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Minireview

Potential Importance of Vitrification in Reproductive Medicine

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ABSTRACT

As early as 1985, ice-free cryopreservation of mouse embryos at −196°C by vitrification was reported in an attempted alternative approach to cryostorage. Since then, vitrification techniques have entered more and more the mainstream of animal reproduction as an alternative cryopreservation method to traditional slow-cooling/rapid-thaw protocols. In addition, the last few years have seen a significant resurgence of interest in the potential benefits of vitrification protocols and techniques in human-assisted reproductive technologies. The radical strategy of vitrification results in the total elimination of ice crystal formation, both within the cells being vitrified (intracellular) and in the surrounding solution (extracellular). The protocols for vitrification are very simple. They allow cells and tissue to be placed directly into the cryoprotectant and then plunged directly into liquid nitrogen. To date, however, vitrification as a cryopreservation method has had very little practical impact on human-assisted reproduction, and human preimplantation embryo vitrification is still considered to be largely experimental. Besides the inconsistent survival rates that have been reported, another problem is the wide variety of different carriers and vessels that have been used for vitrification. Second, many different vitrification solutions have been formulated, which has not helped to focus efforts on perfecting a single approach. On the other hand, the reports of successfully completed pregnancies following vitrification at all preimplantation stages is encouraging for further research and clinical implementation. Clearly, however, attention needs to be paid to the inconsistent survival rates following vitrification.

embryo

INTRODUCTION

The cryopreservation of human oocytes, zygotes, early cleavage-stage embryos, and blastocysts has become an integral part of most every human in vitro fertilization (IVF) program. Since the first report of human pregnancy following cryopreservation, thawing, and transfer of an 8-cell embryo [1], IVF centers have been using traditional slow-rate or equilibrium freezing protocols fairly successfully. The time taken to complete these freezing procedures for human embryos ranges from 90 min to 5 h. Freezing includes the precipitation of water as ice, with the resulting separation of the water from the dissolved substances. Both intracellular ice crystal formation and the high concentration of dissolved substances pose problems. Therefore, a slow rate of cooling attempts to maintain a very delicate balance between those factors that may result in damage, mostly by ice crystallization but also by osmotic and chilling injury, zona and blastomere fracture, and alterations of the cytoskeleton.

Many studies have been undertaken to reduce the time of the freezing procedure and to try to eliminate the cost of expensive, programmable freezing equipment. One way to avoid ice crystallization damage is through the use of vitrification protocols. These cryopreservation methods present an alternative to conventional freezing with equilibration. As early as 1985, ice crystal-free cryopreservation of mouse embryos at −196°C by vitrification was initially reported [2] in an attempted alternative approach to cryostorage. Approximately 8 yr later, the successful vitrification of mouse embryos was demonstrated [3]. In 1996, Martino et al. [4] showed that by using high cooling rates, bovine oocytes after vitrification are still able to develop to the blastocyst stage. With the introduction of open-pulled straws (OPS) in 1997, the successful vitrification of early stage bovine in vitro-produced embryos was reported [5]. In the field of assisted reproductive technologies (ART) in 1999 and 2000, successful pregnancies and deliveries following vitrification techniques and protocols for human oocytes have been reported [6, 7]. Since this time, the number of scientific publications concerning vitrification has clearly risen.

Vitrification as an ultrarapid cooling technique is based on direct contact between the vitrification solution containing the cryoprotectant agents and the liquid nitrogen (LN_2). The protocols for vitrification are very simple, and they allow cells and tissue to be placed directly into the cryoprotectant and then plunged directly into LN_2. In the scientific literature, the terms ''cryopreservation'' and ''thawing'' are commonly used for conventional cryopreservation and the terms ''vitrification'' and ''warming'' for vitrification. Accordingly, this terminological separation will be utilized hereafter.

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VITRIFICATION AS AN ALTERNATIVE TO EQUILIBRIUM FREEZING

Physical Definition

Luyet [8] wrote that crystallization is incompatible with living systems and should be avoided whenever possible. The cooling of small living systems at ultrahigh speeds of freezing was considered to be possible, in that it could eliminate ice formation and create instead a glass-like (vitreous) state [8]. This constituted the origin of the idea of vitrification but not, however, the beginning of the vitrification of organs, which was unthinkable at the rapidity of freezing and thawing demanded by Luyet [8]. It is known that vitrification is used as a natural form of cryoprotection in some arctic plants [9].

