REVIEW ARTICLE

Modern Trends: The Cryopreservation of Human Oocytes

Veronica Bianchi[1], Gabriella Zito[1], Poli Renzo E[1], Furlan Valentina[1], Panayiotis M. Zavos[2]
[1]Future for Family Casa Di Cura Città di Udine, 33100 Udine Italy, [2]Andrology Institute of America, Lexington, Kentucky 40523, USA

Article Information

Received: 18 Nov 2015
Accepted: 08 Dec 2015
Plagiarism software: Turnitin

Keywords:
oocyte,
freezing,
thawing,
pregnancy rate

Veronica Bianchi

ABSTRACT

Oocyte freezing has been considered as an experimental procedure for decade while it would be an important tool to use in IVF routine. In fact, a safe and efficient program would be of substantial benefit for infertile patients and also for women at risk of loosing their fertility due to radio or chemio therapy treatments. Moreover, egg cryopreservation could replace embryo freezing which instead involves important legal and ethical drawbacks. After the initial disappointment due to the low survival rate diverse methods have been developed raising the post thaw recovery. The fertilization and cleavage performance of frozen eggs is now similar to that of fresh sibling oocytes, even though not a great deal is known about the early implantation potential. There is an increased global interest in analyzing intrinsic factors connected to the ability of these embryos to give a live birth. What is clear, up to now is that certain oocyte cryopreservation protocols may affect cell division and thus being associated with low implantation rates. Other freezing methods, instead, seem not to affect the post implantation development and are going to be used in laboratories as an alternative mode to embryo freezing. It would be of immense importance that further randomized studies are carried out which may underline any possible differences between various technical approaches and also to further generate more analytical data on the cumulative pregnancy rates along with long term follow up of the babies born as associated with oocyte cryopreservation.

INTRODUCTION

All cells can be theoretically stored at extremely low temperatures at liquid Nitrogen temperatures (-196°C) for many years. The post cryopreservation survival along with the reappraisal of the competence represented the various challenges. Mazur in 1984[1] described a number of theoretical models to analyse the kinetics of intra-cellular water loss that could allow one to predict the likelihood of intracellular freezing as a function of the cooling rates.

In 1983 for the first time, frozen human embryos were transferred in women that yielded live births.[2] This event has represented an incredible step forward the in vitro fertilization (IVF); multiple pregnancies reduced and repeated stimulation were not necessary due to successful in embryo cryopreservation. This first step aided in the possibility of oocyte cryopreservation even though the cellular characteristics between oocytes and embryos are quite different. The oocyte is just a non-fertilized cell, which could allow overcoming ethical and legal issues associated with embryo cryostorage.

The human oocyte is one of the largest cells of the body and its cytoplasm contains a high amount of water. This characteristic can induce damages in the ultra structures of the cell caused by ice crystal formation during the transition of water into ice. Scientists solved this issue by designing special protocols to dehydrate the eggs before and during the cooling phase to reduce ice crystal formation.

The mouse was the first animal model in which egg freezing yielded positive results almost 30 years ago,[3] this cell survive well low temperature due to its reduce size and content of water. On the contrary the first pregnancy in the human has been achieved only in 1986[4]

Access this article online	
Website: www.actamedicainternational.com	**Quick Response code**
DOI: 10.5530ami.2016.1.35	

Corresponding Author:
Dr. Veronica Bianchi, Casa Di Cura Città di Udine, Centre for Reproductive Health, Viale Venezia 410, 33100 Udine Italy,
E-mail: veronica.bianchi@futureforfamily.it

and afterwards just sporadic success have been reported due to technical issues.

In Italy in 2004, a restrictive law applied to IVF field limiting the number of oocytes to inject. This forced to improve the freezing procedure to avoid repeated stimulation for the patient and, consequently we were able to collect an enormous amount of data on infertile patient.

Nevertheless, there are several benefits associated with oocyte cryopreservation: it represents an important chance for women at risk of loosing their fertility due to malignant disease or premature ovarian failure but also for women who choose to postpone motherhood for personal or professional reasons. The possibility to cryopreserve oocyte as a routine IVF procedure is an important option to maximize egg donation, avoiding donor/recipient synchronization and making quarantine of specimens possible. Moreover, legally, egg freezing bypasses conflicts associated with the ownership of cryopreserved embryos in case of divorce.

