of large intracellular ice crystals is associated with cell death. Although more moderate freezing rates are associated with exposure to smaller ice crystals, these latter can re-crystallize upon re-warming and become lethal (as demonstrated with respect to embryos [39]).

However, if freezing progresses at very slow rates, dehydration will take place over a longer period of time. During this slow freezing, the osmolality of the external medium increases as water-ice is precipitated. The cells will be exposed to high salt concentrations in the external medium and dehydrate to a degree of shrinking associated with serious cellular disruption [36]. Cryoinjury due to the cell's sensitivity to high solute concentrations is referred as to a "solution effect".

Thus, freezing rates should be slow enough to allow the cells to minimize chemical potential and osmolality gradients across the plasma membrane and to dehydrate without being exposed to lethal salt concentrations. This "equilibrium" freezing leads to tolerable cell shrinkage, to an increase in the intracellular chemical potential, and to depression of the freezing temperature. Due to the decreased amount of intracellular water and the freezing point depression, the potential risk of intracellular ice formation is reduced, so that only extracellular ice will be formed. Thus the characteristic shape of the curve plotting cell survival against freezing rate is an inverted "U", with typically a critical cooling rate for maximum survival [36].

The thawing of cells also has to be performed correctly because the warming rate influences the survival of cells. Thawing rates comparable with the freezing rates that induce intracellular ice formation could lead to re-crystallization of small ice crystals resulting in lethal injury. Too slow warming may be lethal depending on cell type, e.g. through over-long exposure to the hypotonic stress that occurs at thawing. At this stage, possible defects in cell volume regulation may be critical. Thus, either slow or rapid warming may be required, depending on biophysical cell properties and on the cooling rates previously used [23].

Osmotic Tolerance

The shrinkage and swelling of cells is limited by their physical capacity to withstand such changes. Damage will occur beyond certain levels of shrinkage or swelling (the overall range between these levels is known as the maximal cell volume excursion). The optimal equilibrium freezing rate found for a given cell type must result in a tolerable volume excursion, i.e. a range of swelling and shrinkage that does not lead to the loss of membrane integrity and impaired cell function (e.g. motility for spermatozoa). Tolerable volume excursions are cell type- and species-specific.

Cryoinjury by Eutectic Formation

Eutectic formation, i.e. the process of crystallization of the unfrozen fraction of the intracellular or extracellular

medium, has thus far been relatively poorly studied in biological systems.

Eutectic crystallization is not usually observable within the normal freezing range if both inorganic salts and organic substances (e.g. cryoprotectants) are components of the system (see [40]). Due to a low concentration of electrolyte and the presence of relatively high concentrations of cryoprotectant, the impact of eutectic crystallization on cryoinjury has therefore not been seen as significant. Hence, despite the theoretical indication that eutectic crystallization may have detrimental consequences on the cell membrane, its effect on cellular systems has been overlooked.

Recently, however, quantitative information on direct cell injury due to eutectic formation has been reported [41]. The post-thaw viability of rat prostate tumor cells decreased dramatically if eutectic crystallization took place. Moreover, cell injury was enhanced by introducing other solutes which had a higher eutectic temperature than NaCl. The authors propose two major mechanisms of cell cryoinjury by eutectic formation: (1) mechanical cell damage to the cell membrane due to extracellular eutectic formation and (2) disruption of cytoplasmic structures by intracellular eutectic formation.

Mechanisms of Cryoprotectant Action

A more complicated system with three or more compounds, e.g. water, sodium chloride and at least one cryoprotective additive, is used in most cryopreservation protocols. The calculation of equilibrium conditions is more complex in this case, because the cryoprotectant serves concurrently as a solvent for sodium chloride and as a solute in water. Such a system can be described by a ternary phase diagram. Melting and eutectic temperatures have been described for the three-compound systems of DMSO-NaCl-H₂O and Glycerol-NaCl-H₂O [42, 43]. These equations are more complex than the analogous equation (equation 10) for a binary system but they allow one to calculate the solution composition at any given temperature. In general, lowering the freezing point is associated with a higher unfrozen fraction and lower salt concentration in the extracellular space. The negative "solution effect" is thereby reduced, and the cells' exposure to hyper-osmolality diminished. Ternary phase diagrams illustrate the protective action of cryoprotectants via their effects on the initial freezing temperature and the eutectic temperature, and it has been shown recently experimentally that addition of various types of cryoprotectant leads to the suppression of eutectic crystallization, both in a simple NaCl-H₂O system and in a more complex phosphate-buffered saline system [41]. One may conclude that one way in which cryoprotective agents protect cells during freezing and thawing is by lowering the initial freezing point and by diminishing injury from eutectic crystallization.

Addition of permeable cryoprotectants will also influence the entire system state because trans-membrane movement of both solute and water will take place, thus changing the transport kinetics, cell dehydration rates and volume excursion.

Cryoprotective agents that diminish cell damage are usually divided into two general classes related to their ability to move across the cell membrane: permeable and

impermeable. Their mechanisms of action are different. Cryoprotection by permeable agents can be explained by their colligative properties: they lower the temperature at which cells are exposed to a critical salt concentration [34, 44]. Impermeable agents, on the other hand, are thought to dehydrate cells, allowing them to be cooled rapidly before lethal injury due to solution effects takes place [44].

