

Cryopreservation of Mature and Immature Oocytes

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Abstract: The aim of this chapter is to evaluate the current situation concerning oocyte freezing. Clinical outcome using slow cooling and vitrification was assessed in the literature and in our clinic to evaluate possible differences using either of the protocols. Both techniques were successfully applied with a comparable number of healthy babies being born using either of the protocols. Nevertheless, slow cooling has been widely applied whereas vitrification has been primarily used in egg donor programs thereby influencing the outcome rates. A randomized study in a comparable group of patients would be appropriate to define the best protocol to apply.

Key words: cryopreservation, slow freezing, vitrification, clinical outcome

Introduction

During the last half of the 20th century, there have been considerable improvements in human reproductive technologies. The inclusion of oocyte cryopreservation into the clinical practice of assisted reproduction has been a major achievement in this field. This technique represents an

advantage to fertile women for several indications. In fact, fertile women may take advantage of this technology to delay childbearing or to preserve their fertility when faced with cancer diagnosis. The necessary treatment for most of the common cancer types occurring in younger women requires either the removal of the reproductive organs or toxic treatment that could partially or definitively compromise reproductive function. Early loss of ovarian function not only puts the patients at risk for menopause-related complications at a very young age, but also is associated with loss of fertility. The remarkable improvement in survival rates because of the progress in cancer treatment necessitated an improvement in the considerations of their quality of life. Oocyte freezing may allow these women to have a chance of having their own baby after total remission.

Other clinical indications for cryopreservation include the rescue of possible hyperstimulated cycles or those with failure to obtain sperm. In this way the patient would not have to repeat the hormonal stimulation as she can preserve her oocytes for later use.

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Moreover, this technique could be a valid alternative to circumvent moral and legal problems associated with embryo freezing, specifically in Italy, where the law (40/2004) has forbidden embryo freezing, but allows insemination of up to 3 eggs and cryopreservation of the remaining number.

Finally, in countries where egg donation program is allowed, oocyte freezing avoids synchronization issues between the donor and the recipient and also reduces the potential for the transmission of infectious pathogens; this makes the application of these techniques more feasible.

Several improvements in assisted reproduction techniques (ARTs) including culture condition, better understanding of cryoprotectant mechanism, and a higher fertilization rate using intracytoplasmic sperm injection (ICSI), have brought about a routine application of oocyte cryopreservation worldwide.

Nevertheless, the human oocyte is 1 of the largest cells in the body, measuring approximately 130 μm , and this has important consequences in the freezing outcome. The surface area to volume ratio is low and, hence, less efficient in the cryoprotectant agent (CPA)-water exchange. The unavoidable consequence is that oocytes are more prone to water retention and consequent damage caused by ice crystals during freezing and thawing procedures. In the last 10 years, several approaches have been made with the attempt to overcome the limits related to this procedure but, to date, there is no consensus on which is the best and the most reproducible protocol for routine use in clinics. Slow cooling and vitrification are 2 primarily available options applied in in vitro fertilization units. Both the methods are still under evaluation in an effort to develop a better methodology and consequently improve the outcomes.

Currently, there is growing interest on other aspects: understanding the importance of oocyte selection before freezing,

the cryobiological fundamentals responsible for survival rates or postthaw recovery, and also possible ultrastructural damages caused by cryopreservation.

The structures that are primarily involved are the membrane, the cytoskeleton, and the zona pellucida. In fact, although the oocytes survive and may seem morphologically normal, there can be several unknown aspects concerning the structural architecture that may influence the implantation potential of the eggs resulting in an unsatisfactory pregnancy rate after thawing.

A multiple approach is now used to evaluate the new protocols and consequentially provide a broad perspective of the real clinical efficiency.

Cryobiology of Slow Cooling

Historically, interdisciplinary researches networked together with the aim of maintaining long-term viability in living cells after cryopreservation. It is well known that biological material show a decrease or loss of viability above -135°C because of biochemical reactions; consequentially the temperature that is generally used for mammalian cell storage is -196°C , the same as liquid nitrogen. This should prevent reactions from taking place, because water at this temperature just exists in a solid state and the only possible alteration may be related to DNA damage caused by background reactions. This does not seem to compromise the chance of survival and development of human oocytes or embryos, anyhow.

