Chapter 8
Oocyte Cryopreservation Technique

Neelke De Munck, Gábor Vajta, and Laura Rienzi

Introduction

Cryopreservation of human oocytes can be performed by both slow freezing and vitrification. In 1986, the first report of a pregnancy from frozen-thawed oocytes was obtained [1]. Since this report, many efforts were made to improve the efficiency of the cryopreservation protocols, both for slow-freezing and vitrification. It was more than one decade later that a live birth was described after oocyte vitrification [2] and it was only in 2005 that a highly efficient and reproducible vitrification protocol for human oocytes was obtained [3]. Both methods are currently still applied although the results obtained with vitrification appear to be superior to the ones obtained with slow-freezing [4].

Indications for Oocyte Cryopreservation

Tremendous increases obtained regarding oocyte survival and clinical pregnancy rates during the last decade led to a widespread application of this technique for many indications. While in the beginning this technique was mainly used in oocyte donation programmes—eliminating the problem of donor-recipient synchronization

N. De Munck (✉)
Centre for Reproductive Medicine, Laarbeeklaan 101, 1090 Brussels, Belgium
e-mail: neelke.demunck@uzbrussel.be

G. Vajta
Central Queensland University, Bruce Highway, North Rockhampton, QLD, Australia

L. Rienzi
GENERA Center for Reproductive Medicine,
Clincia Valle Giulia, via de notaris 2b, Rome, Italy

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and allowing an efficient distribution of oocytes among different recipients—its use could also be beneficial in medical and non-medical fertility preservation programmes.

Women diagnosed with malignant diseases have the opportunity to vitrify oocytes before their gonadotoxic treatment. Depending on how fast the treatment should start and on the hormone receptivity of the tumor, fertility preservation for these patients may be obtained by vitrifying mature oocytes after controlled ovarian stimulation (COS), after *in vitro* maturation (IVM), or after *ex vivo* IVM. In the latter technique, very often used in prepubertal children in combination with ovarian tissue cryopreservation, IVM is performed on immature oocytes retrieved from the extracorporeal ovarian tissue after ovariectomy. Patients with some non-oncological medical conditions including genetic predisposition for premature ovarian failure or endometriosis could also benefit from oocyte vitrification. Finally, women postponing childbirth because of personal ambitions or lack of a partner have the opportunity to vitrify oocytes at a younger age and use them later on if they are confronted with age-related fertility loss.

The Oocyte

By the time the oocyte ovulates, some major oocyte maturation processes have taken place; these include both nuclear and cytoplasmic maturation during which the oocyte grows in size [5]. Nuclear maturation involves completion of the first meiotic division leading to extrusion of the first polar body and initiation of the second meiotic division with an arrest in metaphase II stage of meiosis [6]. Cytoplasmic maturation is indispensable to acquire an oocyte with a high developmental potency; it includes proper spatial and temporal reorganization and redistribution of the cytoplasmic organelles (mitochondria (M), Golgi apparatus, smooth endoplasmic reticulum (SER) and cortical granules) and the cytoskeleton.

Since the oocyte has a specific nuclear and cytoplasmic arrangement, important to achieve fertilization and adequate development, the structural and functional integrity should be maintained during vitrification. However, by exposing the oocyte to the highly concentrated cryoprotective additives (CPAs; often mentioned also as cryoprotectants) some physical and chemical parameters, e.g. osmotic pressure, pH, ionic intracellular content) fluctuate over a wide non-physiological range which may impact structural and genomic integrity [7]. Besides, this exposure also leads to osmotic stress and the repeated volumetric changes may result in a significant loss of functional integrity and even cell death [8]. In order to maintain this integrity during vitrification, a perfect interplay should be applied between (1) cooling and warming rate, (2) CPA choice and their concentration and working temperature, (3) the device and the minimal volume that they allow to load.

This interplay should also take into account the biological variability between oocytes. Different oocytes, even from the same patient, may react differently upon exposure to hyperosmotic solutions. These differences in permeability and inactive volume have been attributed to inherent biological variability [9, 10]. Besides this, the use of different stimulation protocols in assisted reproductive technology (ART) may influence the biological variability even more. The high variations in membrane permeability between oocytes make it hard to establish a fixed highly reproducible cryopreservation protocol. Even if a theoretical model would be established using the mean permeability coefficients, it would be suboptimal for a number of oocytes. Therefore, a more robust protocol, eliminating the effects of the biological variability may deviate from the theoretical optimal protocol.