In contrast to slow-rate freezing protocols, during vitrification the entire solution remains unchanged and the water does not precipitate, so no ice crystals are formed [10]. The physical definition of vitrification is the solidification of a solution (water is rapidly cooled and formed into a glassy, vitrified state from the liquid phase) at low temperature, not by ice crystallization but by extreme elevation in viscosity during cooling [11]. Fahy [10] expressed this as follows: ''. . . the viscosity of the sample becomes greater and greater until the molecules become immobilized and the sample is no longer a liquid, but rather has the properties of a solid.'' However, vitrification is a result of high cooling rates associated with high concentrations of cryoprotectant. Inevitably, this is biologically problematic and technically difficult [12].

Vitrification of water inside cells can be achieved in two ways: 1) increasing the speed of temperature conduction and 2) increasing the concentration of cryoprotectant. In addition, by using a small volume (<1 μl) of high-concentration cryoprotectant, very rapid cooling rates from 15 000 to 30 000°C/min can be achieved (e.g., ΔT from −196 to 25°C = 221°C/0.5 sec = 26 520°C/min) [4, 5, 13]. The radical strategy of vitrification results in the total elimination of ice crystal formation, both within the cells being vitrified (intracellular) and in the surrounding solution (extracellular).

VARIABLES IN VITRIFICATION

Cooling and Warming Rates

The two most important parameters for the success of cryopreservation with equilibration also determine the success of vitrification and have an impact on the biological sample that is cooled from physiological to LN_2 temperature: 1) the speed of freezing (i.e., cooling rate) and 2) the effects of the dissolved substances (i.e., concentration of the cryoprotectants). A practical limit to attainable cooling speed exists, as does a biological limit on the concentration of cryoprotectant tolerated by the cells during vitrification. Therefore, a balance between the maximization of cooling rate and the minimization of cryoprotectant concentration is important. The optimal cooling rate is that which permits the most water to move out of the cells and to freeze/vitrify extracellularly. Therefore, a primary strategy of any vitrification protocol must be to pass rapidly through the critical temperature zone of 15 to −5°C to decrease chilling injuries. The LN_2 at −196°C (point of vaporization) is at the boiling point. As cells are immersed into LN_2, the LN_2 is warmed, and this induces extensive boiling (so that nitrogen gas is produced). At this point, evaporation occurs, and a vapor coat forms around the cells. As a result, the vapor surrounding the cells can create effective insulation that cuts down temperature transfer, and this results in a decreased cooling rate. However, to achieve higher cooling rates, it is better to transfer heat through liquid instead of vapor, because conductive heat transfer in liquid is much faster than in vapor.

Regarding warming, in the most successful experiments high warming rates have been used, which means that warming of cells by directly plunging them into the warming solution (e.g., ΔT from −196 to 37°C = 233°C/3 sec = 4460°C/min). Isachenko et al. [14] obtained highly successful results with the vitrification of ovine germinal vesicle (GV) oocytes in OPS using this combination with quick warming.

Concentration of the Cryoprotectant

To achieve high cooling rates requires the use of high concentrations of the cryoprotectant solution, which depresses ice crystal formation. A critical concentration is required for vitrification. A negative consequence of this is that in some cryoprotectants, this minimal concentration (C_v) can lead to either osmotic or chemical toxicity. Minimizing the toxicity of the cryoprotectant resulting from the high cryoprotective concentration as well as reducing the cooling rate can be achieved in three ways [15].

Substituting an amino group for the hydroxyl (OH^-) group of an alcohol. This increases the vitrifiability of the alcohol [16]. 2-Amino-2-methyl-1-propanol is the best ''glass former'' of all researched amino alcohols.

Increasing the hydrostatic pressure of the solution. Kanno et al. [17] were able to demonstrate that the temperature at which crystallization begins (T_h, the ''ice nucleation temperature'') can be reduced through an increase in the hydrostatic pressure. The ''glass transition temperature'' (T_g, the temperature at which the transition to vitreous condition begins) rises with increased pressure [18]. This allows a transition to smaller cryoprotective concentrations. For example, the T_h in a 35% (v/v) liquid dimethyl sulfoxide (DMSO) solution is −80°C. With an increase in the hydrostatic pressure to 1300 atm, this solution already vitrifies [19]. A downside to this is that the increased pressure can cause damage to the biological system. Dog kidneys, for example, survived a 20-min exposure to 1000 atm [20], whereas rabbit kidneys showed severe damage after only 20 min at 500 atm. However, the increased pressure is only necessary during vitrification. Atmospheric pressure suffices for the subsequent storage.

