The Era of Vitrification in Human-assisted Reproduction

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Abstract

Vitrification has recently been demonstrated to be a highly efficient technique for animal and human gamete and embryo cryopreservation: as a superior approach compared with slow-rate freezing. Vitrification creates a 'glass-like' solidification of solution, including the cells to be cryopreserved, which provides an extreme high survival rate. It is shown to be more efficient for embryo cryopreservation but its superiority the best demonstrated for oocyte cryopreservation. When applied correctly, it provides ≥90% survival rates and embryo development and implantation; pregnancy rates are similar to the use of fresh oocytes. Its use can be indicated for fertility preservation (both for medical and for social reasons), donor oocyte banking, and as an alternative for embryo cryopreservation. Vitrification appears to be not only a highly efficient but also safe technique as demonstrated by the low incidence of congenital malformation of newborns following the application of this approach.

Keywords

Oocyte, cryopreservation, embryo, vitrification, fertilization, implantation, pregnancy

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During the past few decades, two major groups of methods have been developed for cryopreservation of mammalian oocytes, embryos, and ovarian tissue: the more traditional slow freezing and the relatively novel vitrification. Storage, warming, and rehydration, i.e. removal of cryoprotectants differ only slightly between the two procedures (with some exceptions), the main difference exists in the addition of cryoprotectants and cooling.

Slow freezing is an attempt to create a delicate balance between various injuries related to the cryopreservation procedure, including ice crystal formation, fracture, toxic, and osmotic damage. The toxic and osmotic damage caused by the relatively low concentration of cryoprotectant solutions may not be too serious. However, this concentration is insufficient to eliminate ice crystal formation; therefore, an additional manipulation is required to minimize the damage. It is the slow cooling and seeding that results in controlled growth of ice in the extracellular solution. Consequently, a considerable increase of the concentration of ions, macromolecules, and other components—including cryoprotectants—occurs in the remaining fluid containing the sample. Although the concentration of these components—especially of cryoprotectants—seems to be dangerously high at the final phases, it happens at low temperatures (below -20ºC) where the real toxic effect is minimal. On the other hand, this elevated concentration minimizes ice crystal formation allowing solidification of most of the intracellular water in a glass-like form. This phenomenon fulfils the physical criteria of vitrification, i.e. solidification of solutions without crystal formation. However, in cryobiology this definition is mainly reserved for the other major group of cryopreservation methods, where the whole sample (including both the extracellular and intracellular solutions) vitrifies.

Cryopreservation by Vitrification

The first paper describing a successful vitrification method for cryopreservation of a biological sample, namely renal tissue, was published by Fahy et al. in 1984.[1] The first application of vitrification in embryology, namely in mice, ensued approximately a decade after traditional slow freezing.[2] However, in spite of the almost immediate success achieved in domestic animal embryology as well,[3] it was regarded for long as a curiosity developed by experimental embryologists, without any perspectives for practical application. The frighteningly high cryoprotectant concentrations that were required by early vitrification protocols disturbed practitioners, and the industry was anything but enthusiastic to propagate a method that could be performed without any investment. On the other hand, vitrification in these years could not achieve impressive breakthroughs in other important areas, including cryopreservation of human and domestic animal oocytes, early embryos in domestic species, and transferable stage porcine embryos. Eventually the introduction of the high-rate cooling and warming vitrification methods have indeed proved the superiority of this approach, although more than a decade elapsed from the discovery to the acceptance; from the mid 1990s until today.

When discussing the benefits of vitrification, the first and most unquestionable consideration is the total elimination of the major source of injuries at cryopreservation, i.e. that of ice crystal formation. However, to ensure this benefit, technically difficult and potentially harmful conditions have to be established: a radical increase of both the cooling rates and the concentration of cryoprotectants is required. The higher the cooling rate, the lower the required cryoprotectant concentration, and vice versa. The art of establishment of an efficient vitrification method is to find a balance, i.e. to keep the toxic osmotic injury as low as possible while safely providing the required speed of cooling and warming.

Efforts to Improve Vitrification Results

There are several approaches to keeping toxic and osmotic injury low, including application of cryoprotectants with low toxicity and high permeability, to use a mixture of two or more cryoprotectants to decrease the specific toxicity of cryoprotectants. Application of both permeable and non-permeable cryoprotectants, stepwise addition of cryoprotectants, in an increasing concentration to the solutions, and to decrease the temperature when the oocytes and embryos are exposed to the concentrated, final vitrification solution.[4–7] Except for the last strategy (that may be beneficial in several situations, but may also increase the risk of chilling injury, see below), almost all listed approaches are now an indispensable part of a successful vitrification methodology.