However, the effects of cryoprotectants are more complex than can be explained from thermodynamics and solution theory. In high concentrations, they can produce lethal osmotic effects during their addition and removal (hyper-osmolality) and they can be toxic [3]. Initial cellular shrinking (due to dehydration) caused by molar concentrations of cryoprotectant and subsequent swelling during its removal can both result in damage if the critical osmotic excursion is exceeded. Whether the effects of cryoprotective agents are negative or positive is greatly dependent on the kinetics of exposure.

Moreover, it has been shown that the presence of these agents leads to changes in hydraulic conductivity. Various cryoprotectant species affect L_p in different ways, either increasing or decreasing it (see below), an action which leads to changes in the osmotic response of cells according to the mass-transport model. Hence, for optimization of a cryopreservation system, the optimal selection of cryoprotectant, both according to its colligative and permeability properties as well with respect to its effects on other membrane parameters and cell viability, is crucial.

Discrepancies between Theory and Practice in the Cryobiology of Gametes

As outlined above, several quantitative formalisms exist which allow one to predict the movements of water and solutes across cellular membranes during the freezing process. If the major membrane parameters such as hydraulic conductivity and solute permeability as well as the geometrical cell characteristics are known, the optimal equilibrium rates of cell dehydration can be calculated from the mass-transport model equations.

However, several discrepancies have already been found between theoretical approximations and experimental observations with respect to maximum cell volume response to aniso-osmolality. Moreover, empirically determined optimal cooling rates by no means agree with those calculated according to two-parameter models, especially in the case of mammalian spermatozoa. Water permeabilities extrapolated to sub-zero temperatures based on data obtained at supra-zero temperatures in the absence of extracellular ice and cryoprotectants lead to dramatic discrepancies between theoretical and experimental optimal cooling rates [45, 46]. Indeed, whereas observed experimental cooling rates lie in the area of 30–50 °C/min [47–49], the mathematical calculations for an L_p value of 0.5 $\mu\text{m}/\text{min}/\text{atm}$ and an activation energy of 3 kcal/mol imply that very high rates (up to 1000 °C/min) are appropriate for the dehydration of mammalian spermatozoa [45]. In fact, such high rates will be lethal: a major decrease in viability has been observed in a range of sperm species after cooling at no more than 100–300 °C/min. One way to account for this discrepancy between theory and practice is to consider the accuracy of mathematical approximations. For example,

in the two-parameter-model, when the concentration of impermeable salts is low, the concentration of permeable solute is high ($C_{\text{cryoprotectant}} \gg C_{\text{salt}}$), the Boyle Van't Hoff approximation of maximal cell volume excursion V_m (equation 1) is true, and the quotient $P_{\text{cryo}}/P_{\text{salt}}$ approaches infinity (characteristic for freezing in the presence of any applicable cryoprotectant), the product

$$(P_{\text{cryo}}/P_{\text{salt}} - 1)(\pi_0 V_0 - \pi_m V_m)$$

is indeterminate [33]. However, the discrepancy due to this indeterminacy should be not greater than 14 % for maximal volume excursion and therefore cannot essentially account for the discrepancy between theoretically predicted and observed optimal cooling rates. Another explanation lies in the fact that the presence of permeable substances can change the water permeability. Indeed, it has been shown in a large number of species that addition of cryoprotectants such as DMSO, EG and glycerol affects the hydraulic conductivity of many mammalian sperm species (Tab. 1). And, finally, it has been shown recently that the values of membrane parameters of mammalian spermatozoa at sub-zero temperatures in the presence of extracellular ice differ considerably from those measured at supra-zero temperature [48, 49, 57]. Indeed, it has been reported that L_p at sub-zero temperatures is lower by at least one order of magnitude than L_p at supra-zero temperature and that E_a at sub-zero temperatures is higher by a factor of 2 than E_a at supra-zero temperature (Tab. 2). Such divergence between membrane permeabilities at different temperatures may be related to cooling-induced changes in the sperm plasma membrane structure such as lipid phase transition, chilling injury (temperature-dependent alterations in macromolecular structures) or cold-shock [48]. Taking into account these divergences between supra- and sub-zero permeabilities and activation energies, theoretically calculated and experimentally determined optimal rates can be brought into agreement [45, 46]. It is clear that further progress in avoiding the lethal consequences of cryopreservation will depend on more exact experimental determination of membrane water permeability parameters in the presence of cryoprotectant at sub-zero temperatures using differential scanning calorimetry. Unfortunately, this method is limited in that it cannot measure the permeability of cryoprotectants.

There have been attempts to improve the theoretical prediction of cooling rates based on exact solutions for the two-parameter flux model [61] or to introduce a different approach to prevention of intracellular ice formation via restriction of intracellular supercooling [62]. Both these approaches are likely to have crucial biophysical applications in reproductive cryobiology as they will increase the validity of volume change simulations, assuming the membrane parameters can be determined with sufficient accuracy.

Table 1: Supra-zero water and solute permeability in spermatozoa of several mammalian species (at 20–22 °C).