As cells are alive at 37°C and are almost totally inactive at -196°C , the difficult steps to overcome are related to temperature decrease and the rewarming phase. These passages represent the key points responsible for cell survival. Chemically, when water is cooled below its freezing point, it solidifies, thus becoming ice. This can cause damages to the cells mainly because of intracellular ice crystal

formation. As ice takes more space inside the cell than liquid water and spreads through, it may cause stress and subcellular alterations, eventually, resulting in a loss of viability after rewarming. The main issue to overcome in any cryopreservation procedure is to consequently avoid ice crystal formation.

All biological systems respond to lowering of the temperature and water solidification in a different manner. Mazur in 1984¹ was the first author who determined, through specific equations, the kinetics of water exchange in the cell and predicted the likelihood of intracellular freezing as a function of the cooling rate. This quantitative description can be explained qualitatively considering that, by definition, water below its freezing point is supercooled showing higher vapor pressure, activity, and chemical potential at a given subzero temperature than that of ice or of an ice-water equilibrium solution. As a consequence, as far as the cell remains supercooled the vapor pressure or the chemical potential difference will allow the water to leave the cell and freeze externally.

This causes dehydration during cryopreservation with a rate and extent dependent on the permeability of the specific cell to water and the cooling rate. Generally speaking the slower is the cooling rate the higher extent of dehydration will be obtained. Thus, it is obvious that 1 of the most important points in cryopreservation procedures is to dehydrate the cell correctly before or during the freezing procedure to reduce the damages caused by intracellular ice formation. If dehydration is inadequate, large, intracellular ice crystals may form, which can be lethal to the cells.

Although the avoidance of intracellular freezing is important to improve survival rates, it is not the only aspect to overcome. In fact, ice also forms outside the cell leaving the residual unfrozen medium to form channels and increase solute concentration causing shrinkage.

To overcome these issues, CPAs have been introduced in laboratory practices. This family of compounds can be classified into 2 main categories:

- Permeating agents: can enter the cell and includes glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, and 1,2-propanediol (PROH) and generally have a molecular weight lower than 100.
- Nonpermeating agents: cannot enter the cell because of their size and polarity. They include large sugar molecules such as sucrose, ficoll, and raffinose, and also proteins and lipoproteins.

The protective properties of cryoprotectants are related to their ability to lower the freezing point of the solution in a concentration-dependent way and to their chemical property. They form hydrogen bonds with water molecules eliminating ice formation. Moreover, they prevent damages caused by high salt concentration.

The search for suitable cryoprotectants and their appropriate concentrations has been a priority in all the freezing protocols. It seems that low molecular weight agents such as glycerol have a protective effect because of their ability to increase the unfrozen fraction and reduce cell volume excursion during cooling and rewarming procedures. Moreover, they also reduce the toxic effects of high concentrations of other compounds (colligative properties). In slow cooling procedures, PROH or DMSO has been usually used at 1.5 M concentration. The cryoprotectants enter the cells by osmosis while the water leaves. This might cause shrinkage as the water rapidly leaves the cells to dilute the high concentration of extracellular solutes or more serious issues when cryoprotectants need to be removed.

On the contrary, high molecular weight cryoprotectants cannot enter the cells and are, instead, involved in the stabilization of the plasma membrane by solute-specific interactions with the bilayer phospholipids. This is the main reason why in

almost all the slow freezing protocols the cryoprotectant mixture is made up of a penetrating agent and a nonpenetrating agent.

Cryobiology of Vitrification

Vitrification is a recently developed alternative protocol that might become the elective method to freeze oocytes and ovarian tissue. It is based on the concept of avoiding ice crystal formation by using higher cryoprotectant concentrations, which causes the water to form a glassy state around the cell rather than ice crystals. Nevertheless, although these higher concentrations of cryoprotectants may prevent mechanical damages, they can also be toxic for the cell or create an osmotic shock.

