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SYMPOSIUM: OOCYTE CRYOPRESERVATION
REVIEW

Reprint of: Theoretical and experimental basis of slow freezing

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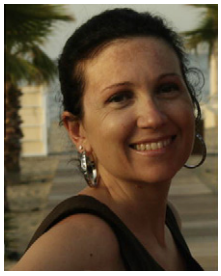

Lucia De Santis graduated in biological sciences at the University of Milan in 1993. During 1989–1994, she worked in the surgical pathology department in the university. In 1995, she established the IVF laboratory of S. Raffaele Hospital, Milan of which she is laboratory director and where she has developed a large IVF programme and introduced basic and more advanced techniques. Lucia has been awarded a Masters in reproductive medicine by University of Padova and is lecturer of the Masters in biotechnology applied to assisted reproduction at Vita-Salute University, Milan. Her current interests concern fundamental and clinical aspects of oocyte cryopreservation.

Abstract In human IVF, cryopreservation of oocytes has become an alternative to embryo storage. It has also shown enormous potential for oocyte donation, fertility preservation and animal biotechnology. Mouse oocytes have represented the elective model to develop oocyte cryopreservation in the human and over several decades their use has made possible the development of theoretical and empirical approaches. Progress in vitrification has overshadowed slow freezing to such an extent that it has been suggested that vitrification could soon become the exclusive cryopreservation choice in human IVF. However, recent studies have clearly indicated that human embryo slow freezing, a practice considered well established for decades, can be significantly improved by a simple empirical approach. Alternatively, recent and more advanced theoretical models can predict oocyte responses to the diverse factors characterizing an entire slow-freezing procedure, offering a global method for the improvement of current protocols. This gives credit to the notion that oocyte slow freezing still has considerable margins for improvement.

KEYWORDS: cryopreservation, cryoprotectants, fertility preservation, oocytes, slow freezing

Introduction

Cryopreservation of oocytes lends itself to numerous applications, some of which have recently become normal practice in many IVF laboratories. In infertility treatments, storage of mature metaphase II (MII) oocytes ensures that the whole reproductive potential of an ovarian stimulation cycle may be conveniently used, at the same time circumventing legal and ethical problems derived from the

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generation of surplus embryos. Oocyte storage has also enormous potential for making oocyte donation safer (by allowing a suitable temporal window for donor screening) and for giving a hope for the preservation of fertility in women at risk of premature loss of ovarian function. In animal biotechnology, oocyte cryopreservation can preserve economically important and genetically modified strains, thereby avoiding the implications of genetic drift.

The first signs of an interest in the low-temperature storage of mammalian oocytes can be traced back as early as the late 1950s, when Sherman and Lin (1958) described the consequences of cooling in mouse oocytes. In 1977, for the first time live births of mice from mature oocytes stored in liquid nitrogen (LN_2) were documented (Whittingham, 1977).

Since these initial achievements, mouse oocytes have represented the elective model to develop oocyte cryopreservation in the human. Experimentally, mouse oocytes are suitable for several reasons. For example, they have plasmalemma permeability characteristics which dictate a rate of shrinkage—swelling response upon exposure to propanediol (PROH) similar to that of human oocytes (Paynter, 2005). Over several decades, their use has made possible the development of theoretical and empirical approaches to oocyte cryopreservation.

In human IVF, in which the first reported pregnancy from cryopreserved oocytes was described in 1986 (Chen, 1986), slow freezing was the method of choice until a few years ago. This approach has generated thousands of births, many of them unreported, and is still being used in numerous laboratories. In recent years, vitrification has gained increasing credit for oocyte cryopreservation, by improving post-storage survival rates and apparently preserving more efficiently oocyte developmental ability (Cobo et al., 2008).

Vitrification has overshadowed slow freezing to such an extent that it has been foreseen that vitrification could soon become the exclusive cryopreservation choice in human IVF (Vajta and Nagy, 2006). However, recent studies have clearly indicated that embryo slow freezing, a practice considered well established for decades, can be significantly improved by a simple empirical approach (Edgar et al., 2009). Alternatively, novel theoretical models have the potential to predict the oocyte response to freezing and thereby indicate protocol modifications which can reduce or eliminate cryoinjury (McGrath, 2009). This gives credit to the notion that slow freezing still includes uncharted territories whose exploration could make oocyte cryopreservation more efficient.