For several years, the unique nature of the human oocyte together with the lack of cryobiological data posed several concerns around this technique. The introduction of intra-cytoplasmic sperm injection (ICSI) as an elective technique for the insemination of thawed oocytes circumvent the zona hardening[5] that limited the fertilization with standard IVF.

SLOW FREEZING

To improve the outcome it is important to avoid intracellular ice crystal formation. To do so, it is necessary to reach a sufficient dehydration achieved thanks to specific molecules called cryoprotectant agents (CPA).

These compounds belong to two distinct groups according to their proprieties:

1) Permeating agents such as glycerol, dimethylsulphoxide (DMSO), ethylene glycol, and 1,2-propanediol (PROH). They all have a low molecular weight and can readily penetrate the lipid bilayer of the cell membrane but at slower rates than water.
2) Non-penetrating agents such as sugars and other macromolecules (ficoll, raffinose, as well as proteins and lipoproteins). They have a high molecular weight so they remain in the extracellular solution.

The basic task of CPAs was to reduce possible damages due to ice crystal formation inside the cell. At the same time, they play a protective effect thanks to their ability to create hydrogen bond achieving high aqueous solubility. In fact, during slow cooling, when the cells are very dehydrated and surrounded by concentrated salts the cryoprotectants appear to reduce possible damages caused by this osmotic milieu.

In slow freezing protocols, there are two steps to follow:

- The first step exposes the egg to a concentration of 1.0–1.5 mol/l of non-permeating cryoprotectant like propanediol (PrOH), dimethylsulphoxide (Me2SO), or ethylene glycol (EG). The presence of CPA in the extracellular media creates an osmotic gradient that draws water out of the cell. The oocyte dehydrates and shrinks then it returns to near the original volume as the cryoprotectant enters the cell replacing the water. This causes a double flux across the membrane (the water exits the cell while the CPA enters) that influences both the intracellular solute concentration and the cell volume.
- The second phase of dehydration a non-permeating molecule is usually added to the mixture. Sucrose is the most commonly used but also trehalose and choline have been applied successfully.[6-8] This new agent creates a second phase dehydration in which the shrinkage rate is faster with a higher reduction of the cell volume before freezing. This phenomenon decrease, further on, the likelihood of intracellular ice crystal formation.

The rewarming phase is usually fast in order to avoid intra cellular small crystals to reach a size that can be detrimental for the cell organelles. Cell rehydration is usually performed by a stepwise dilution of the intracellular CPA present in the freezing media together with a high concentration of the extracellular CPA, such as sugar, which acts as osmotic buffer. This phenomenon allows reducing the influx of water towards the membrane during the thawing phase thus avoiding swelling or bursting of the egg. The analysis of cell volume changes during the freezing thawing procedures is important to evaluate different CPAs and, eventually new approaches to improve freezing protocols. An optimal exposure should aim at minimizing osmotic stress while avoiding chemical toxicity and allow sufficient permeation and dehydration to achieve protection from freezing injury.

Paynter et al,[9,10] tried to evaluate the osmotic response by monitoring the oocytes for 10 minutes during a two-step addition of the permeating cryoprotectant PROH (0.75M and 1.5M PROH). Following this, the oocyte osmotic response to 1.5M PROH and 0.2M or 0.3 M sucrose was measured. Each oocyte shrank during the first exposure to the cryoprotectant (0.75M PROH) as water left the cell and then gradually re-expanded as cryoprotectant entered; this process lasted around 20 minutes. The same happened during the second exposure to an increased cryoprotectant concentration (1.5M PROH) and, in the 1.5M PROH plus sucrose solution the entity of the shrinkage rate was higher

with a reduction of cell volume before freezing to avoid intracellular ice crystal formation.