However, even the best cryoprotectants and the most sophisticated strategies in combining and providing them may be insufficient if the final required concentration proves too high. As mentioned above, according to our current knowledge the only practical approach to keep this concentration on a level that is tolerable for oocytes and embryos is by increasing in the cooling and warming rates. Traditional tools of cryopreservation are insufficient for this purpose: the maximum achievable cooling rate with direct plunging of a standard 0.25ml insemination straw into liquid nitrogen is 2,500ºC/minute[8] and even lower in cryovials, which are manufactured to contain 1 or 2ml of solution.

The main principles of approaches focusing on high-speed cooling and warming are extremely simple. The two obvious possibilities to increase cooling and warming rates are to decrease the volume of the solution and to minimize or completely eliminate the thermoinsulating layer that separates the solution containing the sample from the cooling substance, in most cases liquid nitrogen. Apart from the high cooling rate, the lower volume of the solution offers another benefit: it decreases the chances of heterogeneous ice formation[4] consequently provides the opportunity to decrease further the cryoprotectant concentration required for safe and complete vitrification of the sample.

There were several attempts for practical realization of this strategy, including dropping the sample-containing cryoprotectant solution directly into liquid nitrogen,[9–13] copper electron microscopic grids as carrier tools to hold the sample,[14–18] the Cryoloop originally created for crystallographic experiments,[19–23] and the first purpose-developed tool— the open pulled straw (OPS) technique[24] with all its later subclones, i.e. glass micropipettes (GMP),[25] super-finely pulled OPS (SOPS),[26] gel-loading tips,[27] sterile stripper tip,[28] flexipet denuding pipettes (FDP),[29] fine diameter plastic micropipettes,[30] 100µl pipetting tip,[31] etc. The principles in all these latter devices are the same: a narrow, thin-walled plastic capillary that is usually filled with a tiny (<1µl) amount of solution containing the sample by using the capillary effect, and direct immersion of the tool into liquid nitrogen. The achievable cooling and warming rates with these tools may be as high as 20,000ºC per minute, higher with almost an order of magnitude than the limits of the standard 0.25ml insemination straw. Other versions of the original OPS idea including the closed pulled straws (CPS),[32] sealed open pulled straws,[33] and the more professional and sophisticated form—the Cryotip system[34]—which hermetically isolates the sample from the liquid nitrogen, consequently eliminating the danger of cross contamination (see below). However, this modification resulted in a decrease in the cooling and especially the warming rates, postponed the start of dilution of the cryoprotectnts, and resulted in compromised efficiency of the technique in the case of certain sensitive biological materials, including, for example, human oocytes (Kuwayama, personal communication).

Another group of techniques has also been developed stepwise during the past 15 years using a common principle but entirely different tools. First described by Arav[35] as the minimum drop size (MDS) technique, a very small (<0.5 or even 0.1µl) droplet containing the sample is placed onto a solid surface and immersed into liquid nitrogen. Depending on the tool on which the drop is placed, different vitrification methods have been developed including the minimum volume cooling (MVC),[36] the hemi-straw system,[37] or the latest and probably most practical approach, the Cryotop technique.[39,38,39] The Cryotop method has been found to be efficient for vitrification of human oocytes and blastocysts.

Apart from the small volume and/or direct-contact approach, there have some other attempts described to increase the cooling rates. A logical approach is the elimination of the vapor coat that arises around the sample in the liquid nitrogen at cooling, for example, by using liquid nitrogen slush instead of pure liquid nitrogen for cooling (VitMaster)[40–42] or to place small drops on pre-cooled metal surfaces instead of liquid nitrogen for cooling. (Cryohook CMV, Cryologic, Australia).[43]

Decreased Chilling Injury

The other unquestionable benefit of vitrification is the possibility to dramatically decrease chilling injury, i.e. a type of damage with rather undefined mechanism that destroys for example cytoplasmic lipid droplets, lipid containing membranes, and microtubules. While the damage of the microtubules may be reversible, lipid droplets cannot recover from the injury and the structural change results in death of the cell (in spite of the fact that these lipid droplets are not required for development of oocytes and embryos). Compared with slow-rate freezing, at vitrification the rate samples pass through the dangerous chilling temperature zones are extremely high, and the short exposure period radically decreases the injury of the sensitive structures. Accordingly, the only successful strategy that is currently available for cryopreservation of intact porcine embryos (containing abundant amounts of chilling-sensitive lipid droplets) is the vitrification method.[44,45]

