The current challenges to efficient immature oocyte cryopreservation

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Abstract Oocyte cryopreservation represents an important tool for assisted reproductive technology. It offers the opportunity to preserve fertility in women at risk of loss of the ovarian function for various pathologies. It also represents a treatment alternative for couples that cannot benefit from embryo cryopreservation because of moral, religious, or legal constraints. On the other hand, in vitro oocyte maturation has a range of applications. It can be applied in patients with a contraindication to ovarian stimulation to prevent ovarian hyperstimulation syndrome or to eliminate the risk of stimulation of hormone-sensitive tumours in cancer patients. However, while mature oocyte cryopreservation has found widespread application and oocyte in vitro maturation has a place for the treatment of specific clinical conditions, data on the efficiency of freezing of immature or in vitro matured oocytes are poorer. In this review we will focus on the combination of oocyte in vitro maturation with oocyte cryopreservation with particular emphasis on the biological implications of the cryopreservation of immature or in vitro matured oocytes. The two cryopreservation approaches, slow freezing and vitrification, will be discussed in relation to possible cryodamage occurring to subcellular structures of the oocyte and the functional interaction between oocyte and cumulus cells.

Keywords In vitro maturation · Oocyte cryopreservation · Fertility preservation · Germinal vesicle · Transzonal projections

Introduction

Oocyte cryopreservation can significantly contribute to advances in infertility treatment and reproductive biology. From a clinical perspective, several applications can benefit from the development of an efficient oocyte cryopreservation program. It offers the opportunity to preserve fertility in women who want to postpone childbearing or at risk of loss of ovarian function. For example, this approach makes possible the preservation of fertility in patients with pathologies or dysfunctions affecting the reproductive system, such as premature ovarian failure (as in the case of several genetic disorders), and in patients who need gonadotoxic therapies. Moreover, cryopreservation of the female gamete could simplify oocyte donation programs and it is an important option for infertile couples who cannot benefit from embryo cryopreservation because of moral, religious, or legal constraints [1].

So far, oocyte cryopreservation has been implemented for the storage of mature oocytes. However, many studies have been performed to improve the efficiency of oocyte cryopreservation techniques, focusing mostly on mature oocytes. Less is known about the efficiency and the consequences of cryopreservation on immature and in vitro matured oocytes. The combination of oocyte cryopreservation with in vitro maturation (IVM) represents an emerging concept. In current standard IVF protocols, women are treated with large doses of hormones that can have significant side effects. In the IVM approach, immature oocytes are retrieved from ovaries of untreated women, or following mild gonadotropin priming, and matured in vitro. IVM has a range of applications [2]. It

Capsule The combination of oocyte in vitro maturation with oocyte cryopreservation may find a place in assisted reproduction technology, especially in the field of fertility cryopreservation. In this review, we discuss the biological implications of the cryopreservation of immature or in vitro matured oocytes.

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can prevent ovarian hyperstimulation syndrome (OHSS), resolving cases in which the combined use of GnRH antagonist to suppress endogenous gonadotropin release and GnRH agonist to trigger final maturation is not sufficient to completely avoid the risk of development of the syndrome [3, 4].

It can also be applied in patients with a contraindication to ovarian stimulation eliminating the risk of stimulation of hormone-sensitive tumours, such as breast cancer, especially in countries where the use of aromatase inhibitors is not allowed for IVF treatment [2]. In particular, IVM represents an important tool when gonadotoxic therapies cannot be delayed and there is not enough time to undergo ovarian stimulation [2]. Therefore, in recent years IVM treatment combined with oocyte cryopreservation has gained increasing interest. The aim of this review is to discuss the different cryopreservation approaches for the storage of immature and in vitro matured oocytes. Emphasis will be given to the biological implications of cryopreservation and IVM in regard to the physiology and structure of oocytes at different developmental stages.

Techniques for oocyte cryopreservation

The freezing of aqueous solutions causes a series of phenomena that may affect cell viability, such as formation of intracellular ice, osmotic stress, pH changes and alterations in solute concentration. Oocyte cryopreservation entails specific problems due to the large size, high water content and low membrane permeability of this cell. During freezing and thawing, the interaction of these factors may give rise to the formation of intracellular ice crystals causing extensive ultrastructural damage. Ice crystal formation can be avoided by removing most of water from the cytoplasm. This can be achieved by exposing the oocyte to cryoprotective agents (CPAs) to generate an osmotic gradient inducing oocyte dehydration. Moreover, cryoprotectants interact with water in such a way that the freezing point of the solution is lowered.

Common characteristics shared by CPAs are high water solubility and toxicity directly proportional to their concentration and temperature of use. CPAs are classified in permeating (dimethylsulfoxide, DMSO; ethylene glycol, EG; 1,2-propanediol, PrOH) and non-permeating (sucrose, trehalose) according to their capacity to penetrate cells. DMSO is a low molecular weight substance included in different protocols developed over several decades. EG is widely adopted as a constituent of vitrification solution for its relatively low toxicity [5]. PrOH has been used to cryopreserve cleavage stage embryos and blastocysts both in the human and animals. All these permeating agents may be used in mixture, especially in vitrification protocols, including also non-permeating CPAs [6–8].

The major non-permeating agent routinely used in human oocyte cryopreservation is sucrose. Its presence in cryopreservation solutions involves several advantages during

dehydration for its capacity to limit the exposure time of permeating CPAs, due to its ability to remove water from cells, and during rehydration to reduce osmotic stress.

Slow freezing and vitrification are the two approaches to mammalian oocyte cryopreservation.

The slow-freezing method was the first to be introduced. Slow freezing implies slow cooling rates and relatively low concentrations of CPAs in order to induce a slow dehydration to the aim of reducing chemical toxicity and osmotic shock. Slow-freezing protocols have been mainly studied by Italian researchers. Favourable success rates have been reported, with an increasing number of pregnancies recorded over the years [9–17].