Experiments with ethylene glycol (EG) were carried out in a similar fashion,[11] where during the EG exposure oocytes underwent an abrupt 50% volume reduction and a complete recovery of the initial volume was not observed. Survival rates after freezing with EG were lower than with PROH (51.6 versus 71.5%, respectively, $P < 0.05$). The frequencies of normal spindle configuration were lower in frozen EG and frozen PROH oocytes compared with fresh controls (53.8, 50.9 and 66.7%, respectively, $P < 0.05$).

Selection of the right mixture together with optimal exposure times is mandatory in order to obtain a good post-thaw recovery during slow cooling and freezing procedures.

OOCYTE SELECTION

Oocyte selection prior to freezing represents a difficult task for the embryologist. Beside evident cytoplasm anomalies (a large first polar body, macro cytoplasm, and perivitellin space debris) it is difficult to select which morphological features may affect the outcome. Moreover, post thaw survival is not always a synonymous of viability. Unfortunately, methods to assess the quality of the oocytes are usually invasive and thus not conservative. In addition to surviving the cryopreservation, the oocyte needs to maintain competence to fertilize and develop into a viable embryo to be able to give a pregnancy. Nevertheless, it has been widely demonstrated that, low temperatures during freezing may induce alteration to the cell substructures especially due to the unique nature of the metaphase II oocyte.

At this stage, the chromosomes are in strict relation to the meiotic spindle that is responsible of chromosome segregation during the extrusion of the second polar body.

This structure is made of microtubules that can disassembly and reassembly under certain conditions. In the mouse and in the human, which even a transient decrease in the temperature might lead to a depolymerization of the spindle. Nevertheless, spindle reassemble with returning to normal conditions.[12-14]

During slow freezing, the egg undergoes a drastic change in temperature that can be detrimental for the spindle leading to an increase in aneuploidy; on the other hand, though, the use of cryoprotectant play an important role in protecting the spindle.

Unfortunately, in order to have accurate information, cytogenetic or confocal analyses should be performed on oocytes after thawing with the consequent loss of viability. An acceptable alternative to visualize the recovery of the meiotic spindle is the Polscope which is a microscopy optical system that allows the observation of highly ordered subcellular structures, such as the spindle, through polarized light.[15] Microtubules are responsible for spindle birefringence and their density is measured by the retardance. Polscope offers the advantage of being non-invasive thus preserving egg viability; for this reason it is used as a potential tool for oocyte selection, considering that not all the oocytes showing an extruded polar body and actually mature.

This system can also been used to evaluate spindle recovery after thawing in order to select the best oocytes to inject. Several authors used different slow freezing protocols and try to visualize the reappearance of the spindle after rewarming. Rienzi et al.,[16] used a 1.5M PROH plus 0.1M sucrose mixture and showed a recovery in 37% of the oocytes immediately after thawing and after a transient disappearance the spindle was present in all the eggs survived within 3 h of incubation at 37°C. Similar data were published by Bianchi et al.[17] using a comparable freezing protocol with higher sucrose concentration (0.3M). Immediately after thawing, only 22.9% of oocytes showed a weak birefringence signal, while only 1.2% of oocytes displayed a high signal. After three hours' culture 49.4% of the oocytes showed a weak birefringence while 18.1% a high intensity signal. There was a statistically significant increase in signal restoration after 3 h of culture ($P<0.001$).

A further confirmation was obtained by confocal microscopy[18] where fresh control oocytes were compared with frozen eggs fixe dat different times after thawing (0, 1, 2 and 3 hours). All the control oocytes (100.0%) displayed normal bipolar spindles. Directly after thawing at T=0 it was observed a significant reduction of oocytes with bipolar spindles (59.1%) while after 1 h ofculture (T1) 85.7% regained bipolar spindles. Oocytes cultured for longer (2 or 3 h) displayed 73.7% and 72.7% bipolar spindles, respectively.

