

REVIEW

Vitrification: will it replace the conventional gamete cryopreservation techniques?

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ABSTRACT

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. This may be due to 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 pre-implantation stages is encouraging for further research and clinical implementation.

Keywords: vitrification, cryopreservation, cryoprotectants.

INTRODUCTION

In January 1977, Robert Edward stated: "The storage of human pre-implantation embryos at lower temperature could be valuable in clinical practice for the cure of infertility and possibly to avert inherited defects in children". The same author suggested that it might be beneficial to cryopreserve human oocytes. Since the birth of Louise Brown in 1978, more than one million children have been born as a result of assisted reproductive techniques. Although difficult to document specific numbers, it has been estimated that tens of thousands of children have developed from embryos that had been cryopreserved.

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In 1985, ice-free cryopreservation of mouse embryos at -196°C by vitrification was reported as an alternative approach to traditional slow-cooling/rapid-thaw protocols, vitrification techniques have entered more and more the mainstream of animal reproduction. In addition, the last few years have seen a significant revival of interest in the potential benefits of vitrification protocols and techniques in human-assisted reproductive technologies.

The cryopreservation of human oocytes, zygotes, early cleavage-stage embryos, and blastocysts has become an integral part of almost 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 minutes to 5 hours. 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.

The strategy behind vitrification is more radical: a total elimination of ice formation, then an attempt to reduce toxic and osmotic changes. The physical process of vitrification can be defined as the glass like solidification of solutions at low temperature, without ice crystal formation. This phenomenon could be achieved by increasing the concentration of the cryoprotectant, and/or increasing cooling and warming rates. Other factors that may facilitate vitrification include decreasing the volume of the solutions and increasing the hydrostatic pressure, although the latter has a very few practical consequences in embryology.

Vitrification techniques are mainly used for the cryopreservation of oocytes or blastocysts. The late interest of the technique could be explained by the apprehension of many researchers to expose embryos to high concentrations of cryoprotectants (30% to 50%) necessary to obtain a vitrified state.

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 years 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 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 in nature

Although most living organisms are composed of large amounts of water, it is not inevitable that freezing these organisms results in ice-formation. Among amphibians and insects that can tolerate freezing, there is wide variation in the amount of freezing they can tolerate (8). Woolly bear caterpillars "may spend 10 months of the year frozen solid at temperatures that descend to -50°C ". Species of frogs can spend days or weeks with as much as 65 percent of their total body water as ice. Some amphibians achieve their protection due to the glycerol manufactured by their livers. Glycerol is "antifreeze", it reduces ice formation and lowers freezing point. Such substances are called "cryoprotectants". The sugar glucose is also a cryoprotectant and arctic frogs have a special form of insulin that accelerates glucose release and absorption into cells as temperatures approach freezing. A cryoprotectant can make water harden like glass, with no crystal formation. That phenomenon is called vitrification.

The glycerol, sugars, and other cryoprotectants which are produced naturally in these organisms, are not found in levels that adequately explain (with current knowledge of cryobiology) the remarkable freezing-tolerance. Experiments with cryoprotectants in mammals in the laboratory still produce results far inferior to those observed in nature in frogs and insects.

Approaching Vitrification

Time travel is a solved problem. Einstein showed that if you travel in a spaceship for months at speeds close to the speed of light, you can return to earth centuries in the future. Unfortunately for would-be time travelers, such spacecraft will not

be available until centuries in the future.

Rather than Einstein, nature relies on Arrhenius to achieve time travel. The Arrhenius equation of chemistry describes how chemical reactions slow down as temperature is reduced. Since life is chemistry, life itself slows down at cooler temperatures. Hibernating animals use this principle to time travel from summer to summer, skipping winters when food is scarce.

Medicine already uses this kind of biological time travel. When transplantable organs such as hearts or kidneys are removed from donors, the organs begin dying as soon as their blood supply stops. Removed organs have only minutes to live. However with special preservation solutions and cooling in ice, organs can be moved across hours of time and thousands of miles to waiting recipients. Cold slows chemical processes that would otherwise be quickly fatal.

Some surgeries on major blood vessels of the heart or brain can only be done if blood circulation through the entire body is stopped (9,10). Stopped blood circulation would ordinarily be fatal within 5 minutes, but cooling to +16°C (60°F) allows the human body to remain alive in a "turned off" state for up to 60 minutes (11). With special blood substitutes and further cooling to a temperature of 0°C (32°F), life without heartbeat or circulation can be extended as much as three hours (12).