Ultrastructure

Since the oocyte is the starting point of a new life, the oocyte vitrification procedure should not induce ultrastructural changes that may affect further developmental competence nor health of the liveborn. While immediate survival can be observed by light microscopy, this will not show the ultrastructural changes of the spindle or the cytoplasmic organelles. The spindle is mainly analyzed by confocal microscopy or by Polscope analysis before and after warming. Polscope analysis showed a high spindle re-appearance, both after open or closed oocyte vitrification while confocal microscopy showed comparable results to fresh oocytes [11, 12] or a compromised chromosome alignment [13]. The following differences are observed when fresh and vitrified oocytes are compared by electron microscopy: a slightly higher vacuolization, smaller M-SER complexes, a decrease or abnormality in the microvillar structure and a decrease in the amount and density of the cortical granules [14–17]. When these features are compared between open or closed vitrification devices, the ultrastructure is better preserved in open devices [18]. Besides this, a reduced ATP production in open devices [19] and losses and alterations in the mRNA content in open and closed devices have been observed [20, 21].

These subtle differences between fresh and vitrified oocytes may have consequences for further development. Displacement of the spindle (Fig. 8.1) may result in the potential disturbance in alignment of chromosomes and ultimately aneuploidy [22]. Abnormalities in the mitochondria or M-SER complexes (Fig. 8.2) lead to reduced fertilization potential due to disturbances in Ca^{2+} homeostasis. The increased number of vacuoles (Fig. 8.3) is thought to be responsible for an inward organelle displacement which might have further negative developmental consequences [23]. Finally, the reduction in cortical granules, probably due to the premature release of their content leading to zona hardening, together with the altered microvillar

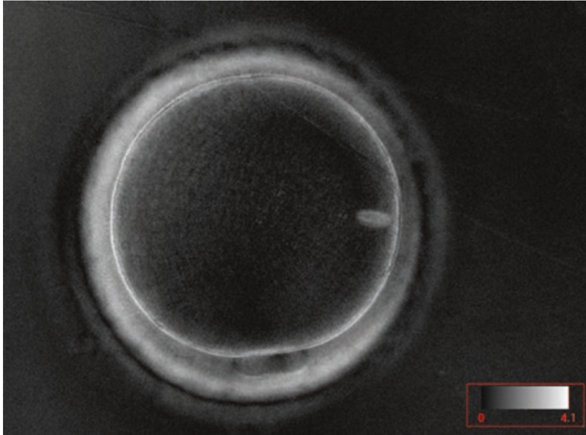

Fig. 8.1 MII human oocyte showing meiotic spindle displacement after warming

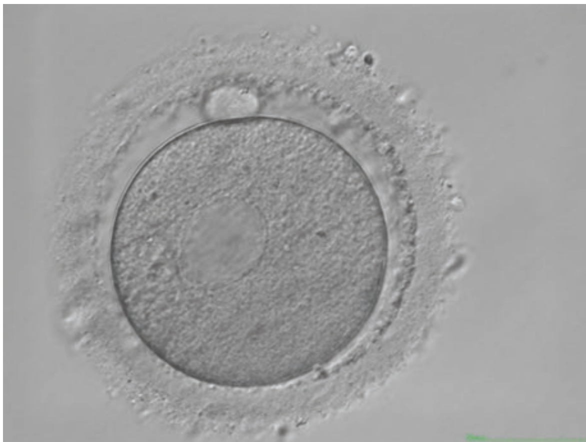

Fig. 8.2 MII human oocyte displaying M-SER complexes

structure leads to an ineffective oocyte-spermatozoon fusion. These differences between fresh and vitrified oocytes and their impact on further development have not only been described in human oocytes, but also in other species [24–26].

Knowing the high successes obtained to date, it is clear that the oocyte tolerates some of the ultrastructural changes induced by the vitrification procedure. However, due to the small number of studies comparing open and closed devices, it is still unclear whether the increased ultrastructural changes observed in closed devices also have a more pronounced effect on the developmental competence and clinical outcomes.

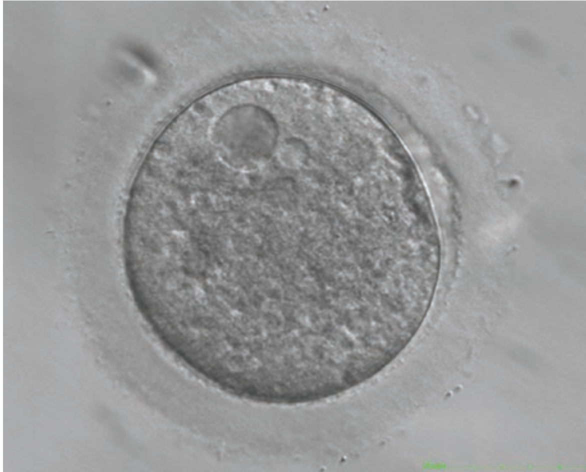

Fig. 8.3 Increased vacuolization in vitrified/warmed oocyte

Biophysical Properties

To be able to understand the volumetric changes of an oocyte upon exposure to one or more CPAs at different temperatures, several biophysical properties should be taken into account [27]. The practical use, however, should also take into account other parameters like the biological variability between oocytes and the CPA toxicity.