REPRODUCIBILITY

One of the advantages of the slow freezing technique is the high degree of reproducibility. This is extremely important since in any laboratories different operators with different skills should be able to reproduce comparable outcomes. This may include different aspects, like the accuracy of making homemade solutions or also the ability to provide strict adherence to the protocol. For these reasons all the procedures should be written by a laboratory director not to leave room for personal interpretation. The storage devices are important as well; in the slow freezing standard plastic straws are usually employed

for the cryopreservation of oocytes and embryos; this limits fluctuation between different procedures. Generally differences in size, volume, and wall thickness between various devices may influence the transmission of heat and potentially affect the freezing–thawing process. For this reason, the volume of the freezing mixture loaded into straws should be standardized.

Almost all the slow freezing protocols generally required a longer operational times then other techniques (i.e. vitrification) so different manual skills can have a lower influence on the outcome. In fact, incubation times either in the freezing or in the thawing procedure range from 5 to 15 minutes instead of few minutes or seconds. Moreover, slow freezing has been applied to embryo cryopreservation for several years; consequently, laboratories with experience in performing embryo cryopreservation would take shorter time to reach an optimal performance with egg slow cooling.

Another important aspect is related to the temperature. It is well known that lower suprazero temperatures reduce the toxicity of cryoprotectants but, at the same time may be detrimental for cell membrane and cytoskeleton. Therefore, the temperature at which dehydration/rehydration solutions are utilized should be precisely regulated. In controlled rate freezing, thanks to the automated machine, the decrease in temperature is finely monitored and recorded. Cooling rates during water to ice transition are slow (-0.3°C/min) and protracted for over 70 minutes, conditions which are believed to ensure good consistency.

SURVIVAL AND INSEMINATION OF FROZEN–THAWED OOCYTE

The controlled rate cooling procedure is based on a slow decreasing temperature rate. Several mathematical analyses have been made to define an optimal curve applicable to oocytes in order to balance the need to obtain a sufficient dehydration and the likelihood of ice crystal formation.

At the very beginning the slow cryopreservation protocol applied worldwide was the same used for embryos.[19] Cryopreservation solutions consisted of a mixture of PHOH (1.5M) and sucrose (0.1M) which was a good recipe for embryo freezing but did not give good outcomes with oocytes.[20,21]

The difference is probably due to the insufficient dehydration of the oocytes that was not optimal with such a lower concentration of sucrose. Generally eggs are incubated for 10 minutes in a 1.5 M PROH solution with 20% protein supplement during the equilibration phase; during this period the PROH enters the cell and the water exits. During the second step (1.5M PROH plus sucrose) the increased dehydration is proportional to the amount of sucrose contained in the solution. Fabbri et al.[22] proved that by increasing the amount of sugar also the loss of water from the oocyte was higher. The survival rate was significantly improved with this modification to the protocol.

Recently Bianchi et al.[23] try to modulate sucrose concentration during freezing–thawing in order to optimise the dehydration/rehydration conditions avoiding excess of shrinkage. In previous osmotic response experiments,[10] it emerged that after around 3 minutes of exposure to 1.5 M PROH in the presence of 0.3 M sucrose, oocyte volume decreases rapidly, reaching values below the 30% threshold excursion, which may be detrimental to cell viability. With a reduced sucrose concentration (0.2 M) the 30% volume change is not reached until after 10 minutes of exposure. Therefore, dehydration may be achieved slower and less traumatically. The steps of this protocol based on a freezing solution containing 0.2 M sucrose and thawing solutions made with 0.3 M sucrose. Beside using the combination of PROH and sucrose, other approaches have been studied to overcome the accumulation of solutes during extracellular ice formation which is believed to affect the stability of the oolemma and the intracellular membranes. Stachecki et al.[8] tried, in the mouse model, to replace sodium with equimolar amounts of choline that does not diffuse through the membrane and should be less toxic. Results were encouraging showing an increase in the rates of survival, fertilization and pre-implantation development. More recently, low-sodium media have been used on human oocytes; nevertheless despite the encouraging survival rate reported initially, studies involving larger numbers of patients have not been reported.

In a clinical study of Boldt et al.[24] used choline plus 0.3M sucrose but survival rates were disappointing, irrespective of the adoption of phosphate-buffered saline (PBS) or HEPES as pH buffers. Other authors have tested the effect of sodium-depleted media on the cryopreservation of human oocytes.[25,26] In all these cases, survival rates remained rather low, generally below 62%, not confirming the beneficial effect of choline.