The Potential Danger of Disease Transmission

According to our knowledge, no infection related to liquid nitrogen-mediated transmission of infective agents has been described after application of embryo technologies including all forms of traditional

slow-freezing, cryopreservation, or vitrification, although these techniques are applied in huge quantity worldwide in humans and in domestic or experimental animal fields. However, the most emphasized argument against the use of new and efficient vitrification techniques is the potential risk of liquid nitrogen-mediated disease transmission. The subject was discussed in detail in a recent reviews,[46] here we summarize the arguments and facts to give an authentic picture about the real risks.

The potential of infection in current reproductive techniques is inherent, as semen and embryo collection protocols are not sterile procedures.[47] Consequently, the contents of virtually all stored straws and cryovials may be sources of infection when transferred to recipients. In the case of an infection occuring in the future, it may be difficult to localize the source: infection prior to cryopreservation or cross-contamination during storage.

On the other hand, in embryology practice there are many other sources for contaminating liquid nitrogen. In the everyday work, the surface of straws, cryovials, racks, and other tools are not handled fully aseptically. According to our knowledge, the systematic and regular cleaning of containers and samples in liquid nitrogen containers is not part of routine practice in any embryology laboratory, and it seems to be technically very demanding, maybe impossible to achieve. Moreover, seemingly sterile containers may not be as safe as supposed, infection may occur through incomplete sealing, and even via the pores of the plastic walls of most commonly used straws (except for some special ones produced to eliminate this problem; for example, CBS straws [IMV, France]). As the result, all storage tanks may contain a number of potentially pathogenic environmental microorganisms.[47]

Liquid nitrogen-mediated disease transmission in other areas of human medicine or food industry is a documented fact, although a very rare event.[48–50] Also, the potential of transfer of infectious agents between open tools used for vitrification has been proved under experimental conditions.[51]

Accordingly, the theoretical danger of liquid nitrogen-mediated disease transfer exists, although it is not restricted to open vitrification methods, as most traditional tools and methods of cryopreservation (probably with a lower level) may be vulnerable as well.

However, it should be noted that the few published disease transmissions happened between blood specimens and carcasses, between volumes approximately 103 to 104 larger than samples in embryology, as the result of huge leaks in the wrapping or because of no wrapping at all. Due to the fact that infection following embryo transfer (with or without cryopreservation) is a rare event, this implies that the oviduct and uterus has an appropriate defence system to eliminate infectious agents in the quantity that may be transferred during routine embryo transfer procedures.

On the other hand, of course our duty is to use systems that are resistant even to the theoretically existing hazards. In the past few years, a considerable effort has been performed in this direction, and as the result of the advancements many vitrification techniques offer now equally high or even a higher level of aseptic handling of samples than common slow freezing procedures.

One approach is to separate the cooling and warming phase from storage: to use a relatively low amount of clean liquid nitrogen for cooling, then to wrap the sample into a pre-cooled sterile container and seal it hermetically before placing it into the common storage tanks. Liquid nitrogen that is low or free of infective agents can be obtained by some producers, or can also be obtained by ultraviolet (UV) irradiation[40,52] and even by filtration through some types 0.2μm filters.[53] Although the latter manipulation does not remove viral particles from the solution, the same is also applicable to most filter-sterilized media. The likelihood of factory-derived, separately stored liquid nitrogen to contain pathogenic viruses is negligible. The effectiveness of UV sterilization may depend on the actual situation (volume, thickness, intensity, wavelength, and duration of irradiation) and can only be regarded as reliable under standardized and controlled conditions.

The choice of storage container depends on the vitrification tool. For the OPS analogues as well as for Cryotop and some other tools the best solution is probably to use large (0.5 or 1ml) plastic straws produced from materials impermeable to any pathogenic agents.[29,53–56] For safe loading, commercial kits are available including the VitSet produced by Minitube. Cryoloop is equipped with a special cryovial, and other tools including electron microscopic grids can also be stored in common cryovials, but for safe application, the instructions from producers should be strictly followed. The same is applicable for approaches where the cooling is performed on metal surfaces (solid-surface vitrification [SSV] technique) or by dropping the sample into the liquid nitrogen.