In recent years, vitrification has represented an attractive alternative for oocyte cryopreservation. Vitrification implies the transition of the aqueous phase into a vitreous- or glass-like state by extremely rapid cooling rates, avoiding the passage through the crystalline state and limiting ice-crystal formation [18]. Although this technique assures high oocyte viability, it involves the disadvantage of the use of very high concentrations of CPAs, which are potentially toxic. So, cells can only be exposed to cryoprotectant solutions for short periods of time [19]. Typically, vitrification procedures involve manipulations of few minutes, a time much shorter in comparison to more than 2 h required by slow-freezing methods, minimizing the time of exposure to sub-physiological conditions.

Protocols have been designed to avoid ice-crystal formation and to reduce osmotic stress during freezing [8]. Solutions with reduced toxicity have been developed using, for example, a mixture of permeable CPAs and elevated concentrations of non-permeating polymers, such as sucrose [20]. Further improvements in vitrification techniques have been achieved by the adoption of multiple steps of equilibration in which oocytes are treated with progressively increasing concentrations of CPAs and then transferred to the vitrification solution [18, 21].

Pros and cons of oocyte cryopreservation at different maturation stages

If performed inappropriately, slow cooling and vitrification may severely affect oocyte survival and physiology [22], compromising irreversibly the ability to develop into viable embryos. In dependence of the maturation stage, oocytes have different specific physiological and biophysical properties that make them more or less sensitive to cryoinjury and CPA toxicity. In this section, such differences are comparatively discussed in relation to the oocyte cellular characteristics.

Cytoskeleton

The oocyte cytoskeleton is formed from complex fibrillar cytoplasmic structures that are necessary to maintain and modify its shape, to allow an appropriate cellular division and

promote the movement of cytoplasmic organelles and membrane proteins.

Moreover, the oocyte and cumulus cell cytoskeleton play crucial roles in the process of maturation, but at the same time it is particularly sensitive to cryopreservation conditions. Understanding its dynamics is therefore fundamental to appraise the possible influences of cryostorage on oocyte quality.

In primordial or growing follicles, immature oocytes are arrested at the diplotene stage of prophase I of meiosis. They are characterized by the presence of a prominent nucleus, the germinal vesicle (GV), in which chromosomes are decondensed. Microtubules are organized in a three-dimensional network of fibres spanning across the entire cytoplasm. When the GV breaks down, the microtubular network becomes more dynamic, establishes contacts with the chromosomes, which in the meantime have condensed, and organizes into a subcortical metaphase I (MI) spindle. This structure assists the progression of chromosome segregation during the first meiotic division, enabling the extrusion of the first polar body in the perivitelline space. In the mature metaphase II (MII) oocyte, chromosomes are evenly aligned at the equatorial plate of the MII spindle. This is a dynamic structure whose microtubules undergo rapid cycles of assembly and disassembly [23]. Actin microfilaments also play an important role, providing a substrate for anchoring the meiotic spindle beneath the oocyte cortex. Likewise the extrusion of the first polar body, the subcortical localization of the MII spindle ensures a highly asymmetric second meiotic division upon penetration of the spermatozoon, limiting the loss of organelles and molecules important for embryo development.

In the mature oocyte, the most characterized cytoskeletal damage caused by cryopreservation concerns disruption of the sub-cortical actin network, microtubule depolymerization, abnormal spindle configuration and chromosome scattering [24–26]. All these effects profoundly modify the oocyte architecture and function, giving rise to cytokinesis abnormalities with subsequent developmental failure. Nevertheless, several studies demonstrate that some cytoskeletal alterations may be reversible. Under specific conditions, spindle fibres may depolymerize and repolymerize after thawing, re-establishing the physical interactions with chromosomes, which can be repositioned at the equatorial plate [27–31].

In immature oocytes microtubules are not organized in the MII spindle. Therefore, in principle the cryopreservation of immature GV-stage oocytes bypasses the risk of chromosome aberrations because at this stage the chromatin is decondensed and protected by a nuclear envelope [32, 33]. However, in some cases loss of DNA integrity may be an implication of the cryopreservation of in vitro matured oocytes. Huang et al. demonstrated that there is no significant difference in post-warming survival, normal spindle morphology, chromosome alignment and incidence of aneuploidy between in-vitro and in-vivo matured murine oocytes [34]. However, they showed that oocyte IVF and vitrification cause a decrease in cleavage

and blastocyst formation rates. They also suggested that this reduced developmental potential is an effect of an increment in DNA fragmentation occurring in in-vitro matured oocytes probably caused by inadequate culture conditions.

Oocyte-cumulus cell contact and interaction

Oocytes are surrounded by, and metabolically coupled with, adjacent cumulus cells. In fact, axon-like microfilament-rich projections referred to as transzonal processes [TZPs] originating from cumulus cells cross the zona pellucida and make contact with the oolemma. In the sites of contact, cumulus cells communicate with the oocyte by means of gap junctions [35]. Gap junctions appear to be critical for the transport of small size [less than 1 kDa] cumulus-derived factors, metabolites and signalling molecules that are necessary for immature oocytes to resume meiosis and acquire full cytoplasmic maturation and developmental competence [36–38]. Moreover, TZPs have a role in maintaining the GV or the meiotic spindle in the correct position in the cortex during oocyte maturation [39–41].

A critical difference between MII- and GV-stage oocytes regards the importance of these intercellular contacts between germinal and somatic cells. While MII-stage oocytes do not rely on support from surrounding cumulus cells and therefore it is irrelevant whether cryopreservation can assure viability of the somatic compartment, cryopreservation of GV-stage oocytes requires maintenance of their communication with viable cumulus cells to preserve a mutual interaction through TZPs that is essential for the process of maturation [42–45]. Firstly, it is essential to guarantee the mere cumulus cell survival after cryopreservation. It is important to optimize cryopreservation protocols considering that the large volume difference between oocyte and cumulus cells leads to diverse responses to CPAs [33]. Secondly, structural and functional integrity of TZPs is very sensitive to cryopreservation [33, 46, 47]. Cryostorage involves several conformational modifications that could severely affect physical interaction and bidirectional communication between germ and somatic cells. In particular, mechanical stress can result in oocyte-cumulus cells membrane injury and TZPs disruption [47]. Also, CPAs can cause cumulus cell disconnection during cooling, as an effect of their toxic effect on the microfilaments and microtubules cytoskeleton forming the TZPs [48–50].