Life is chemistry. When the chemistry of life is adequately preserved, so is life. Preserving the chemistry of life for unlimited periods of time requires cooling below -130°C (-200°F)(13). Below this temperature, any remaining unfrozen liquid between ice crystals undergoes a "glass transition." Molecules become stuck to their neighbors with weak hydrogen bonds. Instead of wandering about, molecules just vibrate in one place. Without freely moving molecules, all chemistry stops.

For living cells to survive this process, chemicals called cryoprotectants must be added. Cryoprotectants, such as glycerol, are small molecules that freely penetrate inside cells and limit the percentage of water that converts into ice during cooling. This allows cells to survive freezing by remaining in isolated pockets of unfrozen solution between ice crystals. Below the glass transition temperature, molecules inside these

pockets lock into place, and cells remain preserved inside the glassy water-cryoprotectant mixture between ice crystals.

This approach for preserving individual cells by freezing was first demonstrated half a century ago (14). It is now used routinely for many different cell types, including human embryos. Preserving organized tissue by freezing has proven to be more difficult. While isolated cells can accommodate as much as 80% of the water around them turning into ice, organs are much less forgiving because there is no room between cells for ice to grow (15).

In 1984 cryobiologist Greg Fahy proposed a new approach to the problem of complex tissue preservation at low temperature (16). Instead of freezing, Fahy proposed loading tissue with so much cryoprotectant that ice formation would be completely prevented at all temperatures. Below the glass transition temperature, entire organs would become a glassy solid (a solid with the molecular structure of a liquid), free of any damage from ice. This process was called "vitrification". Preservation by vitrification, first demonstrated for embryos (2), has now been successfully applied to many different cell types and tissues of increasing complexity.

It is useful to distinguish between reversible vitrification and morphological vitrification. Reversible vitrification is vitrification in which tissue recovers from the vitrification process in a viable state. Morphological vitrification is vitrification in which tissue is preserved without freezing, with good structural preservation, but in which key enzymes or other biomolecules are damaged by the vitrification chemicals.

The physical chemistry of vitrification

Physical Definition

Luyet (17) 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. 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.

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 (18).

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 (16). Fahy 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 (19).

Water is not very viscous, therefore it can be vitrified only by an extremely rapid "flash-freezing" of a small sample. Under such rapid cooling, water molecules don't have time to arrange themselves into a crystalline structure. Viscosity increases very little when water is cooled, but at freezing temperature a sudden phase transition occurs to an ice crystal. Water can be made to vitrify if cooled at a rate of millions of degrees Celsius per second. Water can also vitrify if mixed with cryoprotectants. The cryoprotectant ethylene glycol is used with water as automobile antifreeze. The cryoprotectant propylene glycol is used to minimize ice-crystals in ice-cream. The cryoprotectant glycerol has long been used in vitrifying human sperms and to reduce freezing in human cryonics patients.

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. So, by using a small volume (<1 μ l) of high-concentration cryoprotectant, and very rapid cooling rates from 15 000 to 30 000°C/min, vitrification could be achieved (20).

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

The two most important parameters for the

success of vitrification are:

- 1) The speed of the cooling and warming rates 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.

Cooling and warming rates

The main benefit of an increase cooling and warming rate is the decreased concentration of cryoprotectant solutions, which would subsequently decrease toxic and osmotic injury. Another advantage is the quick passage through the dangerous temperature zones between + 15 and - 5 °C to decrease the chilling injury. This is especially important in the case of sensitive objects such as lipid rich structures (including pig embryos), oocytes and pre-compact stage embryos.

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. The LN2 at -196°C (point of vaporization) is at the boiling point. As cells are immersed into LN2, the LN2 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.

There are three practical ways to increase cooling and warming rates: to minimize the volume of the solution surrounding the oocytes and embryos; to minimize the thermo-insulation, preferably by establishing a direct contact between the cryoprotectant solution and the liquid nitrogen; and to avoid liquid nitrogen vapor formation.

Theoretically, the simplest solution is to drop the cryoprotectant solution directly into the liquid nitrogen. However, this method has several disadvantages. To form a drop, a rather high amount of the solution is required. Moreover, the drop will float for a long period of time over the

surface of the liquid nitrogen, as the result of evaporation of the nitrogen, consequently the cooling rate will be rather low. To avoid these problems, several carrier tools were developed and will be discussed later.