To design a vitrification protocol the main points to consider are the cooling and warming rates, the cryoprotectant concentrations, and the sample volume. Reducing the volume and raising the freezing rate requires a lower concentration of CPAs in the solution leading to lower side damages. On the contrary, if the volume is larger the likelihood of ice nucleation is higher causing the entire specimen to freeze instantaneously.

Yavin and Arav² expressed the probability of vitrification as a direct relationship between the cooling/warming rate and viscosity and, as an inverse relationship with the volume. Increasing the viscosity or the cooling/warming rates or decreasing the volume will raise the probability of vitrification.

It is well known that CPAs at high concentrations might have toxic consequences. This was, in fact, 1 of the main controversial aspects in vitrification procedures. To improve the outcome without using very high concentration of cryoprotectants, it is suitable to lower the cooling rate or to reduce the volume of sample storage to a very small drop that will prevent ice crystal formation.

The cooling rate can be increased using liquid nitrogen slush at -210°C whereas the loading volume can be reduced to less than 1 μL using newly developed devices.

The cryoprotectants in the vitrification procedure are involved in 2 main actions: they should remove the water from the cell and, at the same time, enter the cell to form the amorphous state in the cytoplasm and prevent the cell from damage caused by low temperature.

Initially, only penetrating agents were used in vitrification mixtures but, more recently, the protocols have been changed and the solutions are made by using both penetrating and nonpenetrating agents, and this has increased the survival rate and made significant advancements in the procedure.

Oocyte Selection Before Freezing and After Thawing

The evaluation of oocyte quality before and after cryopreservation is a fundamental aspect to achieve good outcomes. There are universally accepted criteria that define a good quality metaphase II (MII) oocyte like the round shape, the size and position of the polar body, the absence of cytoplasm vacuoles, or abnormality. All these features are related to good prognostic outcomes in embryo or blastocyst development, and consequently it is important to give a close look to the oocyte before cryopreservation as oocyte selection before freezing is the first optimization of any protocol.

Alongside the mere microscopic observation, there are a series of noninvasive evaluations that can be done without compromising the oocyte. The polar body and the meiotic spindle can be visualized using the Polscope, a polarized light microscope that gives the opportunity to visualize the highly ordered tubulin structures of the spindle in a noninvasive

way. The microtubules, in fact, reflect the polarized light generating a phenomenon known as birefringence. There is a quantitative relationship between the degree of birefringence (defined as retardance) and the amount of microtubules of the spindle. The absence of the spindle may compromise the fertilization potential of the egg but it is still under debate if only the Polscope observation is a valid tool to discard oocytes, which do not display an acceptable retardance value.

A recent study³ showed that spindles with an in-range birefringence value did not show a normal spindle organization when observed with confocal microscopy. Normal and abnormal spindles showed the same degree of retardance and a polarized signal, which means that the Polscope can just discriminate between the presence or absence of the spindle but not its quality.

Another noninvasive approach has been suggested by Seli et al⁴ using a technology defined as metabolomics, which can discriminate the metabolic profile of each oocyte by analyzing the media in which it was cultured. This represents the first nonmorphological criteria for oocyte selection.

Although there are several options to select oocytes before freezing, there are no reliable markers that can predict the viability after thawing besides the morphological criteria. It has been shown that oocytes, which apparently survive the freezing/thawing process with no visible sign of degeneration, might be compromised in their developmental potential. Confocal and electronic microscopy did not confirm what the operator was able to assess by standard observation. The meiotic spindle and other structures like the zona pellucida or the cytoplasm had evidence of clear signs of ultrastructural damages but unfortunately, once the egg has been fixed and stained it is not viable and thus not suitable for insemination.