TIMING OF FREEZING AND INSEMINATION

During the routine procedure in the laboratory, the oocytes are cultured for several hours following the retrieval and before being inseminated. The approximate time interval is around 40 hours after human chorionic gonadotropin (hCG) administration; this is actually about the time required

to the oocytes to undergo a nuclear and cytoplasmic maturation. Normally in vivo this process occurred within the follicle unit while once the oocyte is separated from the follicular fluid mainly the cytoplasmic maturation may be compromised. One of the concerns related to egg cryopreservation is the likelihood of creating a discontinuity in oocyte life during the critical period between the recovery and fertilization. The right time in which oocyte should be frozen is still under debate; Parmeggiani et al.[27] in a clinical retrospective study involving 75 patients and 93 oocyte thawing achieved a significantly improved embryo quality and clinical outcome when oocyte cryopreservation was performed within 2 h from the retrieval.

These results were later confirmed by Lappi et al.[28] in a retrospective study on 311 thawing cycles using a slow freezing protocol with 1.5 M PROH and 0.3 M sucrose. Patients had less then 40 years old. Oocytes frozen within 40 hours from the HCG injection showed a significantly higher pregnancy and implantation rates compared to eggs cryopreserved more then 40 hours after the HCG subministration.(22% vs 12% pregnancy/transfer with $P < 0.05$ and 13% vs 7% implantation with $P< 0.05$). No difference was found in the time between end of thawing and micro injection. According to these results, the timing of oocyte cryopreservation seems to play a key role in determining the clinical outcome after thawing. In particular, the hour post-HCG at which freezing is performed is very important in determining the developmental potential of oocytes from frozen cycles. Conversely using a different sucrose concentration in the freezing thawing media[23] this difference was not observed. In a recent study[29] on 325 patients (under 35 years of age) and 375 thawing cycles it was evidenced that the time before freezing does not compromise the final outcome. Instead, a more important role is played by the after thawing culture time. This result can probably be associated with the protective effect that cumulus cells exert on the oocyte limiting its aging thus, denuded oocytes might be more sensitive to damage. Consequently it is suggested to inject the oocytes within two hours after the end of the thawing procedure.

A non invasive tool that can be useful to determine the right time to freeze and to microinject the eggs after thawing is represented by the Polscope. As said above, this system can be useful since it allows to visualize the spindle. Visualization of meiotic spindle occurs more frequently when made at least 38 hours after administration of hCG than when this analysis is performed first (81.5% vs. 61.6%).[30] This can be explained by the fact that oocytes with an extruded polar body may be in a phase that precedes the meiotic metaphase II (telophase I or prometaphase II). Therefore, assessment of oocytes with polarized light can be used prior to cryopreservation to check their proper meiotic stage. The help of the polarized light should increase the effectiveness of freezing and, at the same time can help to improve standardization of the technique.

Moreover the Polscope is a non-invasive method and thus it can preserve oocyte viability while allowing repeated observations over time. In the literature there is a general agreement about correlation between the presence of the spindle and fertilization rate during IVF[31,32] or embryo development potential.[33]

Few information are, instead, available about the time frame needed between the end of thawing and the injection. This time generally is about 3 to 4 hours but, even though still under debate, it seems that approximately 1 to 2 hours should allow a good recovery of the oocytes. The few hours' culture is needed to obtain a correct spindle reorganization after thawing. Nevertheless, in many studies, the duration of this recovery period is not reported.

Confocal microscopy confirmed that the overall structure of the meiotic spindle can be restored after cryopreservation. Stachecki et al,[34] using choline (as a substitute for sodium) and a 0,2 M sucrose concentration observed that the frequency of oocytes with a normal shaped spindle and an array of regular chromosomes do not was statistically different between fresh and thawed (76.7% vs. 69.7% and 76.7% vs. 71.2%, respectively).