Reduction of C_v. This is accomplished through the additional use of polymers that are nonpermeable and, therefore, remain in the extracellular area [11]. In addition, minimizing the toxicity of the cryoprotectant can also be achieved by using a combination of two cryoprotectants and a stepwise exposure of cells to precooled concentrated solutions. By reducing the amount of cryoprotectant required, the toxic and osmotic effects of them are also decreased. Furthermore, by increasing cooling and warming rates, it is possible to reduce the cryoprotectant concentration and, thus, toxicity. A very recent study has shown that the higher cooling rate using the nylon loop allows an apparently beneficial reduction in the concentration of the cryoprotectant (replacement of 5.5 M ethylene glycol [EG] by a mixture of 3.2 M EG/3.2 M DMSO) [21].

Sample Size and Carrier Systems

To improve the chances that the sample is surrounded by liquid and not vapor, the sample size should be mini-

mized so that the duration of any vapor coat is reduced and the cooling rate is increased. Furthermore, to facilitate vitrification by even higher cooling rates, it is also necessary to minimize the volume of the vitrification solution as much as practical. To minimize the volume of the vitrification solution, special carriers are used during the vitrification process. These include OPS [5, 22–26] or the flexipet-denuding pipette (FDP) [21, 27], microdrops [28], electron-microscopic (EM) copper grids [29–32], hemistraw system [33–35], small nylon coils [36] or nylon mesh [37], and the cryoloop [21, 26, 38–40]. These have all been used as carriers or vessels to achieve higher cooling rates. These methods have led to positive results for the vitrification of embryos from species with a high sensitivity to damage from freezing [30, 39, 41] as well as in the equally sensitive human [6, 7, 24] and mouse oocytes [23]. Even the vitrification of human embryonic stem cells in OPS proved to be effective [42].

BUFFERING SOLUTIONS, CRYOPROTECTANTS, AND MACROMOLECULES

Buffering Solutions

Vitrification solutions are aqueous cryoprotectant solutions that do not freeze when cooled at high cooling rates to very low temperature. Therefore, the buffered medium base used for vitrification is either phosphate-buffered saline or Hepes-buffered culture medium such as human tubal fluid.

Cryoprotectant Agents

Cryoprotectant agents are essential for the cryopreservation of cells. Regarding the high concentration of cryoprotectants used for vitrification and in view of the known biological and physiochemical effects of cryoprotectants, it is suggested that the toxicity of these agents is a key limiting factor in cryobiology. Not only does this toxicity prevent the use of fully protective levels of these additives, but it may also manifest in the form of cryoinjury over and above the cryoinjury due to classical causes. Therefore, the main target of any vitrification protocol must be the suppression of toxicity without any loss of effectiveness by the cryoprotective agents.

The most common and accepted cryoprotectant for vitrification procedures is EG. It appears to have a low toxic effect on mouse embryos and blastocysts [3, 43–46] and a rapid diffusion coupled with a quick equilibration of EG into the cell through the zona pellucida and the cellular membrane [46]. Normal pregnancies and live births achieved with cryopreserved oocytes and embryos in animals [3, 44] and in humans [6, 7, 47–52] suggest that this molecule is a good candidate for human embryo vitrification. Interestingly, Shaw et al. [53] observed that mouse pronucleate (PN) embryos and 4-cell embryos can be frozen-thawed in either EG or 1,2-propanediol without significant loss of viability. In contrast, Emiliani et al. [46] obtained results from cryopreservation of pronuclear-stage and 4-cell stage embryos that differed somewhat from those reported by Shaw et al. [53]. In their experience, EG did not seem to be a good cryoprotectant for pronuclear-stage embryos. A common practice to reduce the toxicity of the cryoprotectant, but not its effectiveness, is to place the cells first in a solution of lower-strength EG to partially load the cells with EG before transferring them to the full-strength EG/disaccharide mixture. In addition, the vitrification solution often may contain an almost equimolar combination of EG and DMSO.

Disaccharides

The addition of a sugar (sucrose, glucose, fructose, sorbitol, saccharose, trehalose, or raffinose) to an EG-based vitrification solution influenced the overall properties of the solution [54], so the properties of the sugar in the establishment or modification of a vitrification solution need to be taken into consideration. Additives with large molecular weights, such as disaccharides like sucrose or trehalose, do not penetrate the cell membrane, but they can significantly reduce the amount of cryoprotectant required as well as the toxicity of EG by decreasing the concentration required to achieve a successful cryopreservation of human oocytes and embryos. The incorporation of nonpermeating compounds into the vitrifying solution and the incubation of the cells in this solution before any vitrification helps to withdraw more water from the cells and lessens the exposure time of the cells to the toxic effects of the cryoprotectants. The nonpermeating sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the cryoprotectant after cryostorage. As early as 1988, Freedman et al. [55] used sucrose in this way for the efficient cryorecovery of human pronuclear zygotes.