The *hydraulic conductivity* (L_p) or the permeability of the oocyte to water is the flow of water across each unit of the cell surface as a function of time. When an oocyte is exposed to an extracellular hypertonic solution, the initial response will be a relatively fast shrinkage because water leaves the cell. This initial volume reduction in a short time period will mainly determine L_p [28] and depends on the type and the concentration of the CPA and the exposure temperature. This volume reduction is followed by a gradual entering of the CPA in the oocyte to return to a volume slightly greater than the initial isotonic volume. This re-expansion determines the *solute permeability* (L_{CPA}) or the permeability of the oocyte to CPAs. CPAs with a high permeability will be loaded in the cell more quickly. Therefore the total volume excursion experienced by the cell will be reduced. A higher exposure temperature leads to a less extensive shrinkage/swelling response and reduces consequently the osmotic stress. This higher temperature will unfortunately also increase the unbeneficial effects of CPAs' toxicity [10].

The *activation energy* (E_A) or the temperature dependence of L_p and L_{CPA} [29] gives the minimal amount of energy that is needed to transport water or other molecules through the cell membrane. The lower the activation energy, the faster the

molecules move across the cell membrane. By plotting the values for the hydraulic and solute permeability (Y-axis) at the different temperatures (X-axis), the activation energy (E_A) is indicated by the slope: $E_A = -R*slope$; R = gas constant.

The *inactive volume* (V_b) or the part of the oocyte's volume that is osmotically inactive is defined by exposing the oocyte to a non-permeating hyperosmotic solution. For mature human oocytes this is around 20% of the iso-osmotic volume [30].

The *surface-to-volume ratio* of the oocyte: since the spherical human oocyte has a large diameter, the surface-to-volume ratio is very low. This makes oocytes less efficient in losing water and taking up CPAs. Therefore, oocytes are more susceptible to cryodamage if the exposure to CPAs is not long enough. It is, together with the osmotic inactive volume, an important factor related to the formation of lethal intracellular ice during freezing [31].

The importance of these properties can be summarized as follows: (1) the oocyte has a different membrane permeability for water and individual CPAs which is highly temperature dependent, (2) oocytes cannot shrink to less than 20% of their original volume, (3) oocytes need a long exposure time to CPAs because of their big spherical shape and (4) oocytes will not re-expand to their original volume if they are exposed to partially permeable CPAs.

Vitrification

Principles

The cryobiological definition of vitrification is ice-free amorphous solidification of both intra- and extracellular solutions at subzero temperatures [32]. It can also be regarded as an extremely increased viscosity of these solutions [33]. To induce this phenomenon, special circumstances are required, such as increased cooling rates, and high concentrations of CPAs. However, neither rapid cooling, nor CPAs are indispensable factors: pure water can also be vitrified when extremely rapid cooling rates are applied and vitrification also occurs at low cooling rates when highly concentrated CPAs are used. On the other hand, extremely high cooling rates are difficult to obtain under average embryology laboratory circumstances; and the toxic and osmotic effect of highly concentrated CPAs required for vitrification at slow cooling rates may be detrimental to the biological sample [34]. Accordingly a delicate balance between these two factors is used in current vitrification protocols.

It should also be noted that approaches to prevent ice-crystal induced damage are very similar in both traditional slow-rate freezing and vitrification. Both processes are based on a stepwise increase of permeable and non-permeable CPAs to induce dehydration and a high intra- and pericellular permeable CPA concentration. During slow-rate freezing, this increase is obtained by applying a controlled slow cooling rate, leading to the formation of extracellular ice that will further dehydrate the cell. When the cell is dehydrated before the temperature of intracellular ice formation is reached, an ice-free solidification in and around the sample occurs. On the other

hand, in most vitrification protocols, samples are exposed to increasing CPA solutions at room temperature or at the body temperature of the mammalian species. When samples are subsequently cooled rapidly, the whole solution will solidify without ice formation [35].

Theoretically, both processes can be successful. In practice—and in spite of the low level of standardization and lack of automation that may cause considerable inter-operator variability—vitrification seems to result in more consistent and higher survival and subsequent developmental rates [4]. Also, vitrification is the approach used in most human IVF laboratories for oocyte cryopreservation. Accordingly, the rest of this chapter will focus on vitrification.

Exposure to CPAs

The role of CPAs is crucial in cryopreservation of mammalian cells and tissues since their major function is to guard cells from cryodamage. Dozens of materials were tested, but only a handful was selected, mostly empirically, some of them entirely by chance. CPAs are commonly divided into two categories, permeable and non-permeable ones.