Undoubtedly, the permeability of immature oocytes to cryoprotectant agents depends not only on their membrane lipid composition [51] and cortical microfilament network [52], but also on the presence of a somatic vestment. However, studies on this subject are not consistent. Some reports have highlighted a protective effect of cumulus cells for their ability to make CPAs penetration more gradual during cooling and protect against rapid osmotic changes during CPAs removal in thawing procedure [53], inducing an increment in survival and fertilization rates [54]. On the other hand, other studies have suggested that

cumulus cells do not have protective effects and that they interfere with the CPAs passage through ooplasm [55]. Moreover, intercellular contacts, which establish functional connections between cumulus and oocyte and facilitate communication among cumulus cells themselves, may act as potential nucleation sites that could initiate ice crystals formation between neighbouring cells [56]. However, even if cumulus cells can influence negatively the cryopreservation of GV oocytes, their presence is absolutely required for subsequent IVM. All these considerations underline the importance to maintain the integrity of TZPs during the cryopreservation of immature cumulus oocyte-complex (COCs). Cryopreservation at the immature stage is therefore an extremely problematic practice.

In a study performed on rhesus monkeys, VandeVoort et al. determined the effect of cryopreservation on GV-stage oocytes enclosed within cumulus cells either by slow or rapid cooling [50]. None of COCs cryopreserved by slow freezing using 1.5 mol/L PrOH+0.3 mol/L sucrose exhibited intact TZPs or microtubules within the ooplasm and most of the surrounding cells were shed from oocytes. Cryopreservation by rapid cooling using a mixture of DMSO, EG and sucrose caused disruption of microtubules and TZPs in almost all of COCs, but to a lesser extent than slow freezing [50]. Clearly, all these intra- and intercellular injuries caused by exposure of COCs to freezing solution can affect oocyte maturation.

Luciano et al. tested two different techniques of cryopreservation [slow freezing and vitrification] on feline immature GV-stage oocytes [57]. In most cases (80 %), COCs cryopreserved by slow freezing showed a cytoskeletal distribution similar to control COCs, with microtubules distributed homogenously throughout the ooplasm and microfilaments slightly concentrated beneath the membrane. Instead, the process of vitrification provoked an increment of oocytes with an irregular distribution of cytoskeletal elements that aggregated in the cytoplasm (66.8 %). Moreover, although both cryopreservation procedures caused a decrease in gap junction coupling between the two cellular compartments, the interruption in communication was more pronounced in the vitrification group. This was reflected in an increment in the percentage of oocytes with a higher maturation capability in the slow freezing group compared to vitrified oocytes (32.5 % vs. 14.1 %). The authors explained their results hypothesizing that a controlled cooling rate in presence of CPAs during slow freezing induced a gradual efflux of water from cells minimizing the osmotic shock while the use of more concentrated solutions of CPAs required by vitrification resulted in an increment of chemical toxicity [57]. They proposed an alternative method for COC cryopreservation: the freezing of immature GV-stage oocytes devoid of cumulus cells followed by an appropriate culture system in presence of exogenous cumulus cells, which can support the resumption of meiosis, as demonstrated also in cows [58–60]. However, it is doubtful that co-cultured cumulus cells can confer full maturation ability to denuded cumulus cell-free oocytes.

Our group has investigated the cryopreservation of human COCs retrieved from IVM patients undergoing mild gonadotropin stimulation. After vitrification and warming of immature COCs, we observed massive damage of intercellular contacts (Fig. 1). This interruption of communication between the two cellular compartments is very likely to affect oocyte maturation. Possible solutions to this problem might be: i) to adopt less traumatic protocols for dehydration and rehydration of COCs to preserve TZPs during CPA exposure; ii) to develop a system able to repair TZP structures during the subsequent in vitro culture; iii) to cryopreserve oocytes only after in vitro maturation, regardless the implication that cryopreservation can compromise the integrity of the meiotic spindle.

Oolemma

During the maturation process, oocytes undergo a change in lipid composition of their plasma membrane. It is reported that the oolemma of GV-stage oocytes contains high aquaporin

Fig. 1 Representative confocal images of human immature COCs before (a) or after (b) vitrification. Nuclear chromatin was labeled with Hoechst 33258 [blue signal], while rhodamine phalloidin was used to detect f-actin [red signal]. The process of cryopreservation causes the disruption of cumulus mass vestment and disaggregation of actin filaments, i.e. the main TZPs component

content [51]. In the plasma membrane of mature oocytes there are elevated concentration of saturated fatty acids (79,22 %) and a content of polyunsaturated fatty acids of 6,5 % [61]. This particular composition of lipids makes the mature oocytes membrane more fluid and so more resistant at low temperature in comparison with immature stages, decreasing their sensitivity to chilling injury [51, 62–64].

Moreover, it has been reported a reduction in oolemma-bound aquaporin content in in-vitro matured human oocytes compared to in-vivo matured controls [64]. Therefore, culture conditions may be the cause of decreased membrane permeability of CPAs and consequent increased sensitivity to cryopreservation of in-vitro matured oocytes.

During the process of cryopreservation, a drop in temperature modifies membrane properties with significant consequences for cell integrity. The major components of cellular membranes are phospholipids. The composition of these molecules, the length of fatty acid chains and their degree of unsaturation, the cholesterol level and the protein content influence membrane properties, especially fluidity. For example, low concentrations of cholesterol and polyunsaturated fatty acids increase the fluidity of membrane at low temperatures, decreasing its sensitivity to chilling injury [62, 63, 65].

During cooling, the removal of thermal energy decreases the motility of membrane lipid bilayer. This allows the establishment of various interactions between adjacent lipid molecules. There is a temperature, called “transition temperature” (T_m), at which the membrane modifies its fluidity, passing from a liquid phase, characterized by high lateral and rotational mobility of phospholipids, to a crystal gel phase, with a reduction in mobility.