Another possibility to improve cooling and warming rates is to prevent LN2 vapor formation. Very recently, two solutions for this problem were published. The first possibility is to super-cool the LN2. With a short term application of vacuum over the LN2, the temperature can be decreased to -205 or -210 °C, far below the boiling point. This minimizing the gas coat formation around the sample and consequently increases the cooling rate (21). The alternative solution is to drop the embryos or oocytes onto a metal plate pre-cooled to 150°C (solid surface vitrification)(22).

Concentration of the cryoprotectant

Cell injury and death during freezing and thawing is related to the formation of large amounts of ice crystals within the cells. Cryopreservation aims to remove as much of the intracellular water as is compatible with life, before freezing, so as to reduce the extent of intracellular ice formation to the point where it ceases to constitute a threat to the viability of the cell. Removal of excessive amounts of water, however, will cause cellular injury and possible death through the effect of the resulting highly concentrated intracellular environment on intracellular components, particularly their membranes. This is called the `solution` effect.

Cryoprotectants are compounds that are used to achieve the required intracellular dehydration. They do so either by entering the cell and displacing the water molecules out of the cell (permeating cryoprotectants) or by remaining largely out of the cell but drawing out the intracellular water by osmosis (non-permeating cryoprotectants). Usually, combinations of the compounds are used.

The most common and accepted cryoprotectant for vitrification procedures is ethylene glycol (EG). It appears to have a low toxic effect on mouse embryos and blastocysts (23) and a rapid diffusion coupled with a quick equilibration of EG into the cell through the zona pellucida and the cellular membrane (24).

Normal pregnancies and live births achieved with cryopreserved oocytes and embryos in animals (25) and in humans (26) suggesting that this molecule is a

good candidate for human embryo vitrification.

Interestingly, Shaw et al. (27) 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. (24) obtained results from cryopreservation of pronuclear-stage and 4-cell stage embryos that differed somewhat from those reported by Shaw et al. In their experience, EG did not seem to be a good cryoprotectant for pronuclear-stage embryos.

To achieve high cooling rates, the use of high concentrations of the cryoprotectant solution is required in order to depress ice crystal formation. A negative consequence of this is that in some cryoprotectants, this concentration can lead to either osmotic or chemical toxicity.

Minimizing the toxicity of the cryoprotectant resulting from the high cryoprotective concentration can be achieved in two ways (28).

Reduction of cryoprotectant. This is accomplished through the additional use of polymers that are non-permeable and, therefore, remain in the extracellular area. 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 pre-cooled concentrated solutions.

By reducing the amount of cryoprotectants 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 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 equi-molar combination of EG and DMSO.

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 (29).

Increasing the hydrostatic pressure of the solution.

Kanno et al. (30) 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 (31). This allows a transition to smaller cryoprotective concentrations (32).

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 (33), whereas rabbit kidneys showed severe damage after only 20 min at 500 atm. However, the increased pressure is only necessary during vitrification. Atmospheric pressure is sufficient for the subsequent storage.

With the application of these strategies the vitrification approached or possibly reached the efficiency of traditional freezing, but did not surpass it.

Buffering solutions and Macromolecules

Cryoprotectant solutions contain other compounds that may have protective effects on the cells during freezing and thawing, and they are called extenders. Examples of such compounds include citrates, egg yolk. The cryoprotectant solution is usually made by adding measured amounts of these compounds to physiological solutions similar to gamete and embryo culture media. The pH of the cryoprotectant solution is maintained by using HEPES or phosphate buffers. Egg yolk is only used as an extender in sperm cryopreservation media.

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.

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 (34), 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 non-permeating 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 non-permeating sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the cryoprotectant after cryostorage (35).

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. Early studies evaluated the potential beneficial effects of adding macromolecular solutes to the vitrification solution to facilitate vitrification (36).

These polymers can protect embryos against cryoinjury by extenuating the mechanical stresses that occur during cryopreservation (37). They do this through modifying the vitrification properties of these solutions by significantly reducing the amount of cryoprotectant required to achieve vitrification itself (38). 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 (39, 40).

Sample size and carrier systems

To improve the chances that the sample is surrounded by liquid and not vapor, the sample size should be minimized 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 the open pulled straws (41), the flexipet-denuding pipette (FDP) (42), microdrops (43), electron-microscopic (EM) copper grids (44), hemistraw system (45), small nylon coils (46), or nylon mesh (47), the cryoloop (48), and the minimum volume cooling (MVC) using the cryotop (49).