Theoretically, the role of permeable CPAs is to enter cells and replace a considerable amount of the intracellular water. This simple exchange may decrease the amount of ice formed; an additional effect of these materials is a more complex molecular mechanism that may vary between various CPAs. The final intracellular concentration of permeable CPAs may be rather high, therefore the level of toxicity is an important factor when selecting the right material. It should be noted, however, that the mechanism and tolerance level of toxic effect for cells, including oocytes and embryos, may be different of those for complex organs. Isolated cells may survive with an order of magnitude higher concentrations of permeable CPAs than living mammalian organisms.

Permeable CPAs commonly used for vitrification—selected purely on an empirical basis, –are organic solvents including ethylene glycol (EG), dimethyl sulphoxide (DMSO) and propylene glycol (PG). Since they are characterized by a low molecular weight, these molecules easily penetrate cell membranes. Glycerol, that has resulted in an unexpected breakthrough in sperm cryopreservation in the early 50s was found suboptimal for vitrification. EG, a major component of car coolants was a logical choice and its applicability is widely accepted in embryology (in sharp contrast with its potentially fatal nephrotoxicity). On the other hand, there is an ethernal debate between reproductive cryobiologist between DMSO and PG. Some companies advertise their DMSO-free CPA solutions as “non-toxic”, although this statement is rather controversial; PG, that is used for replacement may have higher toxic and mutagenic effect than DMSO itself (reviewed in [36]).

In fact, except for a very early experiment, no late (*in vivo*) pathological consequences of CPA exposure during vitrification were detected, and today’s commonly used techniques have successfully decreased the required concentration and use two

permeable CPAs instead of one to minimize the specific toxic effect of both of them. Accordingly the main point of selection is the efficiency and not the imaginary toxicity. The majority of published works describing high survival and subsequent *in vitro—in vivo* developmental rates use 50–50% combination of EG and DMSO as permeable CPAs, respectively.

For non-permeable CPAs, their role is to provide a relatively neutral osmotic pressure to expell intracellular water to close of the maximum tolerable level. Different forms of sugars were tested. In early vitrification protocols, Ficoll was a common component, later on replaced by the common sugar, sucrose (saccharose) or trehalose. Although several publications suggest the use of trehalose, no conclusive evidence supports its superiority, and sucrose has remained the most common component of CPA solutions [35].

Although rarely discussed, the role of basic (holding) media cannot be neglected, either. Successful vitrification can be performed by using the simplest buffers including PBS, but more complex media may provide more consistent outcomes. According to our experience TCM-199, one of the most complex media (that has been replaced decades ago by simpler and more appropriate solutions for embryo culture) is uniquely suitable for vitrification purposes. The Hepes-buffered version—in contrast to other buffers—was also found to be more stabile during storage at 4 °C, minimising one factor that may lead to inconsistent outcomes. Supplementation of basic media with biopolymers also seems to be beneficial for survival of cryodamage. The most complex blood serum was a previously indispensable component of vitrification solutions in rather high concentrations. Due to legal restrictions motivated by potential disease transmission and toxicity issues, it has been subsequently replaced with human serum albumin or—preferably—preparations containing both albumin and globulin. Recently, a semisynthetic water soluble polymer hydroxypropil cellulose was suggested to replace blood proteins to further minimize inconsistencies [37], but the conclusive evidence of its superiority is still missing. All these biopolymers may have stabilizing effects on cellular membranes, although the exact mechanism still requires further clarification. It should also be noted that addition of antifreeze proteins, that are part of the surviving strategy of some vertebrates on cold climates, did not fulfill the—otherwise quite resonable—expectations.

Addition of CPAs is usually a stepwise process, with minimum two steps involved (Fig. 8.4). The two steps have two different functions in the protection strategy, even if these differences are slightly overlapping and not realized by most operators. The first step includes equilibration with a relatively low concentration of permeable CPAs (usually half of the final amount), and without addition of non-permeable ones. The relatively short (around 3 min) equilibration phase applied in earlier methods was replaced with rather long (10–21 min) equilibration improving considerably the outcome [38]. For large and osmotically sensitive biological samples including human oocytes, the initial phase of exposition is further distributed to several steps, resulting in a semilinear increase of CPAs. Oocytes are supposed to regain their original shape after each phase; in fact—as the rate of equilibration may depend on the individual physico-chemical characteristics of oocytes—the full