More recently, another study[35] used slow freezing and a mixture of 1.5 M PROH plus different sucrose concentrations (either 0.1 M or 0.3 M) and revealed that while in the fresh control group 73.1 % of oocytes displayed a normal bipolar spindle with equatorial aligned chromosomes the organization of chromatin and spindle was significantly affected (50.8%) after cryopreservation with the lowest concentration of sucrose. These parameters instead remained unchanged (69.7%) with 0.3 M sucrose. It is evident that cryopreservation can induce damage in oocytes but these alterations are acceptable using higher sucrose concentration. The higher dehydration reached with a 0.3 M sucrose limits the amount of intracellular water preventing ice crystal formation. As a consequence the spindle is probably better preserved. Unfortunately not a lot is known on the actual mechanism of depolymerization of the spindle during freezing and thawing. In order to figure out the right time for the microinjection after thawing it is important to balance carefully the recovery time that oocytes need in order to restore the meiotic spindle with oocyte aging that must be avoided. This phenomenon consists of disorders of some oocytes key factors (cell cycle kinase, intracellular calcium, and cytoskeleton) that are apparently still compatible with a normal fertilization, but, if compromised, can lead to lower implantation rates.[36]

Other structures may undergo important modifications during the freezing thawing procedures (cortiacal granules, mitochondria or golgi apparatus) but very few information are available. One recent study focused on ultrastructural features in cryopreserved oocytes[37] showing differences in the mitochondria setting. In fresh samples mitochondria had a regular shape with few short cristae, whereas in the frozen/thawed group a high percentage of oocytes (72%) showed a variable and, in some cases, a very high fraction of mitochondria with decreased electron density of the matrix or ruptures of the outer and inner membranes. Moreover in those oocytes, the mitochondrial damage was associated with SER swelling.

OOCYTE PREPARATION FOR FREEZING

Just after the retrieval, cumulus-corona complexes consist of an oocyte and somatic cells with a highly hydrated extracellular matrix. The presence of the cumulus might influence the exchange of water and CPAs during dehydration and rehydration of the oocytes, and then ultimately have an effect on the cryopreservation procedure. Very few studies are available in the literature about the influence of the cumulus on the freezing outcome. One of the first paper was published by Gook et al.[38] where oocytes with intact cumulus or totally decumulated were frozen using the protocol initially described by Lassalle for embryo cryopreservation.[19] A higher survival rate was observed in the group without cumulus compared to the cumulus enclosed complexes (69% vs. 48% respectively). Conversely these preliminary data were not confirmed by Fabbri et al.[22] The author compared the survival rates in oocytes frozen after complete denudation or partial mechanical removal of the cumulus. Regardless of the concentration of sucrose in the freezing mixture (0.1, 0.2 or 0.3 M) the survival rates were comparable between the two groups of oocytes (39% vs. 31%, 58% vs 60%, and 83% versus 81%, respectively), suggesting that the removal or retention of part of the cumulus is irrelevant to the viability of oocytes after thawing. More recently, Kuwayama et al.[39] showed that, using vitrification procedure, the survival rate was higher in oocytes frozen with the cumulus rather then without (75, 8% vs. 30% respectively). However, the very low number of oocytes included in the groups (33 and 10 eggs) did not allow any conclusions. Therefore, the evidence of the possible effect of the presence of cumulus on the viability of the thawed oocytes remains controversial. In the absence of a clear beneficial effect, the complete removal of cumulus-corona complex before freezing is recommended since the differences in the amount of left cumulus cells prior to freezing would introduce a source of variability that can affect the standardization of the cryopreservation. Moreover, the presence of the cumulus cells do not allow a clear assessment of the nuclear meiotic state of oocytes.

DEVELOPMENTAL PERFORMANCE OF FROZEN–THAWED OOCYTES

The final parameter to consider in order to validate a particular technique is the live birth rate that, as pregnancy depends on the embryo intrinsic viability. Several information are available in the literature about the relation between different freezing conditions and the ability to resume the mitotic cycle. This factor can help predicting the implantation potential of frozen-thawed embryos.[40,41] On the contrary a little is known about the early developmental competence of embryos coming from frozen eggs; possible deleterious effects of oocyte cryopreservation can already appear during pre implantation development. In a previous study[42] we compared frequency of early cleavage, cell number and degree of fragmentation in embryos derived from sibling fresh and frozen-thawed oocytes of patients undergoing IVF treatment to evidenced possible differences.