Species	Cryo-protectant	L_p ($\mu\text{m}/\text{min}/\text{atm}$)	P_{cryo} ($\times 10^{-3} \text{ cm}/\text{min}$)	Method	Reference	
Mouse	None	0.84	–	EPC	[50]	
Mouse	None	0.56	–	EPC	[50]	
Percoll washed						
Mouse	ICR B6C3F1	EG	0.38	3.4	EPC	[50]
			0.68			
Mouse	ICR B6C3F1	Gly	0.38	2.2	EPC	[50]
			0.57			
Mouse	ICR B6C3F1	PG	0.25	1.2	EPC	[50]
			0.22			
Boar	None	1.03	-	EPC	[51]	
Boar	None	0.84	-	SF	[52]	
Boar	DMSO	0.12	0.93	EPC	[53]	
Boar	Gly	0.14	0.48	EPC	[53]	
Boar	EG	0.20	1.98	EPC	[53]	
Bull	None	10.8	-	TL	[54]	
Bull	None	0.69	-	SF	[29]	
Bull	DMSO	0.91	1.72	SF	[29]	
Bull	Gly	0.29	1.75	SF	[29]	
Bull	PG	0.42	2.47	SF	[29]	
Bull	EG	0.39	1.49	SF	[29]	
Ram	None	2.79	-	SF	[52]	
Rabbit	None	0.28	-	SF	[52]	
Rhesus monkey	DMSO	1.09	1.39	EPC	[55]	
Rhesus monkey	Gly	0.91	2.21	EPC	[55]	
Rhesus monkey	PG	1.53	3.38	EPC	[55]	
Rhesus monkey	EG	1.94	6.07	EPC	[55]	
Chimpanzee	EG	4.09	2.18	EPC	[56]	
Chimpanzee	PG	3.91	1.75	EPC	[56]	
Chimpanzee	DMSO	3.49	0.82	EPC	[56]	
Chimpanzee	Gly	2.83	1.42	EPC	[56]	
Man	None	1.84	-	EPC	[30]	
Man	Gly	0.77	2.07	EPC	[30]	
Man	DMSO	0.84	0.8	EPC	[30]	
Man	PG	1.23	2.3	EPC	[30]	
Man	EG	0.74	7.94	EPC	[30]	

Gly: glycerol; DMSO: dimethylsulfoxide; PG: propylene glycol (propane-1,2-diol); EG: ethylene glycol; EPC: electronic particle counter; SF: stopped-flow technique; TL: time-to-lysis technique.

Table 2: Combined best-fit parameter for water transport in mammalian spermatozoa of several mammalian species in the presence of extracellular ice

Species	Cryo-protectant	Sub-zero L_p ($\mu\text{m}/\text{min}/\text{atm}$)	Sub-zero E_a (Kcal/mol)	Supra-zero E_a (Kcal/mol)	Reference
Mouse	None*	0.01	22.5		[48]
Mouse	Glycerol	0.004	15.2	8.5	[48, 50]
Boar	None*	0.02	29.3		[49]
Boar	Glycerol	0.005	18.1	7.8	[49, 53]
Bull	None*	0.036	42.1		[58]
Bull	Glycerol	0.025	30.9		[58]
Dog	None*	0.0029	15.3		[57]
Dog	Glycerol	0.005	29.0		[57]
Dog	DMSO	0.003	12.3		[57]
Stallion	None*	0.02	32.7		[59]
Stallion	Glycerol	0.008	12.1		[59]
Man	None*	0.14	85.5	3.5	[30, 60]
Man	Glycerol – Egg Yolk	0.04	33.2		[60]

None*: membrane parameters were assessed in the presence of standard diluent in the absence of cryoprotectants, though egg yolk was present in some cases.

However, improvements in the accurate determination of membrane permeability parameters and the choice of appropriate mathematical approximations will not suffice in determining cell response to cryopreservation. One of the main reasons for the discrepancy between the

theoretical and experimental behavior of cells during the freezing/thawing process is the biological complexity of the cell. Biological membranes are too complex to justify some of assumptions made from the biophysical point of view. Although it is convenient to consider the cell as a "bag with salt water", there is a great deal of evidence that intracellular water and ions behave in a different way from those in ideal aqueous solutions. The cell cytoplasm does not behave like water with ions dissolved in it; there is much molecular interaction between different cell compartments where different signal transduction mechanisms are involved [17, 18]. Exposure to deep temperatures can affect this interaction and thereby signalling mechanisms; there is already evidence that some sperm intracellular signalling pathways can be affected during cooling and cryopreservation [63–66]. Also, the cell membrane is not a simple semi-permeable barrier. The physical and chemical composition of the membrane is complex and affected by temperature: changes in molecular structure at deeper temperatures will alter solute and water transport through pores, and changes in mobility of lipids will affect diffusion through the lipid bilayer. In particular, in cryopreserved spermatozoa, lipid diffusion in the plasma membrane has been reported to be significantly reduced relative to fresh spermatozoa [67]. These interactions would affect greatly biophysical models based on simplified assumptions. Analogously, the apparently relatively symmetrical external geometry of the oocyte cannot completely justify the use of current biophysical models to optimize cryopreservation protocols on the basis of the mass-transport model [3, 6]. The biochemical and physiological complexity of cellular organization such as cytoskeletal elements and cortical granules must be taken into consideration.