Macromolecules

Cells naturally contain high concentrations of proteins, which are helpful in vitrification. Higher concentrations of cryoprotectants are needed for extracellular vitrification than for intracellular vitrification. The addition of a polymer with a high molecular weight such as polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), or Ficoll is sufficient to vitrify extracellularly with the same cryoprotective concentration used intracellularly. It was demonstrated that in certain circumstances, a polymer can reduce the C_v by 7% on average and by as much as 24% in combination with an increased hydrostatic pressure [11]. Early studies evaluated the potential beneficial effects of adding macromolecular solutes to the vitrification solution to facilitate vitrification [56–60]. These polymers can protect embryos against cryoinjury by mitigating the mechanical stresses that occur during cryopreservation [57]. They do this through modifying the vitrification properties of these solutions by significantly reducing the amount of cryoprotectant required to achieve vitrification itself [58]. They also influence the viscosity of the vitrification solution and reduce the toxicity of the cryoprotectant through lowered concentrations.

Furthermore, the polymers may be able to build a viscous matrix for encapsulation of the oocytes/embryos and prevent crystallization during cooling and warming [59, 60]. Indeed, O'Neill et al. [56] observed that addition of PEG resulted in greatly improved viability of oocytes following cryopreservation and vastly reduced the variability seen with vitrification solution alone. Shaw et al. [58] were able to show that PVP, Ficoll, and dextran interacted with an EG-based vitrification solution with extreme variation. They concluded that Ficoll and dextran had little or no effect on the glass transition of the solutions. In contrast, with the presence of PVP, the melting temperature and T_g increase with the PVP content of the solution. In addition, the results from a very recent study with the use of these polymers showed that the addition of polymers such as PEG or Ficoll to the vitrification solution seem to have no

beneficial effect on the ultimate viability of the oocytes [21].

COMPARATIVE RESULTS OF CARRIER SYSTEMS

The OPS vitrification method [5] has been successfully applied to the cryopreservation of matured bovine oocytes, precompaction- and preimplantation-stage bovine embryos [22], and mature mouse as well as human oocytes [23, 24]. More recently, successful pregnancies and deliveries after using the OPS, cryoloop, or French ministraws in vitrification protocols for human oocytes, Day 3 embryos, and blastocysts have been reported [6, 7, 47–52]. Furthermore, the efficacy of a rapid cryostorage method using the FDP for human PN embryos has also been reported [27]. In this study, the overall survival rate of PN embryos (1PN and 3PN) after warming was 87.5%. The overall percentage of warmed zygotes that cleaved and reached the 2-cell stage did not differ from that in the control groups (77% vs. 85%). Finally, comparing the developmental potential up to cavitation and blastocyst formation on Day 5, the overall outcome of the vitrified PN was 31%, compared to 33% for the control groups [24]. In addition, using EM grids, bovine oocytes and blastocysts [4, 30] as well as human multipronuclear zygotes have been successfully vitrified [31]. Interestingly, a new vitrification device called the VitMaster is able to slightly decrease the temperature of LN_2 to between −205 and −208°C (compared to −196°C). This is achieved by creating a partial vacuum; thereby, it increases significantly the cooling rate by using LN_2 slush. This vitrification device was first introduced by Arav et al. [61] and has been used very successfully for bovine, ovine, and human oocyte vitrification [14, 21, 61].

To date, however, vitrification as a cryopreservation method has had very little practical impact on human assisted reproduction, and human preimplantation embryo vitrification is still largely experimental. Inconsistent survival rates have been reported, and one explanation could be that such a variety of different carriers or vessels have been used for vitrification. Second, so many different vitrification solutions have been formulated that this has not helped to focus efforts on perfecting a single approach. On the other hand, the reports of successfully completed pregnancies following vitrification are encouraging for further research. Clearly, attention needs to be paid to the inconsistent survival rates following vitrification, and work toward continuing improvements should be ongoing.

RECENT DEVELOPMENTS

Intracellular Lipids as a "Stumbling Block" for Vitrification

Data regarding a particularly interesting method of oocyte and embryo cryopreservation have been published [62–65]. This method consists of the polarization and removal of cytoplasmic lipids from oocytes or embryos before vitrification. Nagashima et al. [64], in the first instance, have obtained embryos from porcine GV oocytes that were vitrified following delipidization. Using this method, those authors avoided a negative aftereffect caused by the cooled intracellular lipids. The removal of intracellular lipids did not adversely affect the further development of oocytes and embryos. Successful oocyte vitrification after removal of cytoplasmic lipids leads to the question of changes in the physicochemical properties of cytoplasmic membrane lipids arising at low temperatures [66] being discounted as a significant cause of cryobiological problems for the terms of our experiments.