Between empiricism and theory

In the absence of control, freezing is lethal to cells. Because at temperatures well below 0°C, the freezing of water is the prevailing phenomenon and because water constitutes up to 80% of the living matter, it has long been believed that freezing causes cell death exclusively as a consequence of injuries caused by the formation of ice crystals. In reality, the freezing of aqueous solutions involves a number of phenomena that may affect cell viability, such as changes in solute concentrations, osmotic stress and pH alterations. Therefore, successful cryopreservation requires the control of several detrimental factors which interact in a very complex and often poorly understood fashion.

A twist in cryopreservation history occurred when, in fact rather accidentally, Polge, Smith and Park found that solutions containing glycerol were able to preserve the viability of cock spermatozoa for extended periods of time at −80°C (Polge et al., 1949). Other empirical attempts rapidly followed and soon it was realized that cell survival could be improved by cooling samples very slowly. Collectively, these initial experiences led to the definition of conditions generally necessary, and sometimes sufficient, for the slow freezing of different types of cells: (i) permeation with freezing solutions of 10—20% glycerol or molecules with similar properties, thereafter known as cryoprotective agents (CPA); (ii) cooling at rates of −1°C or slower; (iii) storage at −130°C or lower; and (iv) rapid warming.

Protocols applied for freezing mouse oocytes were generated empirically from those previously developed for embryos of the same species (Whittingham, 1971). Generally, mouse oocytes are exposed at low supra-zero temperatures to 1.5 mol/l dimethylsulphoxide (DMSO), cooled slowly (0.3—0.5°C/min) to temperatures between −30°C and −40°C and finally rapidly cooled and stored in LN_2. Thawing is performed slowly (at about 8—20°C/min) or rapidly (200°C/min or faster) before CPA is diluted and samples rehydrated. Further studies have highlighted the importance of other factors, such as chemical identity of CPA (Nash, 1962), temperature and mode of exposure and removal of CPA (Paynter et al., 1997), dehydration (Fabbri et al., 2001), showing how apparently marginal changes in any of the above steps can have important consequences on oocyte survival and development.

Progress has also been achieved in the theoretical understanding of fundamental principles that govern slow freezing. For example, the hypothesis formulated by Mazur (1963) identified excessive intracellular ice formation (IIF) and high solute concentration, derived from the freezing of water, as the major causes of cell injury during slow freezing. Having stood the test of time, the Mazur hypothesis is essentially correct and has inspired several attempts to improve freezing protocols. However, the fact that other principles are not yet fully understood engenders the belief that cryopreservation still has inherent margins for improvement. A recently developed computer model can predict how, depending on specific sets of operating parameters (e.g. CPA concentration) the probability of IIF changes (McGrath, 2009). Therefore, protocols can be designed to establish conditions that limit injury or stress and maximize cell viability.

Basics of slow freezing

Water and aqueous solutions possess a distinct tendency to cool below their melting point before ice begins to form. For example, despite the fact that the melting point of water is 0°C, in the presence of a permeating CPA in excess of 1.0 mol/l an aqueous sample may be cooled to approximately −40°C before ice is formed. At such low temperatures, once started, conversion of liquid water into ice occurs very rapidly if the intracellular compartment remains hydrated (Mazur, 2010; Mazur et al., 2005). To preserve viability, cells need to be dehydrated before ice may propagate intracellularly,

although the zona pellucida of the oocyte is thought to act as a barrier to impending extracellular ice. CPA are nevertheless crucial to achieve cell dehydration.

Even before cooling is started, the presence of CPA in the freezing mixture generates an osmotic gradient that extracts water from the intracellular environment. CPA classified as intracellular, such as DMSO and PROH, are oligohydroxy low-molecular-weight compounds of relatively low toxicity which can enter the cell across the plasmalemma. Within the cell they interact with biomolecules, acting as 'water replacements'. Because cell membrane permeability to permeating CPA is lower in comparison to water, loss of intracellular water is more rapid than gain of CPA, with the consequence that changes in cell volume over time produce a typical shrink–swell curve. This is of relevance to cell viability because excessive volume reductions, presumably above 40%, may represent a source of cell injury. Mouse and human oocytes have been extensively studied in this respect (De Santis et al., 2007a; Paynter et al., 1997, 1999a, 2001, 2005). On the other hand, the choice of permeating CPA is not without implications. The permeability of the oocyte plasmalemma to DMSO, PROH and ethylene glycol is different, a fact which influences the osmotic response. Glycerol, although having protective properties similar to DMSO and PROH, is not used as a CPA for the storage of oocytes. In fact, plasmalemma permeability to glycerol is so low that a very pronounced shrinkage is generated on exposure to this CPA (Edashige et al., 2003). Low plasmalemma permeability also implies that, to achieve equilibration at the conditions used for other CPA, a few hours would be required instead of minutes.