On the basis that a low lipid phase transition temperature is associated with chilling resistance [66], Ghetler et al. investigated the difference of T_m between human zygotes, in-vitro matured and immature GV oocytes by means of a Fourier Transform Infrared spectroscopy [51]. The T_m of human zygotes is about 10 °C and far below room temperature, thermal condition at which the process of dehydration takes place. This explains the resistance of zygotes to chilling injury. Instead, the T_m of unfertilized oocytes is significantly higher. In particular, the T_m of mature and immature oocytes is closer to room temperature (16.9 °C and 24.4 °C, respectively). This may clarify the higher sensitivity to freezing of mature and immature oocytes compared to zygotes and therefore their poor survival rates after cryopreservation by slow freezing. Similar findings have been reported in case of bovine [67] and mouse oocytes [68, 69].

Based on the above notions, new methods may be developed to increase oocyte tolerance to cryopreservation. For example, artificial incorporation of liposomes could be adopted to modify the composition of the oolemma. A study carried out in sheep showed that a diet enrichment in polyunsaturated fatty acid produced a modification in composition and a change in physical properties of the oocyte membrane

[15, 63, 70]. In particular it was demonstrated a decrease in T_m of sheep oocytes from 15 °C to 4 °C, a temperature much lower than room temperature, with a consequent increase in tolerance to chilling injury.

Another approach to the development of more efficient cryopreservation methods relies on the use of some substances to protect the integrity of the oocyte membrane during cooling. For example, Isachenko et al. found that the addition of egg yolk before and after freezing had a positive effect on both mouse oocytes and cumulus cells integrity [33]. This protective effect of egg yolk was more evident at higher temperatures, probably for an increase in flexibility of oocyte membrane. This effect has not yet been fully clarified, but could be explained taking into account two factors. Firstly, the egg yolk may act by coating and isolating the oocyte membrane from direct contact with cryoprotectant agents, reducing the osmotic shock caused by salt solutions to which the oocyte is subject during freezing. Secondly, some of the yolk egg components may be incorporated into the membranes reducing their predisposition to transition to a gel-like state. Such effect are thought to reduce oocyte internal damage and preserve an intact and functionally coupled cumulus complex. The problem in the possible application of this technology is that the egg yolk is a natural incompletely defined complex mixture of phospholipids and antioxidants that cannot be used in human oocyte cryopreservation. However, it remains a good starting point for future studies aimed at identifying new lipid products that can help to protect the COC during freezing.

Clinical application of immature oocyte cryopreservation

Cryopreservation of immature oocytes from stimulated cycles

In human assisted reproduction, the cryopreservation of left-over immature or in vitro matured oocytes obtained from stimulated cycles has been investigated in several studies. These oocytes are known to be highly incompetent for intrinsic causes. Nevertheless, they represent a widely used model in oocyte IVF.

Recently, Wang and colleagues [71] analyzed sibling GV-stage oocytes cryopreserved before [109] or after [107] in vitro maturation. Survival rate was similar between oocytes frozen before or after IVF (69.7 % vs. 70.5 %), but maturation rate was lower for oocytes cryopreserved at the GV stage (51.3 % vs. 75.7 %). Furthermore, these oocytes showed an increased rate of spontaneous activation, suggesting that it is preferable to freeze MII oocytes.

Similarly, Fasano et al. [72] showed that IVF procedure is more efficient when it is performed before oocyte vitrification. In this study, a total of 184 immature oocytes were randomly divided into two different groups: 100 were vitrified at MII-

stage after IVM and 84 were immediately vitrified at GV-stage and in vitro matured after warming. Survival rate after warming was similar in both groups (86.9 % versus 84.5 %). However, oocyte maturation rate was significantly higher for oocytes matured before vitrification (46 %) than for oocytes vitrified before IVM (23.8 %). Fertilization rate and cleavage rate were similar in both groups. Approximately half of the embryos reached four cells stages in both groups, but no blastocysts were obtained. In conclusion, the study suggested that immature oocytes should be vitrified at the MII stage following IVM because oocyte maturation rates were significantly reduced when oocytes were vitrified at immature stage.

Lee et al. [73] also reported a negative influence of cryopreservation on the efficiency of maturation. In particular, the maturation rate of cryopreserved GV-stage oocytes, was lower than that of fresh controls (25 % vs. 50 %).

Tucker et al. [74] published the first birth from cryopreserved immature oocytes. They collected and cryopreserved 13 germinal vesicle oocytes after conventional ovarian stimulation in a 28-year-old woman. Oocytes were exposed to the cryoprotectants 1,2-propanediol and sucrose at room temperature before being cooled with the slow freezing technique. Three of 13 GV-stage oocytes survived after warming and two of those reached the MII stage after 30 h of in vitro maturation. In vitro matured oocytes fertilized normally after ICSI and developed into cleaving embryos. The following embryo transfer resulted into a pregnancy that progressed successfully to term at 40 weeks, when a female infant was born. The experience of Tucker and colleagues is unique and no other births have been reported from GV-stage oocytes obtained from stimulated cycles.

Cryopreservation of immature oocytes obtained from IVM cycles

Cryopreservation of immature oocytes derived from IVM cycles represents an alternative fertility preservation approach for patients in whom gonadotropin stimulation is not recommended.

In recent years, some experience has been gained in this field. In 2009, Cao et al. [75] carried out a study to determine the efficacy of vitrification of human oocytes cryopreserved before and after IVM, comparing oocyte survival, maturation and fertilization rates and embryonic development after warming. 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 when oocytes were vitrified at the GV stage (50.8 %) in comparison with the control group (70.4 %).

Further studies on oocyte vitrification after in vitro maturation have been carried out. Huang et al. [76] demonstrated that immature oocytes can be retrieved successfully from mid-antral follicles found in follicles of excised ovarian tissue, matured in vitro, and cryopreserved by vitrification, providing

an alternative method of fertility preservation. In particular, in a small series of four patients these authors reported that the mean maturation rate following IVM was 79 % and in total eight mature oocytes were vitrified. Isachenko et al. [77] reported IVM of immature oocytes retrieved during ovarian tissue dissection in two cancer patients. Eighteen immature oocytes were recovered. Ten oocytes reached the MII stage after IVM (maturation rate: 56.3 %) and were cryopreserved using the conventional slow-freezing approach [78].