Freezing of Mature Oocytes

The major issue with the MII oocyte is related to the particular cell characteristics. The mature human oocyte is distinguished by the presence of the first polar body in the perivitelline space and the meiotic spindle in the cytoplasm. Several studies using slow freezing and vitrification protocols have been conducted to analyze the possible damages to the subcellular structures such as meiotic spindle, mitochondria, or cortical granules. It has been shown that the meiotic spindle is a very dynamic and sensitive structure, which is able to disappear and reform during cryopreservation and after thawing. This is regulated by fine polymerization and depolymerization of the tubulin—a very delicate equilibrium; if altered, it can lead to abnormal configuration of the spindle after thawing. The chromosomes are aligned on this structure tightly in contact with the microfilaments and even when the spindle disassembles, they are not found to be dispersed in the cytoplasm. Possible damages to the meiotic spindle are more related to abnormal fertilization than chromosomal abnormalities in the embryos.

Another feature is represented by the zona pellucida and cortical granules, which are responsible for the correct oocyte fertilization by preventing multiple sperm penetration. Normally, the zona pellucida hardens after penetration of 1 sperm to block polyspermy as a consequence of the release of the cortical granules. The sperm, in fact releases a protein that causes an increase in intracellular calcium, which leads to the release of the cortical granules. The cryopreservation affects the normal process because it causes premature release of the cortical granules and zona pellucida hardening, which is why ICSI has been used routinely for the insemination of thawed oocytes.

Although the zona pellucida seems to be compromised by cooling, the

membrane of mature oocytes show a different lipidic composition compared with immature oocytes giving a better resistance to low temperature and cryogenic injuries.

The cumulus is another feature of the mature oocyte—it is involved in the fine communication between the inside and outside of the egg. Even though evidence suggested that its maintenance does not improve the freezing outcome, this is still under debate.

Over the past 10 years several satisfactory results have been published, yet there is not a defined, generally accepted approach that guarantees a safe routine application.

The 2 main protocols applied in the literature are the slow freezing and the vitrification methods.

Slow Cooling

The first protocol used to cryopreserve oocytes was based on a slow cooling/rapid thawing method that had already been applied successfully for embryo cryopreservation.

Since the first pregnancy in 1986, several advancements have been made with the aim of improving the original protocol. Most of the studies were focused around the choice of cryoprotectant and concentration or the exposure time. The freezing curve designed for the programmable freezer is basically unchanged from the original protocol first designed by Lassalle et al⁵ for embryo freezing.

The slow cooling protocol is based on a very slow rate of decreasing temperature ($<1^{\circ}\text{C}/\text{min}$) over time (Fig. 1). Freezing solutions are cooled from room temperature (around 20°C) to -8°C at a rate of $2^{\circ}\text{C}/\text{min}$. Manual seeding of oocytes within straws is performed at near -8°C and this temperature is maintained for 10 minutes to allow uniform ice propagation. This process prevents supercooling and starts the dehydration process. If ice

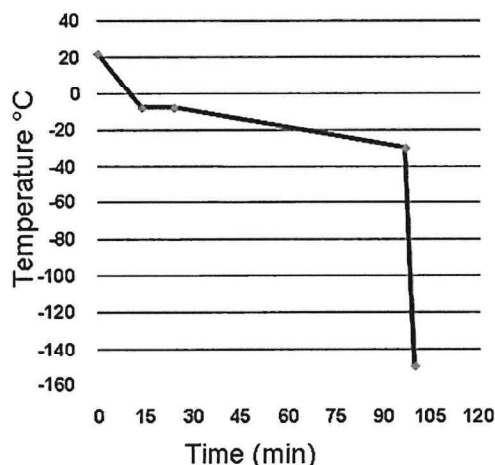


FIGURE 1. Time 0, decrease from room temperature to -8°C , at -2°C per minute. Time 15 minutes, seeding at -8°C , hold for 10 minutes. Time 25 minutes, -8°C to -30°C , change at -0.3°C per minute. Final step, -30°C to -150°C , change at -50°C per minute and plunge into liquid nitrogen.

formation is not initiated by seeding, the solution will remain unfrozen until a much lower subzero temperature is reached, which can be detrimental for oocyte survival. After this hold ramp the temperature is decreased to -30°C at a rate of $0.3^{\circ}\text{C}/\text{min}$ and then rapidly brought to -150°C at a rate of $50^{\circ}\text{C}/\text{min}$. The straws are then directly plunged into liquid nitrogen at -196°C and are stored.