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 as well as in the equally sensitive human and mouse oocytes. Even the vitrification of human embryonic stem cells proved to be effective.

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 (50), and mature mouse as well as human oocytes (51, 52).

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 (34, 7, 26, 53, 54).

Furthermore, the efficacy of a rapid cryostorage method using the flexipet-denuding pipette (FDP) for human PN embryos has also been reported (42). 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 (52).

In addition, using EM grids, bovine oocytes and blastocysts (4, 55) as well as human multipronuclear zygotes have been successfully vitrified (56).

Interestingly, a new vitrification device called the VitMaster is able to slightly decrease the

temperature of LN2 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 LN2 slush. This vitrification device was first introduced by Arav et al. (21) [Arav A. et al, 2000] and has been used very successfully for bovine, ovine, and human oocyte vitrification.

Encouraging results were obtained by using the minimum volume cooling (MVC) technique using the cryotop carrier system created by Kuwayama. They reported that they performed more than 15,000 cases of vitrification over a period of four years on human oocytes, 2PN, 4 cell stage embryos as well as human blastocysts. Survival rates higher than 90% and high pregnancy rates following development of in vitro culture and embryo transfer were obtained regardless of the stage (49, 57).

There was a report of possible embryo infection after exposure to LN2 artificially mixed with high concentrations of virus. Nevertheless, because it is highly unlikely such an adverse environment exists and actual cases of contamination have not occurred in previous surveys, there is hardly any concern in real terms. However, in some countries like USA, legal provisions are beginning to be considered for the future to avoid such a risk.

Viral infection mediated by LN2 can be prevented by completely sealing the cryopreservation container prior to immersing the sample in LN2. Kuwayama et al (57, 58) developed a vitrification method for this purpose, the vitritip method, which is able to realize complete sealing of the container along with ultra-rapid cooling and warming rates comparable to the Cryotop method. Kim et al. (59) used pulled straws for oocyte vitrification and they stated that this method provides a simple, rapid and effective strategy for preventing the risk of LN2 contamination during storage.

To date, however, vitrification as a cryopreservation method has had very little practical impact on human assisted reproduction, and human pre-implantation 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.

Intracellular Lipids as a "Stumbling Block" for vitrification

Data regarding a particularly interesting method of oocyte and embryo cryopreservation have been published (60, 61). This method consists of the polarization and removal of cytoplasmic lipids from oocytes or embryos before vitrification. Nagashima et al. (62), 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 (63).

We do, however, believe that it is impossible to dismiss classic data regarding the role of intracellular lipids as an energy source for oocytes [(64) and as building materials for membranes of future embryos. That is confirmed by the increased volume of mitochondria as well as lipid vesicles increases during oocyte development to the metaphase II stage (65).

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 microtubules 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 (66). 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.

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.

Vitrification of oocytes

Although human oocytes have been successfully cryopreserved using traditional slow-rate or equilibrium freezing protocols and pregnancies reported (67, 68, 69, 70, 71), 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 (72).

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 (73).

However, mouse, bovine, equine, and human oocytes can survive cryopreservation by vitrification (74). Furthermore, human oocytes are able to develop to the blastocyst stage and continue on to birth following vitrification. The results from recent studies highlight that the high cooling rate is an important factor to improve the effectiveness of oocyte vitrification.

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 (42, 56). With respect to survival, cleavage on Day 2, and blastocyst formation, a high survival and cleavage rate of multipronuclear zygotes was also documented (45, 56). Liebermann et al., using 5.5 M EG, 1.0 M sucrose, and an FDP as a carrier for the vitrification, observed 90% of 2PN survival after warming and 82% of 2PN cleavage on Day 2. On Day 3 in the vitrified 2PN group, approximately 80% of embryos

cleaved to become an embryo with four or more blastomeres, and 30% of 2PN embryos eventually became blastocysts.

More recently, successful pregnancies after vitrification of human zygotes have been reported (75, 53).

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

During embryonic development different parameters can be assessed, such as zygote morphology, cleavage speed, embryonic morphology on the second and the third day. The culture of embryos till day 5 allows us to use these parameters to select the embryo(s) with the best implantation chance and allows us to limit the number of transferred embryos to one or two. This policy decreases the number of multiple pregnancies without losing the actual pregnancy rates but confronts us with the task of freezing the more complex blastocyst. Therefore we need an efficient and reliable method to freeze blastocyst.