We do, however, believe that it is impossible to dismiss classic data regarding the role of intracellular lipids as an energy source for oocytes [67] and as building materials for membranes of future embryos. That the volume of mitochondria as well as lipid vesicles increases during oocyte development to the metaphase II (MII) stage [68] indirectly confirms this. Moreover, Sathananthan et al. [69, 70] have shown that in cell complex ''smooth endoplasmic reticulum-lipid globules-mitochondria,'' reticulum-globules-mitochondria connections exist. They have also shown that these connections may be destroyed after oocyte cooling or freezing.

In the overwhelming majority of investigations studying the effect of cooling on mammalian oocytes, a negative cryoinfluence is explained in terms of the effect on cytoskeleton elements. In the opinion of a number of authors [71–73], cooling of mouse oocytes causes depolymerization of cytoskeletal protein structures to take place. Most mouse oocytes cooled to 25°C for 10 min had an abnormal cytoskeleton [73]. After exposure to 4°C for 20 min, completely disassembled spindles were observed. This process of depolymerization is, however, reversible. Spindles of mouse oocytes returned to normal appearance after warming at 37°C for 60 min. Spindles of approximately half of human oocytes exposed to room temperature for 10 min returned to normal after 4 h of culture at 37°C [73]. The negative effect of cooling is also explained as depolymerization of microtubules and microfilaments in other studies performed on human oocytes [74]. Bovine oocytes are also sensitive to decreases in temperature [75]. It has been shown that 56% of oocytes exposed to 25°C and 90% of oocytes exposed to 4°C for 1 min had abnormal spindles [75]. Those oocytes exposed to 0°C for only 2 min had a significantly reduced fertilization rate [76]. Cooling also negatively affected further cleavage of treated oocytes [77]. Martino et al. [78] reported that the developmental rate of bovine GV oocytes is also impaired after exposure to 10°C for 30 min or to 0°C for only 30 sec. Data regarding the sensitivity of porcine oocytes to low temperatures are limited. Didion et al. [79], who examined the viability of pig GV oocytes following cooling or freezing by conventional methods [80], found that the cumulus-intact porcine GV oocytes did not survive cooling to temperatures at or below 15°C. As the authors noted, this was not surprising considering that porcine embryos from the 8-cell to blastocyst stage were killed when cooled below 15°C [81, 82].

Many publications on problems of mammalian oocyte cryopreservation contain information regarding the negative effects of low temperature, including the cytoskeleton depolymerization effect of permeable cryoprotectants [72, 73, 83–86]. We suppose that the negative effect of cooling on porcine oocytes may be explained by way of the effect of cooling lipids on cytoskeletal structures. While performing our preliminary investigations, we found that following centrifugation, redistribution of lipids occurs within 48 h of in vitro culture in oocytes not exposed to freezing/thawing. However, when polarized oocytes are frozen/thawed, the lipid polarization is irreversible. This, in our opinion, suggests that the freeze/thaw process induces an alteration in the physicochemical properties of intracellular lipids. It is known that MII oocytes are more resistant to freeze-damage than GV-stage oocytes. We consider that this may be due to differences in the properties of cytoskeletal elements. One important difference is that the configuration of mi-

crotubules and microfilaments is different during these two stages of oocyte maturation. Cytoskeleton elements in GV-stage oocytes appear straight and rigid, whereas microfilaments and microtubules in MII-stage oocytes appear undulating and flexible [87]. Given the hypothesis that the interaction between the lipid phase of cells and the elements of the cytoskeleton is complex, hardening of these lipids might cause deformation and disruption of the cytoskeleton. In the case of the rigid GV-oocyte cytoskeleton, this apparently results in permanent damage, whereas in the more flexible MII-oocyte cytoskeleton, permanent damage is absent. Cytochalasins have a specific, reversible effect on cytoskeletal elements [88], making them more flexible and less susceptible to the effects of the cooled lipids. This was our reason for testing this substance for the vitrification of porcine GV oocytes [89, 90].

Therefore, on the one hand, the lipids are a ''stumbling block'' during oocyte cryopreservation, but on the other hand, their role in the vital activity of cells as energy and building materials is important. For porcine GV oocytes to be successfully vitrified, the following points should be considered: 1) the prevention of alterations to the physicochemical properties of cooled lipids, 2) the avoidance of irreversible damage to the lipid globule membranes, and 3) the protection of the reticulum-lipid connections from destruction during cooling. Further investigations must address these questions.

Bovine oocytes are considerably more cryostable than porcine ones. Also, information suggests that the diameters of the bovine and porcine intracellular lipid vesicles are different [89]. The question of whether lipid vesicle diameter might be a reflection of the physicochemical properties of lipids and cryostable properties of oocytes of the same and between species, and of the storage rate of intracellular lipid vesicles as a criterion for the effectiveness of testing the cryopreservation regime, are interesting issues. The matter of the character of the intracellular lipid granule membranes is also of great current interest.