The application of a sterile container for storage of vitrified samples may offer another benefit as well. Compared with the samples frozen by using the slow rate method, or vitrified in 0.25ml insemination straws, the small size of samples in recent vitrification methods makes them extremely fragile to temporary warming including transfer of the sample from one container to another. The wrapping applied for sterility measures may also serve as a buffer to avoid accidental damages caused by such transitional warmings. Another possibility is the storage of open devices in the vapor phase of liquid nitrogen.[57] Special containers designed for the purpose are available, and although the price is higher than that of common dewars, the extreme storage capacity compensates the extra expenses. The safety of this storage still has to be proved, as there was a reported fungal transmission through liquid nitrogen vapor.[58]

Achievements

Ovarian Tissue Cryopreservation

One of the most recently emerging techniques of assisted reproductive treatment is cryopreservation of ovarian tissue. Although initially developed in the mouse and sheep models,[59–61] it has the potential to have enormous impact on human fertility preservation, especially for patients with diseases requiring fertility-diminishing treatments, such as cancer, autoimmune diseases, and other indications that require bone marrow transplants or oophorectomies.[62] Furthermore, it is unique in allowing fertility preservation in adolescent patients prior to puberty, where oocyte cryopreservation is not an option.[63]

One of the major advantages of ovarian tissue cryopreservation is that it can be performed immediately, while alternatives, such as oocyte or

embryo cryopreservation, require two to four weeks of stimulation prior to retrieval, which may not be possible when immediate disease treatment is necessary. Primordial follicles are more resistant to cytotoxic cryoprotectants and generally survive cryopreservation better than larger, later-stage follicles.[64] Tissue can be transplanted either heterotopically (subcutaneously or under the abdominal wall) or orthotopically (into the remaining ovary or into the pelvic perinoteum). Resumption of tissue function and oogenesis can be assessed by hormone levels, menstrual cyclicity, and ultrasonography, which is usually first detectable as early as eight to 10 weeks after transplantation.[65,66] Patients with heterotopic transplants have to undergo oocyte aspiration and *in vitro* fertilization (IVF), whereas those that had orthotopic transplants may conceive spontaneously. Alternatively, the transplanted tissue my serve exclusively to re-initiate cyclicity, and not contribute the oocyte(s) to be fertilized.[67]

The first cryopreserved ovarian transplant that resulted in a live birth was reported in 2004 after spontaneous conception,[68] although the source of the oocyte was questioned.[69] Since then, at the time of writing, 12 clinical pregnancies, 12 live births, two biochemical pregnancies and two miscarriages have been reported.[66–68,70–76] It should be noted that the oocyte source varies within these publications, and in some cases the transplanted ovarian tissue was merely the source of endocrine cycle restoration. Twin births were reported after vitrification of oocytes obtained from such grafts, making fertility preservation for these patients another possibility.[75] One of the critical questions is how long the function of the transplanted tissue is maintained, and at present there is not much information exist on it except a report from Silber and colleagues that gives cause for cautious optimism.[77]

As the techniques become optimized and practitioners and patients become more comfortable with the procedures, the overall utilization of this new technology will increase exponentially, possibly making it one of the most common reproductive treatments. It is not hard to imagine that ovarian tissue cryopreservation and transplantation will become part of routine treatments of various diseases.