The slow freezing protocol used in this first approach was based on a 1.5 M PROH and 0.3 M sucrose mixture[22] and ICSI was used as elective insemination technique. Despite the same rate of fertilization 59 % of fresh zygotes showed early cleavage while in the frozen thawed sibling that happened just in a 7.1 % of cases. A statistical significance was evidenced (P<0.001).

Moreover, despite the overall cleavage rate at transfer was similar in the two groups, fresh embryos appeared to cleave with a faster rate, only 39% were at two-cell stage while in the frozen thawed group the majority of cleaved embryos (61%) were at two-cell stage. These data are consistent with the hypothesis that to some extent the implantation potential of embryos developing from frozen oocytes may be affected by freezing with high sucrose concentration.[43]

It has been evidenced by different authors that high cleavage rates is not necessarily related to a high implantation potential.[44-46] In these publications high rates of cleavage (90–93%) resulted in rather scarce or low rate of implantation rates (5–6%).

Conversely when our group[47] compared the developmental ability of embryos derived from sibling fresh and frozen thawed oocytes using a modified slow cooling protocol involving 1.5 M PROH and differential sucrose concentrations in freezing (0.2 M) and thawing (0.3 M) solutions the outcome was different. Embryos from fresh and frozen oocytes were assessed by comparing the frequencies of fertilization, cleavage and number of blastomeres at 42-44 after microinjection.

In 85 fresh cycles, 244 oocytes were inseminated while in 104 frozen cycles, 357 out of 525 oocytes survived after thawing

(68%) and 248 were microinjected. Normal fertilization was high in both fresh and frozen groups (81.9% and 81.4%, respectively). Cleavage rate was 96.5% and 93.1% in fresh and frozen oocytes, respectively. The frequencies of 2-, 3-, 4-, >4-cell embryos were also statistically similar. In particular, the rate of 4-cell embryos was 47.0% and 47.2% in fresh and frozen groups, respectively. The overall implantation rate of embryos developed from frozen oocytes was 15.7%, while this frequency increased to 26.9% in cases in which at least two 4-cell embryos were transferred. Following cryopreservation the early developmental ability of frozen-thawed oocytes does not appear affected in comparison to the one of sibling non-frozen oocytes. These results overlapped perfectly the clinical outcomes observed using this slow freezing protocol.[23] Moreover from these preliminary studies it is possible to affirm that the timing of the first cleavage gives some understandings of the implantation potential of embryos coming from cryopreserved eggs.

CLINICAL EFFICIENCY OF OOCYTE CRYOPRESERVATION

During the last decade a renewed interest in oocyte freezing gave rise to several studies aimed at improving protocols and techniques to make this option reliable. Since the first pregnancy dating back to 1986,[4] several step forward have been made leading to numerous publications.[44-47]

Slow freezing method was the first applied to oocyte cryopreservation exactly as it has been conceived by Lassale et al.[19] for embryo freezing. The freezing mixture, the exposure time and the temperature curve were the same. Unfortunately, the results were not comparable to the embryo outcomes. The main problem was related to the low oocyte survival; the poor recovery did not guarantee any kind of embryo selection. Our group in 2004[21] published clinical data on 68 patients and actually the survival rate was just 37%. The same was for the fertilization rate (45.4%) while, instead, cleavage and pregnancy per patient rates were quite high (86.3% and 22%, respectively).

The turning point occurred in 2001 with the increase in sucrose concentration introduced by Fabbri et al.[22] that drastically improved the survival rate and thus the possibility to have more oocytes to inject and a cohort of embryos among to choose.

The reported post thaw recovery was directly related to the amount of sugar used in the freezing solutions ranging from 34% with 0.1 M sucrose up to 82% when the concentration was increased to 0.3 M. The exposure times were maintained unchanged so was the lowering temperature curve and, consequently, the increase in the survival could be directly correlated to a higher dehydration of the oocyte. The reduced amount of water inside the cell avoided ice crystal formation improving the post thaw recovery. The limit of this study was related to the absence of clinical data so it was not possible to assess the real outcome in terms of pregnancy or live birth rates.