Permeating CPA may also have different intrinsic toxicity. This can obviously affect survival after cryopreservation, irrespective of the events occurring during freezing and thawing. The fine balance between cryoprotective properties and toxicity cannot be established theoretically for each cell type and therefore needs to be found empirically. For instance, PROH has been found to cause a high rate of oocyte activation in mouse oocytes (Shaw and Trounson, 1989), while this manifestation does not seem to occur in the human.

Extracellular CPA are polyhydroxylic and do not diffuse into the intracellular compartment, being unable to cross the plasmalemma. Dehydration therefore is not accompanied by replacement of part of intracellular water by these CPA and osmotic equilibrium is obtained by simple concentration of intracellular solutes.

CPA addition

The conditions at which oocytes are exposed to CPA are also important. Membrane permeability is increased at higher temperatures. When CPA are loaded at 37°C, the rate of exchange of water and CPA between the intra- and extracellular compartments is increased and the shrink–swell response is reduced in comparison to dehydration carried out at room temperature (Paynter et al., 1997, 2001). However, in slow-freezing protocols, cells are rarely exposed to CPA at temperatures above 24°C, because CPA toxicity is enhanced by higher temperatures. Some exceptions to this rule have been reported (Gook et al., 2007; Yang et al., 2007). This gives a clear example of how different factors interact in a complex fashion during the cryopreservation process. However, values of membrane permeability at given temperatures and minimum tolerated volume can be estimated experimentally and integrated in theoretical models that, for each CPA or mixture of CPA, predict the optimum time of exposure and concentration to obtain the desired degree of dehydration without exceeding the minimum tolerated volume. *Vice versa*, it may happen that the required concentration of CPA generates a shrink–swell response beyond the limits of tolerability of the cell (De Santis et al., 2007a). Developmental stage may determine the degree or type of damage from osmotic stress. In immature oocytes, damage may be caused not necessarily by the rate of the shrink–swell response, but rather on the type of shrinkage. At the immature stage, cytoplasmic processes of cumulus cells cross the zona pellucida and make contact with the plasmalemma. During shrinkage, these processes (which remain embedded in the zona even after removal of cumulus cells) may anchor selected areas of the plasmalemma to the zona. As a consequence, shrinkage can occur very asymmetrically instead of isometrically, with possible implications for the cell architecture (Younis et al., 1996).

Protocols may be designed to circumvent excessive osmotic stress. Determination of plasmalemma permeability to water (L_p) and CPA (P_{cpa}) is fundamental to appraise and control the extent of osmotic stress. L_p may be measured by monitoring volume changes induced by non-isotonic conditions which determine water efflux. P_{cpa} may be assessed by measuring oocyte shrinkage and swelling on exposure to a specific CPA. Unlike other cell types, oocytes are extremely large. In addition, their sphericity is often maintained during shrinkage and swelling, allowing simple calculation of their volume on the basis of the equatorial area.

Various approaches exist to measure oocyte volume changes. One of those is based on a microperfusion system (Paynter et al., 1999b). This requires that a single oocyte is placed in a 5-μl droplet of buffered medium. The dish containing the oocyte is placed on the stage of an inverted microscope. A holding micropipette is used to hold the oocyte in the correct position during perfusion by negative pressure applied to the zona pellucida, care being taken not to deform the inner plasmalemma. The oocyte is then perfused by carefully adding an excess volume of perfusate. The oocyte is observed before, during and after perfusion using the inverted microscope and the images are recorded by a video camera. Using computer software and the cross-sectional area, the volume of the oocyte at each time point is calculated and normalized to the volume of the oocyte immediately prior to perfusion. Best-fit plots to the measured data are generated using computer software (Gao et al., 1994). This software generates combinations of values for permeability of the cell to water (hydraulic conductivity) and permeability of the cell to solute, according to the Kedem–Katchalsky model (Kedem and Katchalsky, 1958), of the movement of solutes across cell membranes. This and other approaches have been applied to calculate L_p and P_{cpa} of the plasmalemma in oocytes of different species.