The OPS vitrification method (5) has been successfully applied to the cryopreservation of matured bovine precompaction- and pre-implantation-stage embryos (50).

More recently, successful pregnancies and deliveries after using the OPS, cryoloop, or 0.25-ml French straws in vitrification protocols of human Day 3 embryos and blastocysts have been reported (26, 76, 77, 78).

Hiaso Osada et al. (79) studied the clinical efficiency of vitrification using the Cryotop method by comparing the pregnancy rates after transfer of vitrified and fresh blastocysts from 752 patients. One thousand one hundred fifty six blastocysts were obtained from 3031 oocytes after culture and 580

blastocysts were vitrified, of these blastocysts, 572(98.6%) survived after thawing, and 572 (100%) transferred to the patients. 55.6% (275/495) became pregnant, and the total number of blastocysts per transfer was 1.16. On the other hand, 576 fresh blastocyst were transferred to 483 patients (1.19 blastocysts per patient) and the pregnancy rate was 30.8% (149/483). The pregnancy rate of the vitrification group was significantly higher than those in the fresh blastocyst group.

Factors affecting blastocyst vitrification

Artificial shrinkage

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 blastocoele 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 post-warming survival. Intrablastocoele water, which is detrimental to vitrification, may remain in the cavity after a 3-min exposure to EG solution. Vanderzwalmen et al. 78 [Vanderzwalmen P et al 2002] showed that survival rates in cryopreserved, expanded blastocysts could be improved by artificial reduction of the blastocoele cavity with a needle or pipette before vitrification.

Assisted hatching is beneficial

After thawing and dilution in a sucrose bath, the blastocyst is incubated for 24 hours before transfer. This allows the assessing of their re-expansion and survival. Vanderzwalmen et al. observed that a higher implantation rate of spontaneously hatching or hatched blastocysts as compared to expanded blastocysts which were still surrounded by intact zona pellucida. To facilitate the hatching process they started to mechanically open the zona pellucida after thawing. They observed a higher implantation rate after assisted hatching. That reinforces the hypothesis that vitrification hardens

the zona pellucida and inhibits spontaneous hatching in some cases (80).

Influence of early embryonic quality

The quality of the development of the early embryo determines the quality of the blastocyst and the final result. The blastocysts which originate from a cohort of early embryos of optimal quality had survival rates, implantation rates and ongoing pregnancy rates of respectively 73%, 32% and 19% (80).

In contrast, when the blastocysts came from embryos of sub-optimal quality, the rates of survival, implantation and ongoing pregnancy were only 38%, 9% and 6%. This underlines the importance of following day by day the development of each embryo, and to select the blastocyst with the best potential for vitrification.

Vitrification of ovarian tissue

The problems related to successful cryopreservation increase with the complexity of the sample intended for vitrification. 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 (81).

Various research groups have reported the successful vitrification of ovarian tissue from mice, rats, Chinese hamsters, rabbits, Japanese apes, cows, and human fetuses (82-88). Vital follicles were still detected 4 days after the warming of vitrified fetal rat ovaries. Miyamoto and Sugimoto (89) 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" (90).

Initial studies concerned the vitrification of

human ovarian tissue. Comparable results after vitrification were found in a computer-aided image analysis of cell nuclei (91).

It is known from other areas of research that the vitrification of cornea (92) and vessels (93, 94) is possible. Practical knowledge regarding vitrification of human ovarian tissue by means of direct plunging in LN2 is limited. To our knowledge, only a few publications concern the successful vitrification of human fetal (95) and adult ovarian tissue samples using EG and saccharose.

Rahimi et al. stated that, the histological studies of vitrified human adult ovarian tissue samples (maximum size, 1 mm³) showed that freezing and warming with EG + saccharose + egg yolk in combination with direct plunging of straws or grids in LN2 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 (96).

Vitrification of spermatozoa

The first attempts at cryopreservation of spermatozoa were performed during the 1940s (14). 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. 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 (97-99).

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 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 spermatozoa. Shape and size of the sperm head could be factors that define the cryosensitivity of the cell.

Comparative studies (100) 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 (101).

Nawroth F. et al. (102) 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, 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 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.

CONCLUSION

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. 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 LN₂,
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.

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 and initial trials have been undertaken with human embryos and blastocysts, with births achieved. 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

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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