The thawing procedure consists of rapid rewarming, subsequent stepwise dilution of the cryoprotectants, and finally, return of the oocytes to 37°C for culture.

The solutions that are commonly used during slow cooling procedures are a mixture of a penetrating cryoprotectant (usually PROH) and a nonpenetrating one (usually sucrose). These 2 CPAs have been modified in concentration and exposure time during freezing/thawing procedure in different protocols developed in the last 2 decades. Clinical outcomes have been determined for all the different

protocols. As already said the first protocol used was exactly the same as formulated for embryo freezing. It is based on 1.5 M PROH (equilibration solution) and 1.5 M PROH + 0.1 M sucrose (loading solution) in the freezing mixture and a stepwise dilution of PROH (1.0 M to 0.5 M) with an unvaried 0.2 M sucrose concentration in the thawing solutions.

This protocol applied on 68 patients resulted in poor survival (37%), poor fertilization (45.4%), but good cleavage rate (86.3%). The pregnancy rate was relatively high (22% per patient) even though the implantation rate per oocyte was only 2.3%.⁶

In 2001, Fabbri et al⁷ reported significantly improved postthaw survival rates when the concentration of sucrose was increased from 0.1 M (34%) to 0.2 M (60%) or 0.3 M (82%) in both the freezing and thawing solutions. This increase in sucrose concentration resulted in improved oocyte dehydration, which correlated with higher postthaw survival rates. However, these promising results did not lead to improvements in pregnancy rates. Several groups used this high sucrose freezing-thawing protocol and despite the good outcomes for survival, fertilization, and cleavage rates, the reported implantation and pregnancy rates were low.^{8,9}

From the data it was apparent that, although the low sucrose protocol (0.1 M) compromised the survival rate the developmental potential seemed to remain unchanged, whereas the high sucrose protocol (0.3 M) acted in an opposite way by raising the survival rate but reducing the ability to implant.

A meta-analysis conducted by Oktay et al¹⁰ evaluated the combined outcomes of 26 reports using slow freezing of mature oocytes inseminated by ICSI for approximately 4000 thawed oocytes reported. All the data together, despite the protocol used gave a 2.4% clinical pregnancy per thawed oocyte, and an implan-

tation rate per transferred embryo of 13.1%. In a separate analysis carried out on 7 slow freezing studies, using the 0.3 M sucrose protocol, the clinical pregnancy per thawed oocyte was 2.2%, whereas the implantation rate per transferred embryo was down to 6.5%.^{8,11} These clinical pregnancy rates are low compared with embryo cryopreservation that showed a live birth of approximately 4% to 5% per oocyte inseminated.

More recently, Bianchi et al¹² tried to develop a modified protocol that used a thawing solution in which sucrose concentration was higher than in the freezing solution.

The freezing solution is based on a 1.5 M PROH and 0.2 M sucrose CPA mixture to reduce the impact of shrinkage during cooling procedures, whereas the thawing solution has an increased sucrose concentration (0.3 M) to better stabilize the rehydration of the oocyte. In this study, Borini and Bianchi thawed 403 oocytes of which 306 survived (76.0%); 252 (a maximum of 3 oocytes per patients according to the Italian law) were inseminated by ICSI and 192 fertilized normally, for a rate of 76.2%. One hundred and eighty of the 192 zygotes were cleaved (93.7%) and 178 were transferred. In the end, 17 pregnancies were confirmed by ultrasound assessment, with 24 gestational sacs and 19 fetuses with heartbeats. Pregnancy rates were 21.2%, 18.9%, and 21.8% per embryo transfer, thaw cycle, and patient, respectively, and the implantation rate was 13.4%. Pregnancy rate per cryopreserved-thawed oocyte was 4.9% and, more importantly the implantation rate per oocyte was 6.9% thus improving the one coming from embryo freezing. This improvement in the outcome rates is probably related to the more adequate dehydration achieved with 0.2 M sucrose compared with 0.1 M or 0.3 M sucrose. Moreover, the higher (0.3 M) sucrose concentration used during thawing leads to a better controlled water exchange between

the inside and outside of the cell stabilizing the membrane and thus avoiding shrinkage.