Mouse oocytes are a valuable model (Paynter, 2005). Despite human material available for research being scarce, attempts have been made to establish plasmalemma permeability to water, DMSO, PROH and ethylene glycol (Bernard et al., 1988; De Santis et al., 2007a; Hunter et al., 1992; Newton et al., 1999; Paynter et al., 1999a, 2001, 2005). To control osmotic response during dehydration generated by exposure to PROH and ethylene glycol, protocols have been designed in a multistep fashion and using increasing CPA concentrations (Borini et al., 2007; De Santis et al., 2007a). In some cases, as a confirmation of the difficulty in understanding the cryopreservation process, the multistep approach has not met the expectations of alleviating potential damage from stress. For example, a two-step exposure to PROH does not improve oocyte survival and developmental ability in comparison to a condition in which the same CPA is added in a single step (Bianchi et al., 2007; Borini et al., 2007). Likewise, attempts to develop slow-freezing protocols based on multistep exposure to ethylene glycol, which induces a massive osmotic response when added in a single step, have been frustrated by low survival rates (De Santis et al., 2007a).

Before cooling and after initial exposure to a given permeating CPA, oocyte dehydration can be further obtained by mixtures containing the permeating CPA and a non-permeating CPA, typically sucrose. A low concentration of sucrose (0.1 mol/l) is probably insufficient to ensure adequate dehydration in human oocytes, considering that under this condition survival rates do not exceed 35–40% (Borini et al., 2004). With a higher concentration of sucrose (0.3 mol/l), which determines a higher degree of dehydration (Paynter et al., 2005), survival rates are largely improved and normal fertilization rates by intracytoplasmic sperm injection are re-established. Nevertheless, implantation ability remains compromised (Borini et al., 2006; De Santis et al., 2007b; Levi Setti et al., 2006), a likely consequence of cytoplasmic aberrations (Nottola et al., 2007) and organelle damage (Gualtieri et al., 2009). This further confirms the complexity of cryopreservation and, in particular, the concept that solution of an individual problem does not necessarily guarantee a net improvement in the performance of the procedure as a whole.

Cooling rates

After the initial phase of dehydration is achieved by CPA exposure at supra-zero temperatures, samples are required to be cooled to temperatures at which extracellular ice may be formed. Firstly, the temperature is lowered to a range slightly below 0°C (from −4°C to −9°C). Within this thermal interval, ice nucleation (seeding) is induced deliberately by touching the vessel in which the sample is contained with a LN_2-cooled object. In such a way, the freezing solution does not undergo the above-described phenomenon of undercooling which would cause IIF for the presence of residual freezable water in the intracellular compartment. Seeding initiates extracellular ice formation in a controlled fashion. In water, the growth of the nucleated ice is relatively rapid, but is reduced in the presence of solutes and in particular CPA.

As temperature is slowly lowered further, the controlled conversion of extracellular liquid water into ice has important consequences. Solutes progressively segregate in the liquid phase and this solute accumulation lowers the freezing point of the unfrozen fraction allowing an equilibrium between the two phases and, more importantly, generates an osmotic gradient across the cell membrane that drives dehydration of the intracellular compartment. To a large extent, this supplementary phase of removal of intracellular water is beneficial. This is particularly important for the freezing of oocytes. In fact, these cells have a small surface-to-volume ratio which limits the initial dehydration performed at supra-zero temperatures.

Apart from this dehydration action, high extracellular salt concentration may have significant undesired biological effects, such as membrane destabilization and protein denaturation. Solute accumulation in the unfrozen fraction may be detrimental not only in dependence of the absolute concentration, but as a function of the time of exposure. Relatively shorter exposure to sublethal concentrations of extracellular solutes may be compatible with cell viability, but prolonged periods can be toxic. Thus, once ice nucleation is triggered, the transition of liquid water into ice, and consequently exposure to high solute concentration in the unfrozen fraction, ideally should respond to a fine balance. It should be protracted for a period of time sufficient to achieve extraction of freezable water still present in the intracellular compartment, but at the same time appropriately short to avoid a toxic effect. Cooling rates should be not higher than −1°C/min (Mazur, 1990).