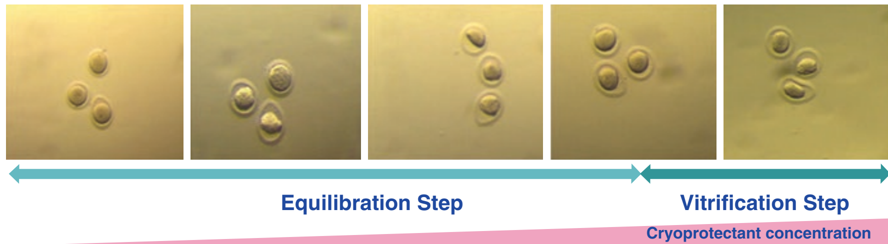

Fig. 8.4 Human oocyte vitrification procedure: the stepwise addition of CPA allows for a gradual dehydration of oocytes. Before exposure to the vitrification solution, oocytes are supposed to regain their original shape

length of the process should be adjusted to the microscopic picture [3]. The recovery of the intracellular volume means that an approximate equilibrium of CPAs and water was achieved between the intra- and extracellular space, respectively. However, this concentration of CPAs is insufficient to protect extra- and intracellular solutions from ice formation. It is only the second step, the exposition of the concentrated CPA solution that will ensure conditions required for ice-free solidification. In this step, ocytes/embryos are exposed to highly concentrated permeable CPAs (15–16% v/v) and approx. 1 M sucrose. This exposition has to be rapid (usually less than 1 min), and aggressive, with vigorous mixing and pipetting. During this short period, only a small amount of permeable CPAs enters the cytoplasm, the rest just contributes in the strong osmotic pressure established together with the high sucrose concentration. As the result of this joint effect, the ball turns into a disc or even more frequently half of it folds into the other half, and the maximum tolerable amount of water leaves the cell. This is the moment when a rapid cooling is required.

Cooling and Warming Rates

According to the empirically established parameters, safe cooling and warming rates for embryological samples should be above 20,000 °C/min for vitrification and warming [39]. Although some publications suggest that lower cooling rates do not compromise efficiency, the experimental basis of these attempts has been established in mouse oocytes that are rather tolerant to cryodamage(s), and developmental competence was not investigated [40–42]. A few recent papers dealing with human oocyte vitrification also argue that cooling rates are less critical [43–45]. However, according to the experience of authors of this paper—and probably thousands of laboratories worldwide—decreased cooling rates may lead to decreased consistency and compromised outcomes.

Submerging samples in liquid nitrogen is the standard and relatively easily available approach for cooling and storage to/at low temperatures. Liquefied forms of

other gasses may offer slightly higher rates and lower storage temperatures, but—due to problems with availability and price, their application is extremely restricted. On the other hand, exposing liquid nitrogen to vacuum decreases its temperature below the standard boiling point, and samples immersed into this "supercooled" liquid nitrogen will cool more rapidly, in lack of a thermo-insulating vapour coat that develops around the sample upon immersion. This option may have considerable perspectives, unfortunately the exploitation is slower than expected.

Most research in the past 20 years has been focused to optimize the sample size and to minimize its insulation. Obviously, a smaller sample may ensure higher cooling and warming rates. Small samples may also decrease the danger of heterogenous ice nucleation, formation of small spots of ice inside the sample [46]. Simple dropping of samples into liquid nitrogen was not found practical, and cooling rates remained relatively high, as these drops were floating on the surface for seconds, due to the evaporation of the liquid nitrogen beneath. Carrier tools obtained from other fields of biology or specially developed for this purpose and holding (preferably) less than 1 μL solution were required. A summary of these tools has been provided earlier [34, 35] and their benefits and disadvantages will be also discussed at the end of this chapter.

With an appropriate carrier tool, warming is a simple task. In optimal systems, vitrified samples are immersed directly into the medium pre-warmed to the core temperature of the mammal. Usually samples may get separated spontaneously from the carrier tool shortly after immersion, and may be processed alone subsequently.

Removal of Cryoprotectants

Direct rehydration, i.e. transferring cryopreserved samples from liquid nitrogen directly into the holding medium without any osmotic buffer is an option for certain embryos after some special slow-rate freezing techniques, and was also applied successfully after vitrification of bovine embryos. However, this approach may be risky for human embryos, and is definitely detrimental for human oocytes. To prevent extreme swelling and lysis, the high intracellular osmotic pressure must be counterbalanced by an osmotic buffer consisting of the concentrated solution of the non-permeable CPA applied for vitrification, i.e. in most cases sucrose or trehalose. The concentration of the osmotic buffer may be carefully decreased in two or three steps. One or two thorough washes in the holding medium are followed by incubation in maturation medium, then ICSI.