A multicenter study recently published by Borini et al¹³ tried to give an exhaustive update of the embryologic and clinical parameters in several in vitro fertilization units in Italy where, according to the law (40/2004) a maximum of 3 oocytes could be inseminated in fresh and frozen thawed cycles. The protocol used was a 2-step PROH-sucrose based solution. In this study, out of 2046 patients the overall survival rate of thawed oocytes was 55.8%. In 940 thaw cycles, the mean numbers of inseminated oocytes and fertilization rates were significantly lower compared with fresh cycles (2.6 ± 0.7 vs. 2.9 ± 0.2 and 72.5% vs. 78.3%, respectively), as were the rates of implantation (10.1% vs. 15.4%), pregnancy rates per transfer (17.0% vs. 27.9%), and pregnancy rates per cycle (13.7% vs. 26.2%). An overall pregnancy rate above 14% was achieved in all the clinics.

More recently,¹⁴ cumulative pregnancy rates using fresh and cryopreserved oocyte cycles have shown the additive value that egg freezing might have in those countries where restrictive laws are in force. A cumulative pregnancy rate of 47.5% was obtained with the transfer of embryos coming from fresh and frozen thawed cycles.

Vitrification

The first report of vitrification in embryology was with mouse embryos in 1985,¹⁵ followed by the successful vitrification of oocytes in 1991,¹⁶ yet the general application of vitrification in assisted reproduction has been rather limited until recently. The use of vitrification has been described in the literature for several mammalian species, including humans, with varying degrees of success depending upon the wide variety of tools and procedures applied.^{17,18} Numerous recent publications

have shown outstanding results for survival and clinical outcomes using vitrification compared with slow cooling.¹⁹ Vitrification methods have been modified over the years to optimize results in humans, by using minimal volumes and very rapid cooling rates, allowing lower concentrations of cryoprotectants to reduce injuries related to chemical toxicity, osmotic shock, chilling sensitivity, and ice nucleation.^{18,19}

The general methodology involves a 2-step sequential exposure of oocytes to vitrification solutions containing 1 or more cryoprotectants in increasing concentrations up to 40% (vol/vol), loading the oocyte in a minimal volume ($< 1 \mu\text{L}$) of solution onto a carrier device (open or closed system), and very rapid cooling by plunging directly into liquid nitrogen. The time and temperature of exposure to such solutions are critical to avoid toxicity. Conversely, warming rates must also be rapid to prevent ice nucleation during the warming process and achieve optimal results. After warming, the oocytes are then moved through at least 3 solutions with decreasing concentrations of sucrose to effectively remove the cryoprotectants and rehydrate the oocytes.

Vitrification can be defined as a physical process by which a highly concentrated solution of cryoprotectants creates a glass-like state during rapid cooling without the formation of ice crystals. This glassy state is an extremely viscous supercooled solution. Vitrification shows certain advantages over conventional slow freezing because it avoids damages caused by intracellular ice crystals and osmotic effects caused by extracellular ice formation. Moreover, it is a very fast procedure that does not require any electronic equipment and allows the freezing of specimens in a very short time.

The most widely used vitrification protocol involves gradual exposure of oocytes to the equilibration solution [7.5% ethylene glycol, 7.5% DMSO, and 20%

serum substitute supplement (SSS) in HEPES buffered medium 199 (M199-H)] for approximately 8 minutes and then in the vitrification solution (15% ethylene glycol, 15% DMSO, 0.5 M sucrose, and 20% SSS in M199-H) for up to 110 seconds. Samples are then loaded onto a carrier device and plunged into liquid nitrogen. The thawing solutions are based on a series of solutions with decreasing sucrose concentrations (1.0, 0.5, and 0 M) with 20% of SSS in M199-H.