In slow-cooling protocols used for human embryos and oocytes a rate of −0.3°C/min is typically adopted until a temperature of −30°C is reached. Subsequently samples are plunged into LN_2. This rate was initially determined empirically for mouse oocytes. Only afterwards, including in a single theory factors for cell volume, membrane water permeability, temperature and osmotic potentials, Mazur (1963) confirmed theoretically that a cooling rate of approximately −0.5°C/min was appropriate to remove the large majority (more than 90%) of freezable water from mouse embryos and oocytes.

It is interesting to note that, in the original experiments conducted on mouse oocytes, slow cooling was protracted to a temperature of about −70°C. The fact that in protocols developed at later stages the temperature of arrest of the slow-cooling phase was increased to between −30°C and −40°C was a consequence of the limits imposed by the technology available in the early days of slow-freezing investigation which made it impractical to cool samples to lower temperatures. In fact, when slow cooling is arrested at temperatures between −30°C and −40°C, the amount of residual freezable water may still generate a risk of IFF during warming.

Once all extracellular water is entirely converted into ice, very little if any freezable water is left in the cell environment. Under such conditions, the complex assortment of proteins, carbohydrates, lipids, nucleic acids and other macromolecules acquire a highly viscous amorphous state which constrains the addition of residual water molecules to small and harmless intracellular ice crystals, if present. Under this state, referred to as quasi-glassy, molecular motions are prevented. Notably, the minuscule ice crystals possibly present in the amorphous state can grow to a dangerous size if warmed too slowly.

Storage

After reaching the stage in which all extracellular water is frozen and the intracellular environment is in the quasi-glassy state, samples may be rapidly transferred and stored at much lower temperatures. Early cryopreservation methods implied storing at −80°C, a temperature which allows long term but not indefinite storage. For safe preservation, samples must be stored at temperatures below the glass transition temperature which correspond to −123°C for solutions including DMSO (Pegg et al., 1997) and −104°C for solutions containing PROH (Wusteman et al., 2003). For practical convenience, storage is normally carried out in LN_2 whose boiling point is at −196°C.

Warming

Very rapid warming of samples cryopreserved in LN_2 is crucial for vitrification. In slow freezing, the rate of warming is less critical. Experimentally, the application of rapid warming for slow-frozen samples has emerged as a golden rule. However, this is correct only in part. Studies in the mouse (Schiewe et al., 1987) have shown that if, after slow cooling, embryos are plunged in LN_2 from temperatures between −25°C to −40°C, in effect rapid warming (approximately 200°C/min) ensures much higher survival rates. The poorer performance of slow warming may be explained by the fact that at these plunging temperatures not all freezable intracellular water is extracted. During slow warming, this water can contribute to the growth of minute ice crystals able to cause cell injury once they reach a certain size. However, if slow cooling is extended to −50°C or even lower temperatures, survival rates obtained after either slow or rapid warming are comparable. It is likely that similar rules are valid for oocytes, although specific studies have not been conducted.

CPA removal

Once samples are returned to room temperature from LN_2, the permeating CPA must be removed from the cell environment and the cell needs to regain its original volume. To achieve this, the cell is exposed to a lower CPA concentration. The osmotic imbalance and the higher hydraulic conductivity for water drives water into the cell more rapidly than the loss of CPA, with the consequence that the cell tends to expand beyond their original size. Afterwards, the slower loss of CPA reduces the cell volume to its original condition. This process of rehydration is critical because cells are more susceptible to swelling than shrinkage. In oocyte slow-freezing protocols, rehydration is carried out in multiple steps involving progressively lower concentrations of permeating CPA (Borini et al., 2004; Fabbri et al., 2001). To control the risk of excessive swelling, dilution mixtures also contain a non-permeating CPA, typically sucrose, which act by reducing the osmotic imbalance between the intra- and extracellular compartments and the rapidity of water efflux. To achieve this, differential concentrations of sucrose are used in freezing and thawing solutions. Despite that, an important proportion of oocytes suffer fatal injury during rehydration or shortly afterwards. Clearly, rehydration is not optimized in current protocols. In effect, concentrations of permeating and non-permeating CPA and time of exposure to rehydration solutions are totally arbitrary. This suggests that a more rational approach to oocyte rehydration after thawing could lead to an improvement in the entire cryopreservation procedure.