These data were later confirmed by several authors, but the worldwide results were often related to a small number of patients since the procedure was considered experimental.[48-50]

Later on, due to the change in the Italian law (40/2004), oocyte cryopreservation became an important tool in the IVF procedures, routinely. In fact, since just three eggs could be inseminated and embryo freezing was not allowed, oocyte cryopreservation was the only option available to avoid repeated stimulation cycles. As a consequence several reports have been published since then. One of the first ones was by our group in 2006[44] regarding 146 patients. Out of 927 oocytes included, the survival rate was good (74.1%) confirming the hypothesis that higher sucrose concentrations can improve the recovery. Nevertheless the implantation was very low (around 5%) suggesting that something could be compromised by this excessive dehydration.

Other reports[43,45,46] confirmed our results; high survival rates were reported (ranging from 65% to almost 80%) but the implantation was in all cases around 5%.

Boltd et al[24] using a sodium-depleted and HEPES buffered freezing media reported a lower survival rate (59.5%) but high implantation (15.9%). Unfortunately these data only included 23 patients and did not have any control group.

In the same year De Santis et al[46] compared different outcomes using either 0.1M or 0.3 M sucrose concentration; the survival was, as expected, significantly improved using 0.3 M sucrose but pregnancy and implantation rates were higher using 0.1 M sucrose.

In 2007 our group[23] modified the freezing protocol in order to balance the need to have a high survival recovery but, at the same time, to achieve an acceptable pregnancy and implantation rates.

Freezing and thawing solutions used different sugar concentration; during the cryopreservation a mixture of 1.5 M PROH and 0.2M sucrose was utilized in order to limit the oocyte shrinkage but a higher sucrose concentration (0.3M) was used during thawing to better balance the rehydration.

This combination resulted in a high survival (76.0%) without compromising the pregnancy/transfer and the implantation (21.2% and 13.4% respectively). Beside the row data about oocyte cryopreservation itself it is interesting to observe the contribution of egg freezing in raising the cumulative pregnancy rate.[51] This technique can actually add value in countries with a restrictive law. The cumulative pregnancy rate obtained with transfer of fresh embryos and embryos coming from frozen thawed eggs was 47.5%.

In 2010 Borini et al[52] published a multicenter study with the outcome of egg freezing in Italy after the application of the law. The protocol used a 2 step PROH – sucrose based solution and, out of 2046 patients the overall survival rate was 55.8%. An overall pregnancy rate above 14% was achieved with a good degree of reproducibility in all the clinics.

Another important aspect, beside the outcome, is the follow up of the babies born from egg freezing. Noyes et al[53] collected results from 58 reports (43 using slow freeze, 12 vitrification and three both methods) between 1986 and 2008. The number of babies born was almost the same using slow freezing and vitrification (308 from slow freezing and 289 from vitrification and 12 from a combination of both protocols). The rate of single pregnancy was 81% compared to 19% for multiples. Moreover, the overall rate of birth anomalies has been reported showing eight anomalies: three ventricular septal cardiac defects, one choanal atresia, one biliary atresia, one Rubinstein–Taybi syndrome, one clubfoot and one skin haemangioma. Fortunately, the offspring arising from frozen eggs have no significant difference in abnormalities compared to naturally conceived children. Consequently it has been proposed to remove the 'experimental' label from oocyte cryopreservation[54] and consider this technique as a routine procedure applicable in IVF laboratories.

FUTURE PERSPECTIVES

It would be of immense importance that further randomized studies are carried out which may underline any possible differences between various technical approaches and also to further generate more analytical data on the cumulative pregnancy rates along with long term follow up of the babies born as associated with oocyte cryopreservation.

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How to cite this article: Bianchi V, Zito G, Renzo PE, Valentina F, Zavos PM. Modern trends: The cryopreservation of human oocytes. Acta Medica International. 2016;3(1):169-177.

Source of Support: Nil, **Conflict of Interest:** None declared.