Finally, cell osmotic response is much more complex than described by the Boyle Van't Hoff relationship. There is a body of evidence that cells are able to moderate or reverse osmotically-induced swelling or shrinking by activation of ion channels and/or transport mechanisms [21]. In spermatozoa particularly, osmotically induced shrinking or swelling is known to activate ion channels, presumably by phosphorylation or dephosphorylation, whence major intracellular ions are allowed to leave or to enter the cell and subsequent water transport takes place [68] (Fig. 2). Such control of cell volume involves finite processes which activate within a time range of a few seconds to several minutes in mammalian spermatozoa ([68] and references therein). Volume regulation has also been reported for oocytes [70, 71]. The impact of these processes on cryopreservation response remains to be elucidated.

Cryobiological Heterogeneity of Reproductive Cells

Because of their central importance in animal breeding and human reproductive medicine, the cryobiology of reproductive cells, especially spermatozoa, oocytes and embryos has attracted a great deal of attention. Over the years major empirical advances have been made to develop successful protocols for their cryopreservation. Similarly, more refined methods for the mathematical prediction of optimal conditions for the freezing of these cells have been established [3, 48, 49, 57, 61, 62]. In this section the cryobiological peculiarities of spermatozoa and oocytes will be considered.

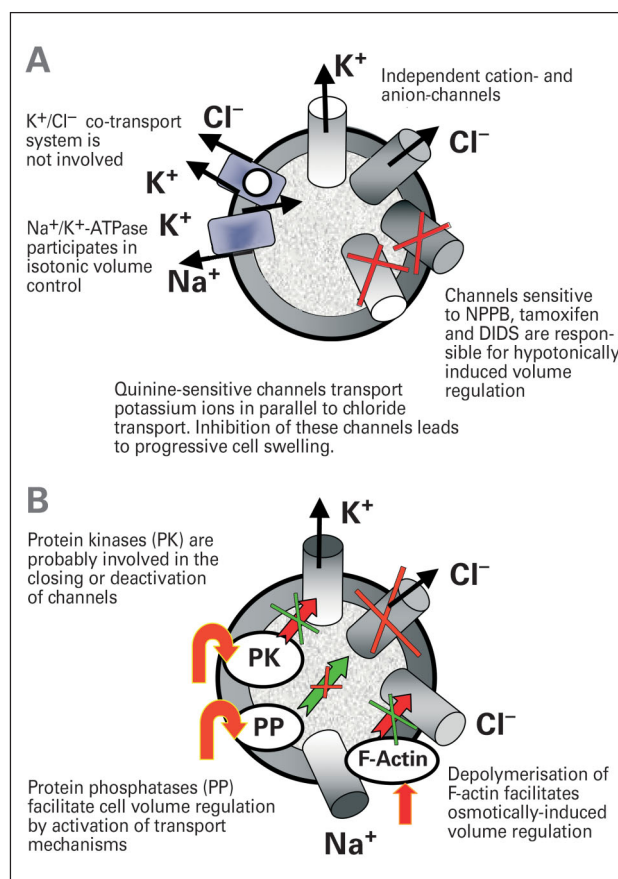


Figure 2: Mechanisms of volume regulation in mammalian spermatozoa (modified after [69]).

A. Transport mechanisms involved. Swelling-activated potassium and chloride channels are involved in osmotically induced volume regulation in mammalian spermatozoa [68, 69]. Inhibition of chloride and potassium channels leads to restriction or loss of volume regulatory function. In contrast, inhibition of K^+/Cl^- co-transport or Na^+/K^+ -ATPase has no negative effect on osmotically induced volume regulation though these systems may be involved in isoosmotic volume regulation, together with other mechanisms related to sodium, potassium, chloride and osmolyte transport. NPPB: 5-nitro-2-(3-phenylpropylamino)benzoic acid; DIDS: 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid. (These and tamoxifen are all inhibitors of chloride channels.)

B. Putative modulation and signalling pathways of volume control. The activation, regulation or deactivation of transport mechanisms can be mediated by modulation of F-actin and cytoskeletal integrity and/or by changes in phosphorylation/dephosphorylation balance (activity of protein kinases and phosphatases). Depolymerisation of F-actin facilitates volume regulation; phosphorylation appears to be involved in the closing or deactivation of channels involved in volume regulation while dephosphorylation leads to activation of ion transport mechanisms (probably chloride channels [68, 69]). Red arrows and lines: negative (inhibiting) action on transport and signalling mechanisms; green arrows and lines: positive (accelerating) action.

Cryobiological Properties of Spermatozoa

Various studies suggest that spermatozoa of different species have specific cryobiological properties and varied degrees of sensitivity to experimental manipulation, cold shock (lipid phase transitions), freezing and osmotic tolerance [3]. Moreover, high degrees of variability with respect to different individuals within a given species have been observed [10, 11]. Whereas species-specific differences in the molecular structure of the plasma membrane could explain differences in osmotic tolerance limits, hydraulic conductivity, cryoprotectant permeability and effects of different extenders on these biophysical properties, the causes of differences between individuals and especially of the heterogeneity within individual ejaculates from a particular male remain unclear.