Data that focused on the ultrastructure of intracellular lipids of the extremely cryosensitive porcine oocytes in comparison to the more cryostable bovine ones are limited [91]. We compared the ultrastructure of lipid droplets and the effect of cooling on intracellular lipid vesicles of bovine and porcine GV oocytes [92]. It was established that lipid droplets of bovine GV oocytes have a homogenous structure. The utilization of lipids takes place directly from these vesicles without formation of interim lipid compounds. In contrast, two kinds of lipid droplets are found in porcine GV oocytes: ''dark'' and homogenous vesicles next to ''gray'' vesicles with electron-lucent streaks. Vesicles of each specific group are connected to each other. After a 12-h culture period, the formation of the cisternal smooth endoplasmic reticulum layer is always associated with gray lipid vesicles. This is evidence that during oogenesis lipolysis takes place in gray vesicles only. It is supposed that cytoplasmic lipolysis has two stages: dark vesicles change into a gray form, followed by a utilization of these gray lipids. Furthermore, both types of lipid droplets in porcine oocytes changed morphologically during cooling; they turned from a round into a spherical form with lucent streaks. Lipid droplets in bovine GV oocytes revealed no visible morphological changes after cooling.

Vitrification of Oocytes

Although human oocytes have been successfully cryopreserved using traditional slow-rate or equilibrium freezing protocols and pregnancies reported [93–97], the inconsistent results have limited the application of clinical cryopreservation of oocytes as a routine technique. To survive cryopreservation, the oocyte must tolerate a sequence of volumetric contractions and expansions. Unlike all stages of preimplantation human embryos, oocytes are more vulnerable to the cryopreservation procedures involving ice crystallization. This can be explained by the decrease in permeability of the cytoplasmic membranes of oocytes [98]. It is well known that the sensitivity of oocytes to osmotic swelling, which can occur during the removal of cryoprotectant from cryopreserved cells, is very high. Furthermore, cryopreserved cells just after warming are more sensitive than fresh ones to osmotic swelling [99]. However, vitrified mouse [23], bovine [22, 25], equine [25, 26], and human oocytes can survive cryopreservation by vitrification [24, 32, 100]. Furthermore, human oocytes are able to develop to the blastocyst stage [32] and continue on to birth following vitrification of human oocytes [6, 7]. The results from recent studies highlight that the high cooling rate is an important factor to improve the effectiveness of oocyte vitrification [21, 35].

To date, whether failed-matured oocytes would have a similar survival potential after vitrification when compared to failed-fertilized oocytes is not clear. Given that the membrane permeability to water determines the dehydration behavior of oocytes during the vitrification process, both uninseminated and inseminated unfertilized oocytes are likely to behave similarly. Indeed, Liebermann et al. [21] noted a postwarming survival that was only marginally less than the survival rate achieved with failed-fertilized, aged human oocytes.

Vitrification of Zygotes

The efficacy of a rapid freezing method using the EM copper grid or the FDP for human PN embryos has already been reported [27, 31]. With respect to survival, cleavage on Day 2, and blastocyst formation, a high survival and cleavage rate of multipronuclear zygotes was also documented [27, 31]. Liebermann et al. [27], using 5.5 M EG, 1.0 M sucrose, and an FDP as a carrier for the vitrification, observed 90% of 3PN survival after warming and 82% of 3PN cleavage on Day 2. On Day 3 in the vitrified 3PN group, approximately 80% of embryos cleaved to become an embryo with four or more blastomeres, and 30% of 3PN embryos eventually became blastocysts. More recently, successful pregnancies after vitrification of human zygotes have been reported [51, 52]. It is stated that the pronuclear stage is well able to withstand the vitrification and warming conditions. Probably, this might be due to the processes during and after the fertilization, such as the cortical reaction and subsequent zona hardening that may give the ooplasmic membrane more stability to cope with the low temperature and osmotic changes. Finally, the low toxicity of EG, together with the good survival, cleavage, blastocyst formation, and pregnancy rates obtained after vitrification of pronuclear zygotes, may satisfy the real need in countries where cryopreservation of later-stage human embryos is not allowed by law or for ethical reasons.

Vitrification of Cleaved Embryos and Blastocysts

The OPS vitrification method [5] has been successfully applied to the cryopreservation of matured bovine precompaction- and preimplantation-stage embryos [22]. More recently, successful pregnancies and deliveries after using the

TABLE 1. Summary of the primary benefits of vitrification.