A place for theoretical modelling in slow freezing

Slow freezing has gained enormous benefit from empiricism. As discussed above, the discovery of the cryoprotective properties of glycerol and similar molecules was empirical, and so were the determinations of cooling rates required to prevent IIF and temperatures at which samples may be plunged in LN_2. On these bases, progress has been impressive. Over several decades, hundreds of thousands of births have been obtained from frozen human embryos, while children born more recently from frozen mature oocytes can be counted in their thousands.

A confirmation of the validity of the empirical approach to slow freezing is offered by a recent study of Edgar et al. (2009). These authors showed that the blastomere survival rate of human embryos cryopreserved by slow freezing can be improved from approximately 70–74% to over 90% by increasing the sucrose concentration in the freezing solution from 0.1 mol/l to 0.2 mol/l and simplifying the dehydration and rehydration steps. This has an obvious reflection in terms of increase in the proportion of embryos that can be recovered from storage fully intact and, ultimately, implantations that can be achieved from a given population of cryopreserved material. In a similar fashion, empiricism could still ensure margins of improvements in oocyte slow freezing.

In effect, the slow-freezing approach requires further development and refinement to improve post-thaw survival rates, standardize procedures to increase reproducibility and expand the understanding of fundamental factors which determine success or failure of low-temperature storage. Theoretical modelling has already contributed decisively to the progress of slow freezing. The Mazur hypothesis (Mazur, 1963, 1977) has demonstrated the potential of theoretical models. Here, it is sufficient to appreciate its significance for the advance of slow freezing. Pioneering experiments carried out by Lovelock (1953) with erythrocytes had identified the increase in solute concentration as the cause of cryoinjury during freezing, but had failed to define IIF as an alternative major source of cell damage. Mazur was able to predict theoretically and demonstrate that in fact solute concentration and IIF have a different relative impact on cell viability depending on the rate of cooling. His two-factor hypothesis implies that, at very low cooling rates, the major element responsible for cryodamage is the accumulation of solutes, because cells are exposed to high salt concentration for an excessive period of time at relatively higher temperatures. This effect may be mitigated and survival rates may be improved by increasing cooling rates and therefore inducing a faster ice formation. However, with further increase in cooling rates, survival rates decrease again instead of improving. The Mazur hypothesis explains this bi-phasic survival change in

terms of inability of higher cooling rates to allow sufficient time for cell dehydration driven by accumulation of solutes. Therefore, for each cell type, cooling can be more safely achieved within a specific rate range. This is particularly valid for cells such as oocytes in which extraction of water is constrained by a low surface-to-volume ratio.

Over the years, other models have been developed to predict the response of human oocytes to the addition and removal of permeating CPA, taking into consideration oocyte size, type and concentration of CPA, temperature and exposure time. Modelling can also be applied to the action of non-permeating extracellular solutes to assess oocyte volume and intracellular concentrations of water changes as a function of time. The effect of seeding temperature and the equilibration time at the seeding temperature may be predicted in terms of subsequent response during freezing and warming. These are only examples of the range of applications of theoretical modelling.

Recently, it has been suggested that modelling of individual steps may be connected together in a way that for a given factor, for example oocyte volume, the end-point of a step represents the starting point of the following. This exercise can be extended to an entire protocol and to the different factors believed to have an influence on oocyte survival and viability, in order to identify more appropriate cryopreservation conditions. In a recent study, McGrath (2009) has applied this approach to model a slow-freezing protocol extensively used for the storage of human oocytes (Fabbri et al., 2001). For example, taking into consideration volume changes as a factor of interest, he modelled the oocyte osmotic response to the sequential addition of 1.5 mol/l PROH and 1.5 mol/l PROH/0.3 mol/l sucrose, steps by which an initial dehydration prior to cooling is obtained. Afterwards, he linked these changes to the ones that are predicted to occur during CPA removal after thawing. In such a way, he was able to generate a complete history of the oocyte osmotic response throughout the procedure (**Figure 1**) and identify those steps which should more carefully controlled to prevent excessive volume changes.