Marked differences in sperm cell size and shape, as well in the fraction of osmotically inactive cell volume, exist between 10 mammalian species (Tab. 3). The iso-osmotic cell volume varies over a relatively narrow range (between 20–28 μm^3) in human, primate and large domestic animal species like boar and bull (Tab. 3). Wider variability was observed for the sperm volume of wildlife species; murine spermatozoa, in particular, have an exceptionally high volume, reported to be about 54 μm^3 as measured by the Coulter technique or about 73 μm^3 when measured by magnetic resonance (Tab. 3). Mammalian spermatozoa appear to act as perfect osmometers according to the Boyle Van't Hoff relationship over a wide range of osmolalities (Tab. 3). Osmotic tolerance varies between species: porcine and bovine spermatozoa are rather sensitive to osmotic changes (maximal cell volume excursion $V_{e,\text{max}}$ for maintaining motility is about 0.97–1.03), whereas murine spermatozoa have broad tolerance limits ($V_{e,\text{max}} \sim 0.76\text{--}1.24$) [53, 75, 78].

It is worth noting that there are considerable differences in sperm shape, cell volume and osmotic tolerance between mouse and man, which raises the reflection as to whether the mouse is the best model for studies on cryobiology and physiological cell function regulation in the field of human andrology.

Since freezing and thawing cause very large changes in the osmotic environment of cells, L_p appears to be the most important parameter in determining the response to exposure to deep temperatures in a given cell type. It is apparent that there is a large variation in L_p in mammalian species (Tabs. 1, 2). Estimates of L_p vary, depending upon the methodology used: e. g. time-to-lysis, stopped-flow or cell-sizing. The most accurate estimations for supra-zero permeability are given by cell sizing (particle counter technique) whereas differential scanning calorimetry is adequate for measuring sub-zero permeability [30, 48, 49, 57].

metry is adequate for measuring sub-zero permeability [30, 48, 49, 57].

The use of different cryoprotectants as components of semen extenders has led to considerable improvements in the cryopreservation of spermatozoa. The selection of a suitable cryoprotectant depends on (a) its membrane permeability in the given species and (b) its possible toxic effects. Not only do different cryoprotectants vary in their permeability but also a given cryoprotectant's permeability varies considerably between species (Tab. 1). Moreover, different cryoprotectants produce effects of different degrees on membrane hydraulic conductivity. In man, boar and mouse, the addition of cryoprotectants reduce the hydraulic permeability of sperm membranes; the degree of change is species-dependent. Moreover, even sperm from different mouse genotypes appear to have different water and cryoprotectant permeabilities and therefore require specific cryopreservation protocols (Tab. 1). Hence the development of new protocols or their modification by transfer to other species should be based on knowledge of specific cell osmotic and thermodynamic characteristics and the effects of different solutes and their colligative properties on these characteristics.

Cryobiological Properties of Oocytes

The successful freezing of oocytes will play an important role in both animal and human assisted reproduction. Use of oocyte cryobanks will provide the necessary basis for availability of large number of frozen oocytes in different state of maturation, and will allow, for example, female cancer patients to retain their fertility [3]. The use of frozen oocytes in assisted reproductive technologies will represent a major breakthrough as an alternative to frozen embryos, as it will avoid the ethical and legal objections related to embryo cryopreservation [79–81].

Generally, oocytes behave as perfect osmometers with an inactive cell volume fraction in the range of about 20 %. This parameter appears to vary relatively little between mammalian species, although a high degree of individual variation has been reported [82]. In fish oocytes, in contrast, higher osmotic inactive volumes have been reported than in mammalian species.

The oocyte is one of the largest mammalian cell types: the mean isotonic volume of human oocytes is about $2.65 \times 10^6 \mu\text{m}^3$ [83]. Being essentially spherical in shape, their area-to-volume ratio A/V is very low, < 0.01 ; their surface area is in the range $10\text{--}100 \times 10^3 \mu\text{m}^2$, e.g. $16 \times 10^3 \mu\text{m}^2$ for murine oocytes [84]. They have a relatively low hydraulic conductivity and low membrane permeability towards cryoprotectants (Tab. 4), thus opportunities for improvements of cryopreservation protocols are limited. Transport of water and permeable cryoprotectants, critical for dehydration of the intracellular environment and minimisation of intracellular ice formation and osmotic stress, is less efficient than in embryos (whose surface-to-volume ratio is much higher due to their multi-cellularity [97]) and in spermatozoa (where water and solute permeabilities are higher): for comparison see Tables 1 and 4.

One of the main focuses of cryobiological research on oocytes is the understanding of changes in permeability characteristics of the membrane. The reported hydraulic

Table 3: Osmotically inactive fraction of cell volume, iso-osmotic volume, and osmotic range of Boyle Van't Hoff response of several mammalian sperm species.