As it is a relatively new technique, the literature and available data for vitrification is more limited than that for slow cooling; Kuleshova et al²⁰ reported the first human live birth with vitrification, using ethylene glycol and sucrose as CPAs and an open-pulled straw device.

In the meta-analysis, Oktay et al¹⁰ included 4 reports of vitrification regarding the outcome of 503 thawed oocytes. The live birth per thawed egg was 2.0% with a total of 10 live births.

More recently, Antinori et al²¹ reported similar results using the Cryotop vitrification method. In this study, 330 oocytes were thawed with a survival rate of 99.4%. The fertilization, pregnancy, and implantation rates were 92.9%, 32.5%, and 13.2%, respectively.

Cobo et al²² compared the outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. The fresh group included 219 MII oocytes whereas the vitrified group included 231 MII oocytes. The survival rate after thawing was very high (96.9%), the fertilization and the day 3 cleavage rates were higher in the fresh group but not significant (fertilization rate 82.2% vs. 76.3% and cleavage rate 84.6% vs. 77.6% in fresh vs. frozen thawed respectively). The blastocyst rate was almost the same for the fresh and frozen thawed groups (47.5% and 48.7%, respectively). The pregnancy rate in the frozen/thawed group was very high if compared with other clinics, and so were the implantation rates (65.2% and 40.8%, respectively).

In general, it is very challenging to define egg freezing clinical outcomes for many different reasons including the mean age of the patients, different culture conditions, or different protocols/techniques used to freeze. A relatively new concept is the implantation rate per thawed/inseminated oocyte and thus the rate of babies born out of 100 eggs. This idea was originally developed for embryo freezing by Gook Debra and Edgar David²³ who estimated 4 heartbeats out of 100 oocytes; this rate has likely increased in the years since this report. If the same approach is applied to the various oocyte slow freezing protocols, it is possible to observe that, using the first 0.1 M sucrose protocol this rate is 2.3% and thus very low, and does not significantly improve using a higher sucrose concentration (0.3 M) to achieve rates of 2.6%. A significantly better rate (5.9%) was observed with the protocol based on differential concentration of sucrose in the freezing (0.2 mol/L) and thawing (0.3 mol/L) solutions.

As mentioned earlier, the data for vitrification are less exhaustive as it is a more recent technique. Using the Cryotop vitrification method, Cobo et al²² achieved an implantation rate per embryo transferred above 40%. This resulted in an implantation rate/oocyte of 8.6%. It is important to remember that these patients were donors and, consequentially very young (mean 26.7 y). The rate of 8.6% implantation/oocyte is not incredibly higher if compared with an age-related group of patients using the slow cooling method¹² in whom this rate was 7.3%.

Cryopreservation of the Immature Oocytes

To circumvent issues associated with the MII structures, immature oocyte cryopreservation may represent an alternative. Oocytes arrested at prophase I of

the meiotic process have different features than MII oocytes: they display a prominent nucleus called germinal vesicle (GV) that contains the chromosomes that are still decondensed even though they are transcriptionally active. Immature oocytes do not have microtubules organized in the spindle but rather dispersed mainly around the GV; this avoids possible damage during freezing procedures as opposed to MII oocytes. The cumulus and granulosa cells play a key role here because they are still tightly connected to the egg through transzonal projections and the plasma membrane is characterized by a reduced content in cholesterol and fatty acids so its permeability to CPAs is very different than that of MII oocytes.

Immature oocyte cryopreservation may represent an alternative for fertility preservation in a selected group of patients who cannot undergo ovarian stimulation because of cancer-related issues. Generally speaking, this technique has not been extensively used because of the low success rates. This might be related either to the difficulties to overcome during freezing procedure that probably affect the transzonal projections negatively leading to irreversible damages or the poor results obtained with *in vitro* maturation (IVM) methods after freezing/thawing. The immature oocytes are, in fact, characterized by this strict communication between the cumulus and the egg and consequently, the freezing process can cause stress that destroys the tight gap junctions and corresponding communication. The penetration of cryoprotectants and any extracellular ice formation can result in cumulus-granulosa cell loss.