Chian et al. reported the first live birth of a healthy baby after vitrification of in vitro matured oocytes retrieved in a natural menstrual cycle after hCG triggering [79]. In this study, 16 of 18 retrieved germinal vesicles matured in vitro with maturation rate of 88.89 %. After warming only 4 oocytes survived and three embryos were transferred, reaching a single healthy live baby.

In a previous study, Chian et al. [80] showed that vitrification of in vitro matured oocytes is less effective than vitrification of mature oocytes from ovarian stimulation treatment in terms of oocyte survival (67.5 % vs. 81.4 %) and fertilization rate (64.2 % vs. 75.6 %). However, the differences in the implantation rate per embryo (9.6 % vs. 19.1 %), clinical pregnancy rate per cycle started (20.0 %, vs. 44.7 %), and live-birth rate per cycle started (20.0 % vs. 39.5 %) were not statistically significant. They concluded that the combination of IVM treatments and oocyte vitrification could be applied to selected women to eliminate the risk associated with ovarian stimulation, such as estrogen-receptor-positive breast cancer patients.

Moreover, Imesh et al. [81] demonstrated that oocytes matured in vitro have limited developmental potential after cryopreservation and artificial activation. Sixty-three immature oocytes from seven unstimulated patients were collected from ovaries during laparoscopy, cultured in IVM medium and vitrified at the MII stage. After warming, oocytes were subjected to parthenogenetic activation and cultured for 6 days. They achieved a maturation rate of 61.9 % and a survival rate of 61.5 % after vitrification and warming. About 75 % of the surviving oocytes responded to activation. More

Table 1 Survival rate of oocytes vitrified at different maturation stages. Survival rates of oocytes cryopreserved at different stage of development: in-vivo matured oocytes, GV-stage oocytes before and after in vitro maturation. It is also reported the maturation rate of immature oocytes after vitrification and warming. All these oocytes are derived from IVM cycles

Cryopreserved oocytes	MI In-vivo matured	Immature (GV-stage)	MI In-vitro matured
Warmed oocytes	30	97	49
Survival rate	73.3 % (22/30)	88.7 % (86/97)	65.3 % (32/49)
Maturation rate (48 h)	—	51.2 % (44/86)	

than half of the activated oocytes arrested at the pronucleus stage and only one embryo reached blastocyst stage (1/18 embryos, 5.6 %). So, they conclude that the outcome of IVM and cryopreservation without ovarian stimulation is poor, but it is a considerable alternative to the ovarian tissue cryopreservation for women for whom ovarian stimulation is not applicable.

Recently, we carried out a pilot study to determine the efficacy of vitrification of human oocytes derived from IVM cycles that were cryopreserved before or after IVM (Table 1) comparing their survival rates. The survival rate of oocytes vitrified at the GV stage was 88.7 % (86/97). This rate was higher than that of in-vivo matured oocytes (73.3 %; 22/30) and oocytes recovered at GV stage and cryopreserved at MII stage after IVM (65.3 %; 32/49). Moreover, maturation rate of oocytes vitrified at the GV stage was slightly lower (51.2 %; 44/86) in comparison to a fresh control group (62.3 %; 200/321). These data suggest that the vitrification of GV-stage oocytes can assure high rates of survival. However, immature stage cryopreservation may involve a partial loss of the ability to mature in vitro in comparison to fresh controls, in addition to the previously described damage to the oocyte-cumulus cells intercellular communication.

Recently, we published a case report [82] that offers the proof-of-principle of the possibility to associate oocyte IVM and cryopreservation. The case involved the recovery of immature oocytes from antral follicles during a laparotomic conservative surgery for ovarian cancer. This practice did not have any additional implication for the patient because laparotomy was independently dictated by the pathology, while oocytes were obtained without previous gonadotropin stimulation. Three recovered oocytes were matured in vitro and cryopreserved by vitrification at the mature stage. Two of the three cryopreserved oocytes survived after warming. Only one fertilized normally after ICSI and 48 h post-insemination one embryo was transferred into the uterus. Even if no pregnancy was achieved, our experience demonstrates that retrieval of immature oocytes during surgery is another possibility to preserve female reproductive potential.

Conclusions

In conclusion, current evidence does not suggest an advantage in cryopreserving oocytes at the GV stage.

The original hypothesis was that the presence of the nucleus and the absence of a spindle could be an advantage for cryopreserving oocytes at GV stage because the risk of chromosome aberrations could be bypassed. Recent data have shown that other important cellular structures may be damaged. In particular, as discussed above, the damage to the cumulus cell compartment and TZPs may explain the poor performance of

oocyte cryopreserved at the GV stage in terms of maturation, fertilization and embryo developmental capacity [83–85].

Therefore, it is our opinion that cryopreservation of in vivo or in vitro matured MII-stage oocytes is a better option than cryopreservation at the GV stage.