Devices

In the initial period, vitrification was performed in 0.25 mL straws or cryovials developed for slow-rate freezing in embryology or cell-tissue culture, respectively. Subsequently, to decrease the volume to the required level various devices obtained

from other fields of science were used including electron microscopic grids [47]. The open pulled straw (OPS) was the first device developed for embryo/oocyte vitrificationpurposes [39] followed by other tools including the Cryotop, Cryotip, Cryoleaf, Cryohook, etc. (see reviewed in [34, 35]). In a short period, almost every scientist working in reproductive cryobiology has developed his own method, and faced troubles not only in proving its superiority over the previous ones, but also in finding a relevant name. Eventually about a dozen of different devices remained and reached the level of commercial production. These devices are commonly sorted into two groups. In the first group, samples are exposed directly to the cooling and warming solutions allowing the highest possible cooling and warming rates, but the lack of a barrier layer means a potential danger of contamination from the infected liquid nitrogen [48]. In the other group, samples are isolated from liquid nitrogen, decreasing both the cooling rate and the danger of contamination. It has to be clarified whether some of the so-called closed devices are either not safely closed or result in a compromised situation at warming. The problem including the possible consequences has been discussed in detail in a recent review [49].

In some areas of reproductive biology, the cooling rate provided by several closed devices may be satisfactory. However, for human samples, especially human oocytes the compromised cooling rate may be insufficient. While inventors and producers of certain closed devices emphasize their superiority, the number of relevant publications is still insufficient to talk about conclusive evidence(s). On the other hand, the vast majority of groups continued to use the highly successful open devices for oocyte vitrification, in spite of the existing or potential legal restrictions. After more than a million of babies and several million of transfers after vitrification in open devices, without a single documented case of infection caused by liquid nitrogen mediated disease transmission, professional and legal authorities are more or less convinced not to interfere and let one of the most successful inventions in human embryology be applied properly. On the other hand, inventors, producers and clinics have implemented measures to minimize even the theoretical danger, using sterile containers for storage and/or contamination free liquid nitrogen for cooling [50–52].

Standardization, Safety and Automation

Due to various devices, CPAs and parameters, vitrification in human reproduction cannot be considered as a method or technique, it is rather an approach with some common principles but extremely diverse realization. Moreover, the rapid spreading of methods all over the world has resulted in an inappropriate education and application. Manuals and videos without personal teaching are insufficient; personal teaching without a qualified instructor, performed by poorly informed marketing agents and recently involved colleagues may be inadequate. Hands-on vitrification workshops are held worldwide, but offer access only to a limited number of embryologists, and many of them serve predominantly marketing and not educational

purposes. Accordingly, results achieved in an average clinic may be below the expectations and the intrinsic capability of the given technology.

Moreover, vitrification in embryology is performed by the alternative use of the microscopes, delicate micropipetting and the liquid nitrogen placed in open containers close to the operator. Strict safety rules determine the required clothes, gloves and protective glasses while working with liquid nitrogen. Practically none of them are, and very few of them can be followed in the routine process of embryo/oocyte vitrification. The situation was more or less tolerable in a research laboratory where ad hoc solutions are common, but vitrification is now part of the everyday practice in any reproductive laboratory, with staff improperly informed about potential hazards.

As vitrification is now a key element of human assisted reproduction, to resolve problems related to standardization and work safety is indispensable. It should also be realized that all actually used manual vitrification techniques are extremely primitive. There are attempts to change this situation by introducing devices that are capable to automate some isolated phases of the process. However, future directions should focus on more complex and more intelligent solutions including machines capable to perform both equilibration and cooling, or both warming and dilution, respectively. This advancement may require a significant investment including intellectual input and financial support.

However, considering the past achievements and the future perspectives, the required investment does not seem to be disproportional.

Key Message

1. Oocyte vitrification affects the oocyte ultrastructure
2. High cooling and warming rates are a pre-requisite
3. No contamination has been observed when using open devices
4. Safe, standardized, automated vitrification systems may become a reality.

References

1. Chen C. Pregnancy after human oocyte cryopreservation. Lancet. 1986;1(8486):884–6.
2. Kuleshova L, Gianaroli L, Magli C, Ferraretti A, Trounson A. Birth following vitrification of a small number of oocytes: a case report. Hum Reprod. 1999;14(12):3077–9.
3. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online. 2005;11(3):300–8.
4. Paramanantham J, Talmor A, Osianlis T, Weston GC. Cryopreserved oocytes: update on clinical applications and success rates. Obstet Gynecol Surv. 2015;70:97–114.
5. Guraya SS. Cellular and molecular biology of human oogenesis, ovulation and early embryogenesis. New Delhi: New Age International Publishers; 2008.
6. Seidler GE. Mammalian oocytes and preimlantation embryos as methodological components. Biol Reprod. 1983;28:36–49.
7. Kopeika J, Thornhill A, Khalaf Y. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. Hum Reprod Update. 2015;21(2):209–17.
8. Chen SU, Lien YR, Chao KH, Ho HN, Yang YS, Lee TY. Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications on oocyte freezing-a review article. Mol Cell Endocrinol. 2003;202(1–2):101.7.