Clinical Outcome

Immature oocyte retrieval during the follicular phase represents an option for women who need chemotherapy and therefore are restricted to a very short

time frame and must avoid ovarian stimulation.

To date, the data for cryopreservation of immature oocytes are limited because of poor results. This can be either because of direct damage on the egg induced by the cryopreservation procedure or because of the lack of consistency with the IVM protocols.

Theoretically, oocytes at the GV stage should be more feasible to cryopreserve because they do not have the meiotic spindle or other structures sensitive to low temperature.

Immature oocytes can either be recovered from stimulated or unstimulated cycles. Toth et al^{24,25} slowly cooled GV oocytes obtained after ovarian stimulation and, even though the results were acceptable in terms of oocyte maturation, postthaw fertilization and developmental potential remained scarce. In addition, the results could not be reproduced for immature oocytes obtained from unstimulated ovaries.

To date, the slow freezing of immature oocytes has resulted in 1 pregnancy²⁶ whereas limited information is available on vitrification of GV stage eggs.

Recently, Cao and Chian²⁷ found that survival rates between oocytes vitrified at GV or MII stage were not significantly different, nevertheless, the maturation rate of oocyte vitrified as GVs and then *in vitro* matured (IVM) after thawing was significantly lower if compared with IVM oocytes that were not vitrified. Nevertheless, no difference in fertilization, cleavage, and blastocyst development rates was observed in these 2 groups. Consequently, vitrifying mature oocytes gives better outcomes than immature oocytes.

The available alternative is to freeze the immature oocyte that is retrieved without ovarian stimulation. Such oocytes are subsequently matured *in vitro* and inseminated. This protocol has yielded a 35% clinical pregnancy rate. From a clinical point of view, freezing immature oocytes

has several benefits including the avoidance of expensive medications, ovarian hyperstimulation syndrome, and, last but not least, this novel approach has the greatest potential to help some cancer patients. For example, in estrogen receptor-positive breast cancer patients, the IVM protocol eliminates the risk of stimulating hormone-sensitive cancers.

These data show that healthy live births can be achieved from the combination of IVM oocytes and vitrification, even though vitrification of IVM oocytes is less effective than vitrification of in vivo matured oocytes. The results suggest that oocytes should be vitrified at the mature MII stage after IVM rather than at the immature GV stage because the potential of oocyte maturation is reduced by the vitrification of immature oocytes at the GV stage.

Cao et al²⁸ wanted to assess the efficacy of vitrification of human oocytes before and after IVM. The recovered immature oocytes (n = 472) were divided into 2 groups: (i) immature oocytes (n = 219) vitrified at the GV stage and (ii) immature GV stage oocytes (n = 253) that were first matured in vitro (MII stage oocytes; n = 178), then vitrified (n = 79). The remaining oocytes (n = 99), which were not vitrified, were used as controls. After thawing, survival, maturation, fertilization, and cleavage the rates were evaluated and the results showed no significant difference between the survival rates of the oocytes vitrified at GV stage and those vitrified at MII stage (85.4% vs. 86.1%). However, oocyte maturation rates were significantly reduced ($P < 0.05$) when oocytes were vitrified at the immature GV stage followed by IVM (50.8%) in comparison with the control group (70.4%). No difference was found in the fertilization (62.1% vs. 58.8%) and cleavage rates (69.5% vs. 67.5%) in the 2 groups.

Results of the study showed that freezing oocytes after IVM improved the chance of success compared with freezing

GV stage oocytes and maturing them in vitro after thawing. This might be either because of the poor IVM methods available to date or because of the possible damage induced by cryopreservation of GV stage eggs, even though they have a higher survival rate and lower incidence of meiotic spindle damage that could impact their subsequent development.

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