Sperm species	V_t/V_{iso}	V_{iso} (μm^3)	Osmotic range (mosmol/kg)	Reference
Mouse	0.13*	73.4	250–900	[72]
Mouse	0.61**	54.5	75–1200	[73]
Boar (ejaculated)	0.23*	22–25.4	210–1500	[74]
Boar (ejaculated)	0.67	26.3	185–900	[51]
Boar (ejaculated)	0.63	24.1	150–900	[22]
Boar (epididymal)	0.64	25.0	150–900	[22]
Bull	0.61	23.5	150–1200	[75]
Warthog (ejaculated)	0.59	16.5	58–870	[76]
Warthog (epididymal)	0.36	21.9	58–870	[76]
Impala (ejaculated)	0.45	27.8	58–870	[76]
Impala (epididymal)	0.36	28.9	58–870	[76]
Elephant (ejaculated)	0.16	30.8	58–870	[76]
Elephant (epididymal)	0.35	27.3	58–870	[76]
Lion (ejaculated)	0.25	24.9	58–870	[76]
Rhesus monkey	0.77	36.8	75–900	[77]
Rhesus monkey	0.51	27.7	160–860	[55]
Chimpanzee	0.69	21.7	160–860	[56]
Man	0.50	28.2	145–900	[30]

*These volumes correspond only to the fractional volume of osmotically inactive water in spermatozoa as determined by paramagnetic resonance. The total fraction of intracellular water was assumed to be 0.59 in mammalian spermatozoa.

**This value is a combined one for murine spermatozoa from ICR and B6C3F1 strains.

permeability L_p of oocytes in the absence of cryoprotectants lies in the range of about 0.4–0.5 $\mu\text{m}/\text{atm}/\text{min}$ at 20 °C and shows little variation between species. Cryoprotectants affect oocyte L_p as they do in spermatozoa. However, in contrast to their effects in the latter cell type, cryoprotectants seem to increase L_p in several oocyte species, thereby accelerating and facilitating water flux; this effect was especially marked in human and bovine oocytes when DMSO was used as cryoprotectant. The maturation state of the oocyte also seems to have a crucial bearing on L_p (Tab. 4). In rat oocytes water permeability decreases during maturation whereas in bovine oocytes it increases [91, 92, 98]. Such changes could be associated with disappearance or occurrence of transcripts for the broadly selective channels responsible for solute and water transfer in cells, e. g. aquaporin-9 transcript in rat oocytes [98].

The optimal choice of cryoprotectants for oocyte cryopreservation varies between oocytes of different species, dependant on protectant permeability characteristics (Tab. 4). The type of cryoprotectant, its concentration, and the temperature of exposure have been shown to affect greatly changes in cell volume. Even small changes in duration of exposure to cryoprotectant prior to cooling can result in drastic changes in cellular hydration [99]. Studies have been performed on both mouse and human oocytes, using the protectants PG (propylene glycol), EG (ethylene glycol) and DMSO commonly used for cryopreservation of these cells [79, 80, 83, 96]. It has been demonstrated in particular that DMSO has a lower permeability than PG and thus would probably not be a suitable alternative. Similar permeability studies like those on spermatozoa are required to establish procedures for oocytes that will yield acceptable rates of post-thaw survival.

Cooling and Warming Rates

The survival of spermatozoa and oocytes and the maintenance of their fertilization potential require the use of optimal cooling and warming rates. Not surprisingly, it has been found that these rates vary between species and between cell type.

The greatest advances in sperm cryopreservation have been achieved for bull and man. This success can be explained partly by the relatively favourable fundamental cryobiological properties of these cell species (see Tables), and partly by the general application of empirically determined slow freezing rates (1–5 °C/min) during initial cooling, followed by more rapid cooling rates after initial

Table 4: Supra-zero water permeability in absence and presence of cryoprotectant and solute permeability in mature and immature oocytes of several mammalian species

Oocyte species	Cryo-protectant	Stage	L_p ($\mu\text{m}/\text{min}/\text{atm}$)	P_{cryo} ($\times 10^{-3} \text{ cm}/\text{min}$)	Temp	Reference
Murine	None	Mature*	0.36	-	21 °C	[85]
Murine	None	Mature*	0.40	-	22 °C	[86]
Murine	DMSO	MII	0.41	0.96	23 °C	[87]
Murine	PG	MII	0.36	1.44	23 °C	[87]
Murine	EG	MII	0.51	0.54	19 °C	[88]
Murine	DMSO	MII	0.4	1.07	20 °C	[84]
Murine	Gly	MII	0.98	0.02	24 °C	[89]
Rat	DMSO	GV	0.68	1.29	20 °C	[90]
Rat	DMSO	MII	0.45	0.56	20 °C	[90]
Hamster	None	Mature*	0.45	-	22 °C	[86]
Bovine	None	Immature*	0.45	-	20 °C	[91]
Bovine	None	In vitro matured*	0.84	-	20 °C	[91]
Bovine	EG	GV	0.5	1.32	22 °C	[92]
Bovine	EG	MII	0.83	2.22	22 °C	[92]
Bovine	DMSO	GV	0.73	1.6	20 °C	[93]
Bovine	DMSO	MII	1.16	2.6	20 °C	[93]
Goat	PG	GV	0.97	0.87	20 °C	[94]
Goat	PG	MII	0.60	1.2	20 °C	[95]
Rhesus monkey	EG	Immature*	1.08	1.08	30 °C	[95]
Rhesus monkey	EG	MII	1.14	0.84	30 °C	[95]
Rhesus monkey	DMSO	Immature*	0.96	3.60	30 °C	[95]
Rhesus monkey	DMSO	MII	1.08	6.00	30 °C	[95]
Human	None	Mature*	0.65	-	21 °C	[85]
Human	None	Ff	0.84	-	21 °C	[85]
Human	None	Mature*	0.40	-	20 °C	[82]
Human	None	Ff	0.64	-	20 °C	[82]
Human	DMSO	MII+GVBD	0.70	1.5	24 °C	[83]
Human	PG	Mature*	0.53	1.68	24 °C	[96]

PG: propylene glycol (propane-1,2-diol); DMSO: dimethylsulphoxide; Gly: glycerol; EG: ethylene glycol; GV: germinal vesicle stage; GVBD: germinal vesicle breakdown; MII: metaphase II stage; Ff: failed to fertilize.