1. Direct contact between cells/tissue and liquid nitrogen
2. No ice crystallization
3. Utilizes higher concentration of cryoprotectant that allows shorter exposure times to the cryoprotectant
4. Rapid vitrification/warming
5. Small volume used provides a significant increase in the cooling rate
6. Cooling rates from ~15 000 to 30 000°C/min
7. Minimizes osmotic injuries
8. Reduces the time of the cryopreservation procedure (duration from 2 to 10 min)
9. Very simple protocols
10. Eliminates the cost of expensive programmable freezing equipment

OPS, cryoloop, or 0.25-ml French straws in vitrification protocols of human Day 3 embryos and blastocysts have been reported [47–50]. A major factor that can affect the survival rate of blastocysts is that the blastocyst consists of a fluid-filled cavity called the blastocoele. The likelihood of ice crystal formation is directly proportional to the volume and inversely proportional to the viscosity and the cooling rate. A decrease in survival rate after vitrification was noted when the volume of the blastocoelic cavity increased. Therefore, it should be assumed that an insufficient permeation of EG inside the cavity might allow ice crystal formation during the cooling step, reducing the postwarming survival. Intrablastocoelic water, which is detrimental to vitrification, may remain in the cavity after a 3-min exposure to EG solution [50]. Vanderzwalmen et al. [50] showed that survival rates in cryopreserved, expanded blastocysts could be improved by artificial reduction of the blastocoelic cavity.

Vitrification of Ovarian Tissue

The problems related to successful cryopreservation increase with the complexity of the sample intended for vitrification (cell → tissue → entire organ). The main problems in the vitrification of large samples are fracturing as well as crystallization during cooling. Fracturing can be mostly prevented through careful handling of the sample, so that crystallization remains the more serious problem [101].

Various research groups have reported the successful vitrification of ovarian tissue from mice, rats, Chinese hamsters, rabbits, Japanese apes, cows, and human fetuses [102–108]. Vital follicles were still detected 4 days after the warming of vitrified fetal rat ovaries [104]. Miyamoto and Sugimoto [109] vitrified rat ovaries and removed the cryoprotectant stepwise. The histological examination of the follicles yielded positive results in surface area but revealed degenerative changes, such as pyknosis, vacuolization, and cell swelling, in the other remaining tissue. Therefore, ''slow cooling'' was considered to be superior, even though the tissue showed a partial vitality. The comparison of conventional freezing and vitrification of bovine ovarian tissue demonstrated, however, that a vitrification protocol (exposure to 5.5 M EG at 22°C for 20 min) could be just as effective as ''slow freezing'' [110]. Initial studies concerned the vitrification of human ovarian tissue [111]. Comparable results after vitrification (i.e., after slow freezing of human ovarian tissue) were found in a computer-aided image analysis of cell nuclei [111].

TABLE 2. Variables of vitrification that can profoundly influence its effectiveness.

1. Type and concentrations of cryoprotectant (almost all cryoprotectants are toxic)
2. Media used as base media (holding media)
3. Temperature of the vitrification solution at exposure
4. Length of time cells/tissue are exposed to the final cryoprotectant before plunging into liquid nitrogen
5. Variability in the volume of cryoprotectant solution surrounding the cells/tissue
6. Device used for vitrification (size of the vapor coat and cooling rate)
7. Technical proficiency of the embryologist
8. Quality and developmental stage of the tested cells/tissue
9. Direct contact of the liquid nitrogen and the vitrification solution containing the biological material can be a source of contamination [128-131]; to eliminate this danger, using sterile liquid nitrogen for cooling and storage is essential

It is known from other areas of research that the vitrification of cornea [112] and vessels [113, 114] is possible. Practical knowledge regarding cryopreservation of human ovarian tissue by means of direct plunging in LN_2 is limited. To our knowledge, only a few publications concern the successful vitrification of human fetal [102, 106, 115] and adult ovarian tissue samples [107] using EG and saccharose.

Our own results (unpublished data) from histological studies of vitrified human adult ovarian tissue samples (maximum size, 1 mm^3) showed that freezing and warming with EG + saccharose + egg yolk in combination with direct plunging of straws or grids in LN_2 did not influence the ovarian tissue morphology or the follicle morphology significantly. In combination with suitable long-term cultures of human ovarian tissue, the subsequent in vitro maturation could complement treatment in planned transplants, for example. In a long-term culture of native ovarian tissue, we were already able to show that no significant apoptosis occurred [116].