References

1. Mature oocyte cryopreservation: a guideline. *Fertil Steril*. 2013; 99: 37–43.
2. In vitro maturation: a committee opinion. *Fertil Steril* 2013; 99: 663–6.
3. Iliodromiti S, Blockeel C, Tremellen KP, Fleming R, Tournaye H, Humaidan P, et al. Consistent high clinical pregnancy rates and low ovarian hyperstimulation syndrome rates in high-risk patients after GnRH agonist triggering and modified luteal support: a retrospective multicentre study. *Hum Reprod*. 2013;28:2529–36.
4. Seyhan A, Ata B, Polat M, Son WY, Yarali H, Dahan MH. Severe early ovarian hyperstimulation syndrome following GnRH agonist trigger with the addition of 1500 IU hCG. *Hum Reprod*. 2013;28:2522–8.
5. Songsasen N, Buckrell BC, Plante C, Leibo SP. In vitro and in vivo survival of cryopreserved sheep embryos. *Cryobiology*. 1995;32:78–91.
6. Baka SG, Toth TL, Veeck LL, Jones Jr HW, Muasher SJ, Lanzendorf SE. Evaluation of the spindle apparatus of in-vitro matured human oocytes following cryopreservation. *Hum Reprod*. 1995;10:1816–20.
7. Porcu E, Fabbri R, Seracchioli R, Ciotti PM, Magrini O, Flamigni C. Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Fertil Steril*. 1997;68:724–6.
8. Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Hum Reprod*. 2001;16:411–6.
9. Borini A, Bianchi V, Bonu MA, Sciajno R, Sereni E, Cattoli M, et al. Evidence-based clinical outcome of oocyte slow cooling. *Reprod Biomed Online*. 2007;15:175–81.
10. Borini A, Lagalla C, Bonu MA, Bianchi V, Flamigni C, Coticchio G. Cumulative pregnancy rates resulting from the use of fresh and frozen oocytes: 7 years' experience. *Reprod Biomed Online*. 2006;12:481–6.
11. Borini A, Bonu MA, Coticchio G, Bianchi V, Cattoli M, Flamigni C. Pregnancies and births after oocyte cryopreservation. *Fertil Steril*. 2004;82:601–5.
12. La Sala GB, Nicoli A, Villani MT, Pescarini M, Gallinelli A, Blickstein I. Outcome of 518 salvage oocyte-cryopreservation cycles performed as a routine procedure in an in vitro fertilization program. *Fertil Steril*. 2006;86:1423–7.
13. Levi Setti PE, Albani E, Novara PV, Cesana A, Morreale G. Cryopreservation of supernumerary oocytes in IVF/ICSI cycles. *Hum Reprod*. 2006;21:370–5.
14. De Santis L, Cino I, Rabellotti E, Papaleo E, Calzi F, Fusi FM, et al. Oocyte cryopreservation: clinical outcome of slow-cooling protocols differing in sucrose concentration. *Reprod Biomed Online*. 2007;14:57–63.
15. Bianchi V, Coticchio G, Distratis V, Di Giusto N, Flamigni C, Borini A. Differential sucrose concentration during dehydration (0.2 mol/l) and rehydration [0.3 mol/l] increases the implantation rate of frozen human oocytes. *Reprod Biomed Online*. 2007;14:64–71.
16. Parmegiani L, Cognigni GE, Bernardi S, Ciampaglia W, Infante F, Pocognoli P, et al. Freezing within 2 h from oocyte retrieval increases the efficiency of human oocyte cryopreservation when using a slow freezing/rapid thawing protocol with high sucrose concentration. *Hum Reprod*. 2008;23:1771–7.
17. Bianchi V, Lappi M, Bonu MA, Borini A. Oocyte slow freezing using a 0.2–0.3 M sucrose concentration protocol: is it really the time to trash the cryopreservation machine? *Fertil Steril*. 2012;97:1101–7.

18. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, et al. Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev*. 1998;51:53–8.
19. Kasai M, Zhu SE, Pedro PB, Nakamura K, Sakurai T, Edashige K. Fracture damage of embryos and its prevention during vitrification and warming. *Cryobiology*. 1996;33:459–64.
20. Kasai M, Komi JH, Takakamo A, Tsudera H, Sakurai T, Machida T. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *J Reprod Fertil*. 1990;89:91–7.
21. Chian RC, Kuwayama M, Tan L, Tan J, Kato O, Nagai T. High survival rate of bovine oocytes matured in vitro following vitrification. *J Reprod Dev*. 2004;50:685–96.
22. Gardner DK, Sheehan CB, Rienzi L, Katz-Jaffe M, Larman MG. Analysis of oocyte physiology to improve cryopreservation procedures. *Theriogenology*. 2007;67:64–72.
23. Gorbisky GJ, Simerly C, Schatten G, Borisy GG. Microtubules in the metaphase-arrested mouse oocyte turn over rapidly. *Proc Natl Acad Sci U S A*. 1990;87:6049–53.
24. Eroglu A, Toth TL, Toner M. Alterations of the cytoskeleton and polyploidy induced by cryopreservation of metaphase II mouse oocytes. *Fertil Steril*. 1998;69:944–57.
25. Rho GJ, Kim S, Yoo JG, Balasubramanian S, Lee HJ, Choe SY. Microtubulin configuration and mitochondrial distribution after ultra-rapid cooling of bovine oocytes. *Mol Reprod Dev*. 2002;63:464–70.
26. Mandelbaum J, Anastasiou O, Levy R, Guerin JF, de Larouziere V, Antoine JM. Effects of cryopreservation on the meiotic spindle of human oocytes. *Eur J Obstet Gynecol Reprod Biol*. 2004;113 Suppl 1:S17–23.
27. Coticchio G, De Santis L, Rossi G, Borini A, Albertini DF, Scaravelli G, et al. Sucrose concentration influences the rate of human oocytes with normal spindle and chromosome configurations after slow-cooling cryopreservation. *Hum Reprod*. 2006;21:1771–6.
28. Cobo A, Perez S, Santos MJ D I, Zulategui J, Domingo J, Remohi J. Effect of different cryopreservation protocols on the metaphase II spindle in human oocytes. *Reprod Biomed Online*. 2008;17:350–9.
29. Larman MG, Minasi MG, Rienzi L, Gardner DK. Maintenance of the meiotic spindle during vitrification in human and mouse oocytes. *Reprod Biomed Online*. 2007;15:692–700.
30. Ciotti PM, Porcu E, Notarangelo L, Magrini O, Bazzocchi A, Venturoli S. Meiotic spindle recovery is faster in vitrification of human oocytes compared to slow freezing. *Fertil Steril*. 2009;91:2399–407.
31. Bromfield JJ, Coticchio G, Hutt K, Sciajno R, Borini A, Albertini DF. Meiotic spindle dynamics in human oocytes following slow-cooling cryopreservation. *Hum Reprod*. 2009;24:2114–23.
32. Cooper A, Paynter SJ, Fuller BJ, Shaw RW. Differential effects of cryopreservation on nuclear or cytoplasmic maturation in vitro in immature mouse oocytes from stimulated ovaries. *Hum Reprod*. 1998;13:971–8.
33. Isachenko EF, Nayudu PL. Vitrification of mouse germinal vesicle oocytes: effect of treatment temperature and egg yolk on chromatin and spindle normality and cumulus integrity. *Hum Reprod*. 1999;14:400–8.
34. Huang JY, Chen HY, Park JY, Tan SL, Chian RC. Comparison of spindle and chromosome configuration in in vitro- and in vivo-matured mouse oocytes after vitrification. *Fertil Steril*. 2008;90:1424–32.
35. Gilula NB, Reeves OR, Steinbach A. Metabolic coupling, ionic coupling and cell contacts. *Nature*. 1972;235:262–5.
36. Vanderhyden BC, Armstrong DT. Role of cumulus cells and serum on the in vitro maturation, fertilization, and subsequent development of rat oocytes. *Biol Reprod*. 1989;40:720–8.
37. Tanghe S, Van Soom A, Mehrzad J, Maes D, Duchateau L, de Kruif A. Cumulus contributions during bovine fertilization in vitro. *Theriogenology*. 2003;60:135–49.
38. Thompson JG, Lane M, Gilchrist RB. Metabolism of the bovine cumulus-oocyte complex and influence on subsequent developmental competence. *Soc Reprod Fertil Suppl*. 2007;64:179–90.
39. Albertini DF. The structural basis of oocyte-granulosa cell communication. *Ernst Schering Res Found Workshop* 2002: 101–10.
40. Albertini DF, Barrett SL. Oocyte-somatic cell communication. *Reprod Suppl*. 2003;61:49–54.
41. Barrett SL, Albertini DF. Cumulus cell contact during oocyte maturation in mice regulates meiotic spindle positioning and enhances developmental competence. *J Assist Reprod Genet*. 2010;27:29–39.
42. Albertini DF. Regulation of meiotic maturation in the mammalian oocyte: interplay between exogenous cues and the microtubule cytoskeleton. *Bioessays*. 1992;14:97–103.
43. Fulka Jr J, First NL, Moor RM. Nuclear and cytoplasmic determinants involved in the regulation of mammalian oocyte maturation. *Mol Hum Reprod*. 1998;4:41–9.
44. Carabatsos MJ, Sellitto C, Goodenough DA, Albertini DF. Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Dev Biol*. 2000;226:167–79.
45. Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction*. 2001;122:829–38.
46. Cha KY, Chung HM, Lim JM, Ko JJ, Han SY, Choi DH, et al. Freezing immature oocytes. *Mol Cell Endocrinol*. 2000;169:43–7.
47. Ruppert-Lingham CJ, Paynter SJ, Godfrey J, Fuller BJ, Shaw RW. Developmental potential of murine germinal vesicle stage cumulus-oocyte complexes following exposure to dimethylsulphoxide or cryopreservation: loss of membrane integrity of cumulus cells after thawing. *Hum Reprod*. 2003;18:392–8.
48. Vincent C, Pickering SJ, Johnson MH, Quick SJ. Dimethylsulphoxide affects the organisation of microfilaments in the mouse oocyte. *Mol Reprod Dev*. 1990;26:227–35.
49. Navarro-Costa P, Correia SC, Gouveia-Oliveira A, Negreiro F, Jorge S, Cidado AJ, et al. Effects of mouse ovarian tissue cryopreservation on granulosa cell-oocyte interaction. *Hum Reprod*. 2005;20:1607–14.
50. Vandevoort CA, Shirley CR, Hill DL, Leibo SP. Effects of cryoprotectants and cryopreservation on germinal vesicle-stage cumulus-oocyte complexes of rhesus monkeys. *Fertil Steril*. 2008;90:805–16.
51. Ghetler Y, Yavin S, Shalgi R, Arav A. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. *Hum Reprod*. 2005;20:3385–9.
52. Le Gal F, Massip A. Cryopreservation of cattle oocytes: effects of meiotic stage, cycloheximide treatment, and vitrification procedure. *Cryobiology*. 1999;38:290–300.
53. Miyake T, Kasai M, Zhu SE, Sakurai T, Machida T. Vitrification of mouse oocytes and embryos at various stages of development in an ethylene glycol-based solution by a simple method. *Theriogenology*. 1993;40:121–34.
54. Imoedemhe DG, Sique AB. Survival of human oocytes cryopreserved with or without the cumulus in 1,2-propanediol. *J Assist Reprod Genet*. 1992;9:323–7.
55. Mandelbaum J, Junca AM, Plachot M, Alnot MO, Salat-Baroux J, Alvarez S, et al. Cryopreservation of human embryos and oocytes. *Hum Reprod*. 1988;3:117–9.
56. Acker JP, Larese A, Yang H, Petrenko A, McGann LE. Intracellular ice formation is affected by cell interactions. *Cryobiology*. 1999;38:363–71.
57. Luciano AM, Chigioni S, Lodde V, Franciosi F, Luvoni GC, Modina SC. Effect of different cryopreservation protocols on cytoskeleton and gap junction mediated communication integrity in feline germinal vesicle stage oocytes. *Cryobiology*. 2009;59:90–5.
58. Modina S, Beretta M, Lodde V, Lauria A, Luciano AM. Cytoplasmic changes and developmental competence of bovine oocytes cryopreserved without cumulus cells. *Eur J Histochem*. 2004;48:337–46.
59. Luciano AM, Lodde V, Beretta MS, Colleoni S, Lauria A, Modina S. Developmental capability of denuded bovine oocyte in a co-culture system with intact cumulus-oocyte complexes: role of cumulus cells, cyclic adenosine 3',5'-monophosphate, and glutathione. *Mol Reprod Dev*. 2005;71:389–97.
60. Godard NM, Pukazhenthil BS, Wildt DE, Comizzoli P. Paracrine factors from cumulus-enclosed oocytes ensure the successful