* These values were obtained in mature/immature oocytes without specification of the exact maturation stage.

ice formation (50 °C/min). Human spermatozoa in particular show a very broad survival curve in response to different cooling rates, with negligible differences in post-thaw viability between 1–100 °C/min (optimum about 10 °C/min) [100]. Despite a relative lack of detailed cryobiological investigation, the use of sperm processed in this way for artificial insemination or in vitro fertilization has been successful, even though maintenance of viability is sub-optimal (~50 % or less). However, less progress has been achieved in other species, as the simple transfer of protocols has not proven appropriate. It is clear that the various physical changes that take place in sperm cells undergoing freezing must be considered in detail in order to improve the methodology in each particular case. In general, optimal cooling rates for successful freezing of spermatozoa from many large and domestic animal species range narrowly between 30 and 50 °C/min; careful control of these rates is essential for maximum recovery of viable and functional cells [49, 57, 101].

Slow equilibrium freezing of oocytes is crucial to prevent the lethal consequences of intracellular ice formation.

However, the exposure to high solute concentration that necessarily accompanies this slow freezing needs to be optimized with the help of a theoretical cryobiological approach so as to avoid lysis during thawing [79–84]. Currently, optimal cooling rates lie in the range 0.3–0.5 °C/min in order to promote extracellular ice nucleation at higher sub-zero temperatures (not far below zero). After the cells became dehydrated, they are plunged into liquid nitrogen with almost no risk of intracellular ice formation. Successful freezing protocols for oocytes, derived empirically as well as on the basis of known effects of medium components on membrane permeability parameters, nevertheless result in survival rates of only 40–50 %, which for practical purposes is low [79].

Particular Aspects of Gamete Cryobiology

Cellular Cryoinjury of Gametes

Freezing has several severe consequences for gamete function. The overall consequence is cell death and/or limited fertilization ability. In spermatozoa, extensive damage occurs to the plasma membrane and the acrosome, and to the tail configuration, with the result that motility is impaired or destroyed [9–11]. The degree of damage is related to the cold-shock sensitivity of the particular species resulting from differing biochemical and biophysical membrane characteristics: while porcine spermatozoa are very sensitive, bovine and equine are less sensitive, and human are least sensitive [102].

There are reports about the lethal effects of intracellular ice formation during freezing on the viability of both spermatozoa and oocytes [100, 103]. These effects are amplified at high cooling rates. Cell injury during cryopreservation is also due to the toxic effects of cryoprotectants. Cryoprotectants in high concentration have detrimental effects on sperm motility in a number of mammalian species [104, 105]. This may be due in part to poor volume regulation, resulting in post-thaw lysis and reduced sperm viability after cryopreservation [106, 107]. Single-step addition of cryoprotectants at supra-zero temperatures may result in critical volume excursions close to the limits beyond which lethal damage can occur. These limits may be only 2–7 % of isotonic volume in the spermatozoa of some species and about 30 % of isotonic volume in oocytes [53, 80]. If they suffer critical volume excursion, the cells are more vulnerable to subsequent freezing and thawing, thus this situation should be avoided [108].

In addition to the detrimental physical and colligative effects of cryoprotectants, these compounds can have direct effects on intracellular structures and mechanisms. In murine oocytes the cortical actin network was disrupted by exposure to molar concentrations of DMSO [109]. In zebrafish oocytes, the viability decreased and germinal vesicle breakdown was observed with increasing molar concentrations of PG, DMSO, EG, sucrose and glucose [110]. From these studies, it is clear that cryoprotective agents should be present at minimal possible concentrations, and a multi-step approach should be used for their addition and removal.

For oocytes in particular, reduced fertilization ability and cell death are likely to be due very largely to the disruption of cytoskeletal elements and cortical granules at low temperatures [109, 111, 112]. Cooling of oocytes resulted

in a progressive disassembly of the spindle and dispersal of the chromosomes. The extent of the changes observed was dependent upon temperature and time of exposure [109]. It should be noted that lipid phase transition in human oocytes in different maturation states appears to take place at higher temperatures than in zygotes, which explains the increased sensitivity of oocytes to cooling and cryoinjury [113].

Particularities of Sperm Physiology and their Relevance for Cryopreservation

Apart from lethal changes produced by cryoinjury in a general way, e.g. via intracellular ice formation or solution effects, there may be sub-lethal changes in spermatozoa that take place at a molecular level, impacting on their cellular function and impairing fertilizing capacity.