Vitrification of Spermatozoa

The first attempts at cryopreservation of spermatozoa were performed during the 1940s. However, it was not until Polge et al. [117] added glycerol as a cryoprotectant that recurring problems were solved. The empirical methods developed during the 1950s are still used today. The motility of cryopreserved/thawed spermatozoa normally falls to approximately 50% of the motility before freezing, wherein interindividual fluctuation can be considerable. Despite routine application, the problem of toxicity due to osmotic stress during saturation and dilution of the cryoprotectant as well as the possible negative influence on the genetic material is as yet unresolved [118–121]. Stepwise saturation and dilution can minimize the negative consequences of osmotic stress. In practice, current results are acceptable, but the procedures are still altogether relatively difficult and simplification desirable. Besides the possible savings in time, it should also be considered that cryoprotectants as well as appropriate equipment are necessary. Most laboratories use programmable freezers. The entire procedure (saturation, freezing, and dilution) lasts approximately 30–60 min, and in some circumstances even longer.

Compared to the slow-freezing method, vitrification has economic advantages, because no freezing instruments are needed and vitrification/warming requires only a few seconds. Classical vitrification requires a high percentage of permeable cryoprotectants in medium (30%–50%, compared to 5%–7% with slow freezing) and is unsuitable for the vitrification of spermatozoa due to the lethal osmotic effect. No data exist regarding the vitrification of sperma-

tozoa. Shape and size of the sperm head could be factors that define the cryosensitivity of the cell. Comparative studies [122] on various mammalian species (boar, bull, ram, rabbit, cat, dog, horse, and human) showed a negative correlation between the size of the sperm head and cryostability. Among the above-mentioned species, human spermatozoa possessed the smallest size with maximal cryostability [123].

Our own studies [124] demonstrated that in the vitrification of human spermatozoa, the same concentration of cryoprotectant as used in the conventional method showed severe toxic effects. Vitrification yielded the best results with swim-up prepared spermatozoa without cryoprotectant. In comparison to conventional freezing with cryoprotectant (native and prepared samples), the vitrification of prepared spermatozoa without cryoprotectant led to significantly higher motility. The differences in morphology, recovery rate of motile spermatozoa, viability, and acrosome reaction between the two freezing methods (with and without cryoprotectant) were irregular but, in most cases, not significant. Spermatozoa vitrified without cryoprotectant maintained the ability after warming to fertilize human oocytes, which developed further into blastocysts.

SUMMARY AND FUTURE DIRECTIONS

Vitrification as a cryopreservation method has many primary advantages and benefits, such as no ice crystal formation through increased speed of temperature conduction, which provides a significant increase in cooling rates. This permits the use of less concentrated cryoprotectant agents so that the toxic effect is decreased. Additionally, chilling injuries are considerably reduced (Table 1). Many variables in the vitrification process exist that can profoundly influence its effectiveness and the potential to improve the survival rates of vitrified cells. These include 1) the type and concentration of cryoprotectant (almost every kind of cryoprotectant is toxic), 2) the temperature of the vitrification solution at exposure, 3) the duration of exposure to the final cryoprotectant before plunging into LN_2, 4) the type of device that is used for vitrification (which influences the size of the vapor coat and cooling rate), and 5) the quality as well as the developmental stage of the tested cells/tissue (Table 2).

Increasing the speed of thermal conduction and decreasing the concentration of cryoprotectant is an ideal strategy for cryostorage of cells/tissue with vitrification methods. The vitrification of water inside cells/tissue is achieved efficiently in two main ways. One is to increase the temperature difference between the samples and vitrification medium. The second is to find materials with rapid heat transfer. However, the actual rate of heat transfer during vitrification procedures may vary extremely depending on the device used, technical proficiency, and the specific movement at immersion. In addition, it is very important to mention that every cell has its own optimal cooling rate (i.e., oocytes are cells that are more prone to chilling injury than other developmental stages, such as cleavage-stage embryos or blastocysts). To date, the ''universal'' vitrification protocol has yet to be defined. In light of this, it is important for researchers to achieve more consistent results from existing protocols and, thereby, to establish a standardized vitrification protocol that can be applied for cryopreservation of different developmental stages.

Toward this end, it should be noted that vitrification protocols are starting to enter the mainstream of human ART. Protocols successfully applied for bovine oocytes and embryos have been used initially with human oocytes [6, 125], and initial trials have been undertaken with human embryos and blastocysts [38], with births achieved [47–50]. Vitrification is relatively simple, requires no expensive programmable freezing equipment, and relies on the placement of the cell/tissue in a very small volume of vitrification medium that must be cooled at extreme rates not obtainable in regular enclosed cryostraws and cryovials. The more convenient protocols of ultrarapid freezing and vitrification, which eliminate the use of expensive controlled-rate freezers, await crossover from use in other species, and they require validation from more extensive experimental study in humans [22, 27, 31, 126, 127]. Despite this, we suspect that the convenience of vitrification will push the development of this technique to higher levels of clinical efficiency and utilization.

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