Oocyte and Embryo Cryopreservation

The vast majority of the approximately 800 publications dealing with this area finds vitrification more efficient than traditional slow-rate freezing, especially for oocytes, but also for embryos (and it is expected to be more efficient for ovarian tissue cryopreservation as well). Vitrification has proved its superiority even in areas where traditional slow-rate freezing was successful, i.e. cryopreservation of bovine blastocysts and morulae or early cleavage stage human embryos; and has resulted in impressing breakthroughs in cryopreservation of early stage bovine embryos, bovine mature and immature oocytes,[24,78] and cloned domestic animal embryos.[79,80] Probably the most significant success has happened in the field of cryopreservation of human human blastocyst, human oocytes,[35,81] and human embryonic stem cells.[82] For day three-stage embryos, Desai and colleagues[83] reported 270 transfers with clinical pregnancy and implantation rates for patients under 38 years of age (n=200) of 45 and 24%, respectively. Corresponding rates in patients 38–42 years of age were 29 and 13% (n=70) and no congenital malformation of newborns were observed. For day five-stage embryos (blastocyst), there are several publications showing the high efficiency of vitrification, also for embryos that were biopsied prior to cryopreservation[21,84–87] achieving significantly improved results compared to slow-freezing (survival and pregnancy rates of 83 and 17% by slow rate freezing and 100 and 50% by vitrification)[88] and no adverse effects have been associated so far.[89] Although vitrification proved to be more efficient for embryo cryopreservation for all stages of development, the power of this technique is clearly shown when comes to oocyte cryopreservation. For the first two decades, oocyte freezing had extreme low efficiency using slow-rate cooling (it has been estimated to require between 20 and 100 oocytes to achieve a pregnancy).[90] Only after the introduction of vitrification in human[91] when interest turned to this technique and recently was reported that consistent high survival rates of oocyte are possibly achieved.[28,38,39] Indications for oocyte cryopreservation using the highly efficient vitrification have been extended and include donor oocyte banking, ethical/moral egg freezing (instead of embryo freezing for IVF patients); 'rescue' egg freezing (when no sperm obtained on the day of oocyte collection), social reasons for oocyte freezing besides medical indications for egg cryopreservation.[92] Current studies demonstrate that efficiency of vitrification can be compared with results of using fresh oocytes in terms of fertilization and embryo development and pregnancy rates.[93,94] When the highest quality oocytes were used, obtained from egg donors, outstanding 55% implantation and 75% clinical pregnancy rates were reported by Nagy and colleagues.[92] Cobo and colleagues have also confirmed the efficiency of oocyte vitrification for donor egg banking both at the level of fertilization and embryo development and at the level of pregnancy outcomes.[81,95] The excellent vitrification outcomes described above are performed using mature (metaphase-II) oocytes; at the same time, some studies are evaluating the possibility to use immature (and optionally *in vitro* matured) oocytes for cryopreservation with vitrification as well.[96,97] Vitrification as a technique proved to be much more 'powerful' that it demonstrated excellent survival, even for those embryos that originated from vitrified oocytes. In these cases, the 'double vitrification' (once at the M2 oocyte stage and secondly at the blastocyst stage) seemed to have no impact on viability of the oocyte/embryo, which demonstrates the efficiency of this technique.[98] Proving the efficiency of a newly applied technique, such as vitrification is essential; however, it is equally critical to demonstrate the safety of the technique as well. So far, infants born after oocyte cryopreservation, and specifically from vitrification, have not shown any higher incidence of congenital malformations,[97,99] which emphasizes that vitrification is not only efficient but also safe.

Future Tasks

One may regard this achievement as a great triumph; however, on closer inspection of this seemingly inventive area, the production of various vitrification tools is not an overwhelmping success. With all our efforts and all our creativity, these tools are rather 'primitive'. Even with rapidly increased interest, vitrification has failed to meet the basic requirement of a medical technology, including standardization, complexity, practicality, legal approval, and widespread public acceptance. In a broader sense, this handicap is typical for the whole mammalian embryology: our tools and approaches do not differ much from those applied 25 to 30 years ago, the rate of advancement is simply miserable if we compare it with the skyrocket development of other branches in biology including applied genetics and molecular biology. To meet the

requirement of the second decade of our millennium, we have to quickly eliminate these formal errors while maintaining or rather increasing the great value of vitrification, the high overall efficiency.

Conclusion

Vitrification is often referred to as a new technology, although it was first applied successfully in embryology more than 20 years ago. After a long lag phase, an exponential growth of application both in animal and human embryology has been recently observed. This impressive spreading of the technology is due to technical improvements, and also the fact that vitrification may be used efficiently for cryopreservation of samples that have low survival rates after traditional slow freezing, for example, *in vitro* produced embryos of domestic animals, or human oocytes and blastocysts. Currently, as usual in emerging technologies, numerous diverse tools and technologies have been developed for application in vitrification, with varying individual benefits and disadvantages. A predictable tendency for the future evolution is a standardization of the technology based on merging of the simplest, safest, and most efficient procedures. A realistic perspective is that—based on this standardization—vitrification will replace traditional slow-rate freezing for the cryopreservation of oocytes and all stages of embryos of mammals, including both domestic and experimental animals and humans. ■

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