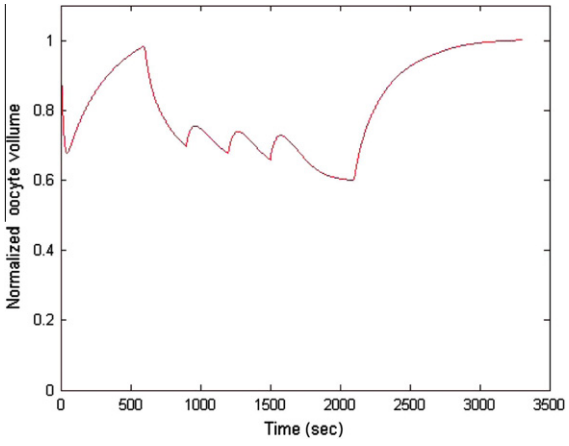

Figure 1 History of normalized human oocyte cell volume in response to the addition and removal of propanediol and sucrose according to the protocol described by Fabbri et al. (2001). With permission from: McGrath (2009) Predictive models for the development of improved cryopreservation protocols for human oocytes. In: Borini A, Coticchio, G (eds) *Preservation of Human Oocytes*. Informa Healthcare, London, 62–82.

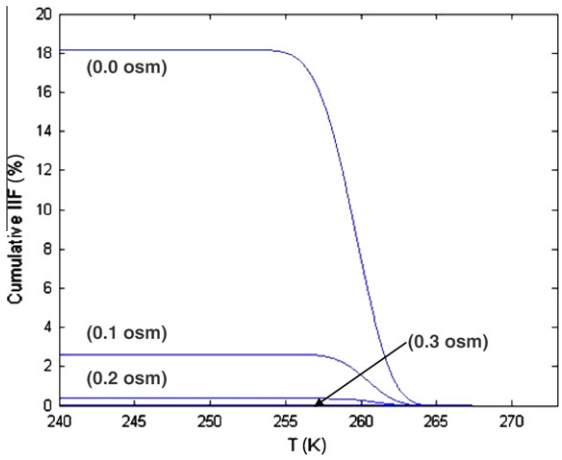

Figure 2 Cumulative percentage of intracellular ice formation as a function of temperature for a human oocyte cooled at 1°C/min. The human oocyte was equilibrated with 1.5 osm propanediol and 0.3 osm salt with no sucrose in one case. Three other cases with different concentrations of sucrose were also considered. The oocyte was seeded at the respective equilibrium freezing temperature of each solution and allowed to equilibrate fully prior to cooling. With permission from: McGrath (2009) Predictive models for the development of improved cryopreservation protocols for human oocytes. In: Borini A, Coticchio, G (eds) *Preservation of Human Oocytes*. Informa Healthcare, London, 62–82.

The potential of the modelling described by McGrath is considerable. In a different analysis (**Figure 2**), he predicted how the addition of different sucrose concentrations can influence the amount of IIF, a factor directly involved in the determination of cell survival. In this model, sucrose concentrations of 0.2 and 0.3 mol/l were shown to be associated with very little – if any – risk of IIF, while a concentration of 0.1 mol/l still involved a more significant chance of IIF. Interestingly, these predictions are in agreement with what was previously found in human oocytes in an empirical fashion. Survival rates of human oocytes, presumably dictated by IIF, are relatively high (70–75%) by freezing with solutions containing either 0.2 or 0.3 mol/l sucrose (Borini et al., 2006; De Santis et al., 2007b; Fabbri et al., 2001), while survival rates are reduced (35–40%) when the same CPA is present at a concentration of 0.1 mol/l (De Santis et al., 2007b). However the relatively poor subsequent development with the use of 0.3 mol/l sucrose and evidence of cytoplasmic aberrations following cryopreservation indicates that survival is only the first step. The future also requires an understanding of the impact of cryopreservation on intracellular components.

Conclusions

In recent years, oocyte cryopreservation has become an important option in human IVF. Slow freezing has been the cryopreservation standard for the storage of embryos

and, subsequently, oocytes. This approach to cryopreservation was initially founded on purely empirical experiences. At later stages, predictive models were suitably developed to better understand the cryopreservation process and above all identify and solve criticalities that affected cell survival and viability. Theories were developed to control in various cell types factors causing cryoinjury, such as IIF and solution effect. These theories were extended to the cryopreservation of mammalian oocytes. The mouse model, which shares important properties with the human, has been essential for the development of protocols specifically designed for oocytes. Recent and more advanced theoretical models can predict oocyte responses to the diverse factors characterizing an entire slow-freezing procedure, offering a global approach to the improvement of current protocols. Regardless, empiricism can still play a determinant role in slow freezing, as suggested by the fact that human embryo freezing has recently been perfected by an entirely empirical approach.

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