9. Hunter J, Bernard A, Fuller B, McGrath J, Shaw RW. Plasma membrane water permeabilities of human oocytes: the temperature dependence of water movement in individual cells. J Cell Physiol. 1992;150(1):175–9.
10. Paynter SJ. A rational approach to oocyte cryopreservation. Reprod Biomed Online. 2005;10(5):578–86.
11. Cobo A, Pérez S, De los Santos MJ, Zulategui J, Domingo J, Remohí J. Effect of different cryopreservation protocols on the metaphase II spindle in human oocytes. Reprod Biomed Online. 2008;17(3):350–9.
12. Cao YX, Xing Q, Li L, Cong L, Zhang ZG, Wei ZL, Zhou P. Comparison of survival and embryonic development in human oocytes cryopreserved by slow-freezing and vitrification. Fertil Steril. 2009;92(4):1306–11.
13. Coticchio G, Bromfield JJ, Sciajno R, Gambardella A, Scaravelli G, Borini A, Albertini DF. Vitrification may increase the rate of chromosome misalignment in the metaphase II spindle of human mature oocytes. Reprod Biomed Online. 2009;19(Suppl 3):29–34.
14. Nottola SA, Coticchio G, Sciajno R, Gambardella A, Maione M, Scaravelli G, Bianchi S, Macchiarelli G, Borini A. Ultrastructural markers of quality in human mature oocytes vitrified using cryoleaf and cryoloop. Reprod Biomed Online. 2009;19(Suppl 3):17–27.
15. Gualtieri R, Mollo V, Barbato V, Fiorentino I, Iaccarino M, Talevi R. Ultrastructure and intracellular calcium response during activation in vitrified and slow-frozen human oocytes. Hum Reprod. 2011;26(9):2452–60.
16. Bianchi V, Macchiarelli G, Borini A, Lappi M, Cecconi S, Miglietta S, Familiari G, Nottola SA. Fine morphological assessment of quality of human mature oocytes after slow freesing or vitrification with a closed device: a comparative analysis. Reprod Biol Endocrinol. 2014;12:110.
17. Palmerini MG, Antinori M, Maione M, Cerusico F, Versaci C, Nottola SA, Macchiarelli G, Khalili MA, Antinori S. Ultrastructure of immature and mature human oocytes after cryotop vitrification. J Reprod Dev. 2014;60(6):411–20.
18. Bonetti A, Cervi M, Tomei F, Marchini M, Ortolani F, Manno M. Ultrastructural evaluation of human metaphase II oocytes after vitrification: closed versus open devices. Fertil Steril. 2011;95(3):928–35.
19. Manipalviratn S, Tong ZB, Stegmann B, Wildra E, Carter J, DeCherney A. Effect of vitrification and thawing on human oocyte ATP concentration. Fertil Steril. 2011;95(5):1839–41.
20. Chamayou S, Bonaventura G, Alecci C, Tibullo D, Di Raimondo F, Guglielmino A, Barcellona ML. Consequences of metaphase II oocyte cryopreservation on mRNA content. Cryobiology. 2011;62(2):130–4.
21. Monzo C, Haouzi D, Roman K, Assou S, Dechaud H, Hamamah S. Slow freezing and vitrification differentially modify the gene expression profile of human metaphase II oocytes. Hum Reprod. 2012;27:2160–8.
22. Smith GD, Silva E, Silva CA. Developmental consequences of cryopreservation of mammalian oocytes and embryos. Reprod Biomed Online. 2004;9(2):171–8.
23. Ebner T, Moser M, Sommergruber M, Gaiswinkler U, Shebl O, Jesacher K, Tews G. Occurrence and developmental consequences of vacuoles throughout preimplantation development. Fertil Steril. 2005;83(6):1635–40.
24. Hyttel P, Vajta G, Callesen H. Vitrification of bovine oocytes with the open pulled straw method: ultrastructural consequences. Mol Reprod Dev. 2000;56(1):80–8.
25. Wu C, Rui R, Dai J, Zhang C, Ju S, Xie B, Lu X, Zheng X. Effects of cryopreservation on the developmental competence, ultrastructure and cytoskeletal structure of porcine oocytes. Mol Reprod Dev. 2006;73(11):1454–62.
26. Boonkusol D, Faisaikarm T, Dinnyes A, Kitiyanant Y. Effects of vitrification procedures on subsequent development and ultrastructure of in-vitro matured swamp buffalo (Bubalus bubalis) oocytes. Reprod Fertil Dev. 2007;19(2):383–91.
27. Paynter SJ, O'Neil L, Fuller BJ, Shaw RW. Membrane permeability of human oocytes in the presence of the cryoprotectant propane-1,2-diol. Fertil Steril. 2001;75(3):532–8.