- maturation and fertilization in vitro of denuded oocytes in the cat model. *Fertil Steril*. 2009;91:2051–60.
61. Matorras R, Ruiz JJ, Mendoza R, Ruiz N, Sanjurjo P, Rodriguez-Escudero FJ. Fatty acid composition of fertilization-failed human oocytes. *Hum Reprod*. 1998;13:2227–30.
 62. Arav A, Pearl M, Zeron Y. Does membrane lipid profile explain chilling sensitivity and membrane lipid phase transition of spermatozoa and oocytes? *Cryo Letters*. 2000;21:179–86.
 63. Zeron Y, Sklan D, Arav A. Effect of polyunsaturated fatty acid supplementation on biophysical parameters and chilling sensitivity of ewe oocytes. *Mol Reprod Dev*. 2002;61:271–8.
 64. Van den Abbeel E, Schneider U, Liu J, Agca Y, Critser JK, Van Steirteghem A. Osmotic responses and tolerance limits to changes in external osmolalities, and oolemma permeability characteristics, of human in vitro matured MII oocytes. *Hum Reprod*. 2007;22:1959–72.
 65. Zeron Y, Ocheretny A, Kedar O, Borochoy A, Sklan D, Arav A. Seasonal changes in bovine fertility: relation to developmental competence of oocytes, membrane properties and fatty acid composition of follicles. *Reproduction*. 2001;121:447–54.
 66. Arav A, Rubinsky B, Fletcher G, Seren E. Cryogenic protection of oocytes with antifreeze proteins. *Mol Reprod Dev*. 1993;36:488–93.
 67. Kim JY, Kinoshita M, Ohnishi M, Fukui Y. Lipid and fatty acid analysis of fresh and frozen-thawed immature and in vitro matured bovine oocytes. *Reproduction*. 2001;122:131–8.
 68. Schroeder AC, Champlin AK, Mobraaten LE, Eppig JJ. Developmental capacity of mouse oocytes cryopreserved before and after maturation in vitro. *J Reprod Fertil*. 1990;89:43–50.
 69. Candy CJ, Wood MJ, Whittingham DG, Merriman JA, Choudhury N. Cryopreservation of immature mouse oocytes. *Hum Reprod*. 1994;9:1738–42.
 70. Zeron Y, Tomczak M, Crowe J, Arav A. The effect of liposomes on thermotropic membrane phase transitions of bovine spermatozoa and oocytes: implications for reducing chilling sensitivity. *Cryobiology*. 2002;45:143–52.
 71. Wang H, Racowsky C, Combelles CM. Is it best to cryopreserve human cumulus-free immature oocytes before or after in vitro maturation? *Cryobiology*. 2012;65:79–87.
 72. Fasano G, Demeestere I, Englert Y. In-vitro maturation of human oocytes: before or after vitrification? *J Assist Reprod Genet*. 2012;29:507–12.
 73. Lee JA, Barritt J, Moschini RM, Slifkin RE, Copperman AB. Optimizing human oocyte cryopreservation for fertility preservation patients: should we mature then freeze or freeze then mature? *Fertil Steril*. 2013;99:1356–62.
 74. Tucker MJ, Wright G, Morton PC, Massey JB. Birth after cryopreservation of immature oocytes with subsequent in vitro maturation. *Fertil Steril*. 1998;70:578–9.
 75. Cao Y, Xing Q, Zhang ZG, Wei ZL, Zhou P, Cong L. Cryopreservation of immature and in-vitro matured human oocytes by vitrification. *Reprod Biomed Online*. 2009;19:369–73.
 76. Huang JY, Buckett WM, Gilbert L, Tan SL, Chian RC. Retrieval of immature oocytes followed by in vitro maturation and vitrification: a case report on a new strategy of fertility preservation in women with borderline ovarian malignancy. *Gynecol Oncol*. 2007;105:542–4.
 77. Isachenko E, Rahimi G, Isachenko V, Nawroth F. In-vitro maturation of germinal-vesicle oocytes and cryopreservation in metaphase I/II: a possible additional option to preserve fertility during ovarian tissue cryopreservation. *Reprod Biomed Online*. 2004;8:553–7.
 78. Gook DA, Osborn SM, Johnston WI. Cryopreservation of mouse and human oocytes using 1,2-propanediol and the configuration of the meiotic spindle. *Hum Reprod*. 1993;8:1101–9.
 79. Chian RC, Gilbert L, Huang JYJ, Demirtas E, Holzer H, Benjamin A, et al. Live birth after vitrification of in vitro matured human oocytes. *Fertil Steril*. 2009;91:372–6.
 80. Chian RC, Huang JY, Gilbert L, Son WY, Holzer H, Cui SJ, et al. Obstetric outcomes following vitrification of in vitro and in vivo matured oocytes. *Fertil Steril*. 2009;91:2391–8.
 81. Imesch P, Scheiner D, Xie M, Fink D, Macas E, Dubey R, et al. Developmental potential of human oocytes matured in vitro followed by vitrification and activation. *J Ovarian Res*. 2013;6:30.
 82. Fadini R, Dal Canto M, Mignini Renzini M, Milani R, Fruscio R, Cantu MG, et al. Embryo transfer following in vitro maturation and cryopreservation of oocytes recovered from antral follicles during conservative surgery for ovarian cancer. *J Assist Reprod Genet*. 2012;29:779–81.
 83. Toth TL, Baka SG, Veeck LL, Jones Jr HW, Muasher S, Lanzendorf SE. Fertilization and in vitro development of cryopreserved human prophase I oocytes. *Fertil Steril*. 1994;61:891–4.
 84. Toth TL, Lanzendorf SE, Sandow BA, Veeck LL, Hassen WA, Hansen K, et al. Cryopreservation of human prophase I oocytes collected from unstimulated follicles. *Fertil Steril*. 1994;61:1077–82.
 85. Son WY, Park SE, Lee KA, Lee WS, Ko JJ, Yoon TK, et al. Effects of 1,2-propanediol and freezing-thawing on the in vitro developmental capacity of human immature oocytes. *Fertil Steril*. 1996;66:995–9.