Spermatozoa are not able to fertilize an oocyte immediately after deposition in the female genital tract. They must first undergo changes collectively known as capacitation, during which they acquire fertilizing ability; capacitation is a prerequisite for the acrosome reaction and fusion with the oocyte. In murine spermatozoa, these changes have been shown to involve massive BSA-mediated cholesterol efflux from the plasma membrane [114]. This efflux takes place under the action of bicarbonate, which is a component of artificial capacitation media. It has been found that in boar spermatozoa the bicarbonate acts to stimulate a form of adenylyl cyclase and initiate cAMP-dependent pathways, one of which brings about remodelling of the phospholipid distribution in the plasma membrane, whence the cholesterol can be extracted by BSA [115]. Further slower changes occur during capacitation, such as remodelling of the sperm surface, protein tyrosine phosphorylation, increased internal pH and Ca^{2+} levels, and membrane polarization [116–119]. An understanding of the capacitation process can be very important in explaining the detrimental effects of cryopreservation on sperm fertilizing capacity, and will therefore have a bearing when improvements are sought. Although sperm are subjected to cryopreservation soon after ejaculation or collection, i.e. before the onset of capacitation, the physiological characteristics of thawed sperm have been found to be similar in certain respects with those of sperm that are in advanced stages of capacitation [9, 120], although they do not correspond completely at the molecular level. This phenomenon is known as cryo-capacitation [63–65]. In particular, frozen-thawed sperm have decreased survival ability, increased acrosomal changes, shortened time interval to undergo further membrane destabilization and develop oocyte penetrating ability, and a decreased ability to bind to the oviductal epithelium [9, 10].

Knowledge regarding the physiology of capacitation is therefore highly relevant to understanding the mechanisms by which cryopreservation alters sperm function. Although millions of sperm are deposited in the female reproductive tract, only a small number are trapped in the distal portion of the oviductal isthmus serves as a protective storage region [121]. Numerous studies using *in vitro* oviduct-sperm binding assays in several species have shown that viable uncapacitated sperm are selectively bound by the oviductal epithelium and that there is suppression of capacitation-related events such as the influx of calcium ions into the cell and tyrosine phosphorylation of sperm proteins during such binding [122–127].

This sperm storage site in the mammalian oviduct is designated a functional sperm reservoir since it fulfils the following functions: 1) selection of the fertilization-competent sperm population, 2) modulation of sperm capacitation, and 3) regulation of sperm transport to minimize polyspermic fertilization [128–131]. Since the capacitation process is understood as a controlled on-going destabilization process which reduces the life span of sperm, the maintenance of sperm viability within a certain window of time and the control of capacitation are mutually associated events [132, 133]. To provide sufficient competent spermatozoa at the time of ovulation, capacitation should not be completed until after ovulation has occurred. At this time, conditions within the oviduct seem to reverse the inhibitory influence of the oviduct on sperm function, initiating capacitation and hyperactivation in order for the sperm cells to release and to progress toward the site of fertilization [133]. It should be noted that sperm populations appear to undergo capacitation at different rates, thereby providing a continuous supply of “primed” cells ready to fertilize the ovulated eggs.

Assessment of Frozen Semen

The morphological and functional changes of sperm that result from cryoinjury require assessment [9–11]. Whereas gross aspects such as changes in morphology and motility can be readily assessed using phase-contrast microscopy, there is a need to establish more sophisticated spermatological methods that will allow rapid and sensitive assessment of sperm function in frozen-thawed semen. Given the similarity between frozen-thawed and capacitated spermatozoa, and the fact that ejaculated spermatozoa are not able to fertilize an oocyte, it is logical to conclude that assessment of those sperm function parameters that are modified during the capacitation process must be performed under fertilizing conditions [134]. It is known that animal fertility varies not only between males but also between ejaculates within individual males, therefore there is a need to design assays sensitive enough to detect intra- as well as inter-individual differences and even the subtle heterogeneity within one ejaculate of a given individual [10, 11, 135, 136].

Assays which have been shown to be sufficiently sensitive to assess the influence of cooling and/or cryopreservation on semen quality (and thereby to predict its freezability) include:

- inducibility of the acrosome reaction by calcium ionophore
- measurement of changes in intracellular calcium content
- measurement of volume regulatory ability
- assessment of plasma membrane lipid disorder (merocyanine assay)
- computer-assisted motility analysis
- assessment of sperm chromatin stability
- assessment of ability to bind to oviductal explants.

These and other assays have been reviewed in recent reviews of clinical and laboratory assessment of sperm function [107, 137–139]. Details of the assays are to be found in the many original studies cited in these reviews.

Conclusions

A basic understanding of the cellular processes which accompany cryopreservation is necessary for successful

development of its applications in reproductive biomedicine. Physico-chemical conditions inside and outside the cell are controlled by three main parameters: extracellular solute concentration, the presence of permeable and impermeable solutes, and the cooling and warming rates. Optimization of a cryopreservation protocol requires a three-step approach: optimization for a particular cell type, for that cell type within species, and for that cell type among individuals in the given species. For reproductive medicine in particular, the heterogeneity between cells of the same type within a single sample (ejaculate) from an individual male must be taken into consideration. Perhaps the next decade will reveal the development and evolution of profound cryobiological approaches which will highlight the cell response produced by this subtle but crucial kind of heterogeneity. Better means of assessing the level of damage produced by cryopreservation are needed. From a technological point of view, future research should be focussed on improving techniques for the evaluation of biophysical and osmotic parameters and on establishing or developing alternative freezing technologies e. g. isochoric freezing or cryoprotectant-free vitrification [140, 141].

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