28. Kleinhans FW. Membrane permeability modelling: Kedem-Katchalsky vs a two-parameter formalism. Cryobiology. 1998;37(4):271–89.
29. Leibo SP. Water permeability and its activation energy of fertilized and unfertilized mouse ova. J Membr Biol. 1980;53(3):179–88.
30. Newton H, Pegg DE, Barrass R, Gosden RG. Osmotically inactive volume, hydraulic conductivity, and permeability to dimethyl sulfoxide of human mature oocytes. J Reprod Fertil. 1999;117(1):27–33.
31. Mazur P. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. J Gen Physiol. 1963;47:347–69.
32. Fuller B, Paynter S. Fundamentals of cryobiology in reproductive medicine. Reprod Biomed Online. 2004;9:680–91.
33. Arav A. Cryopreservation of oocytes and embryos. Theriogenology. 2014;81(1):96–102.
34. Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. Reprod Biomed Online. 2006;12:779–96.
35. Vajta G. Vitrification in human and domestic animal embryology: work in progress. Reprod Fertil Dev. 2013;25:719–27.
36. Vajta G, Reichart A, Ubaldi F, Rienzi L. From a backup technology to a strategy-outlining approach: the success story of cryopreservation 2013. Expert Rev Obst Gynecol. 2013;8:181–90.
37. Mori C, Yabuuchi A, Ezoe K, Murata N, Takayama Y, Okimura T, Uchiyama K, Takakura K, Abe H, Wada K, Okuno T, Kobayashi T, Kato K. Hydroxypropyl cellulose as an option for supplementation of cryoprotectant solutions for embryo vitrification in human assisted reproductive technologies. Reprod Biomed Online. 2015;30:613–21.
38. Papis K, Shimizu M, Izaike Y. Factors affecting the survivability of bovine oocytes vitrified in droplets. Theriogenology. 2000;54:651–8.
39. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev. 1998;51:53–8.
40. Seki S, Mazur P. Effect of warming rate on the survival of vitrified mouse oocytes and on the recrystallization of intracellular ice. Biol Reprod. 2008;79:727–37.
41. Seki S, Mazur P. The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure. Cryobiology. 2009;59:75–82.
42. Mazur P, Seki S. Survival of mouse oocytes after being cooled in a vitrification solution to −196 degrees C at 95 degrees to 70,000 degrees C/min and warmed at 610 degrees to 118,000 degrees C/min: a new paradigm for cryopreservation by vitrification. Cryobiology. 2011;62:1–7.
43. Stoop D, De MN, Jansen E, Platteau P, Van den Abbeel E, Verheyen G, Devroey P. Clinical validation of a closed vitrification system in an oocyte-donation programme. Reprod Biomed Online. 2012;24:180–5.
44. Papatheodorou A, Vanderzwalmen P, Panagiotidis Y, Prapas N, Zikopoulos K, Georgiou I, Prapas Y. Open versus closed oocyte vitrification system: a prospective randomized sibling oocyte study. Reprod Biomed Online. 2013;26:595–602.
45. Panagiotidis Y, Vanderzwalmen P, Prapas Y, Kasapi E, Goudakou M, Papatheodorou A, Passadaki T, Petousis S, Nikolettos N, Veletza S, Prapas N, Maroulis G. Open versus closed vitrification of blastocysts from an oocyte-donation programme: a prospective randomized study. Reprod Biomed Online. 2013;26:470–6.
46. Yavin S, Arav A. Measurement of essential physical properties of vitrification solutions. Theriogenology. 2007;67:81–9.
47. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod. 1996;54:1059–69.
48. Bielanski A, Vajta G. Risk of contamination of germplasm during cryopreservation and cryobanking in IVF units. Hum Reprod. 2009;24:2457–67.

49. Vajta G, Rienzi L, Ubaldi FM. Open versus closed systems for vitrification of human oocytes and embryos. Reprod Biomed Online. 2015;30:325–33.
50. Vajta G, Lewis IM, Kuwayama M, Greve T, Callesen H. Sterile application of the Open Pulled Straw (OPS) vitrification method. Cryo Letters. 1998;19:389–92.
51. Cobo A, Romero JL, Perez S, de los Santos MJ, Meseguer M, Remohi J. Storage of human oocytes in the vapor phase of nitrogen. Fertil Steril. 2010;94:1903–7.
52. Parmegiani L, Accorsi A, Bernardi S, Arnone A, Cognigni GE, Filicori M. A reliable procedure for decontamination before thawing of human specimens cryostored in liquid nitrogen: three washes with sterile liquid nitrogen (SLN(2)). Fertil Steril. 2012;98:870–5.