The Efficacy and Safety of Human Oocyte Vitrification

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ABSTRACT

Vitrification is now a widely applied and highly successful approach for cryopreservation in reproductive biology. Rapidly increasing data prove that it is also a highly efficient technique for low-temperature storage of human oocytes. The latest approaches with appropriately selected cryoprotectants, tools and techniques, and properly adjusted parameters allow close to 100% morphological survival rates, and in vitro embryo development, as well pregnancy and implantation rates, comparable with those achieved with fresh oocytes. With standardization of the technique and elimination of biosafety problems by preserving all the positive features, vitrification may become a common part of the everyday routine in a human embryo laboratory, and it may offer a solution for various medical and social situations as well as for simple logistic problems commonly occurring in assisted reproduction.

KEYWORDS: Cryopreservation, sterile, DMSO, oocyte, vitrification

Cryopreservation of human gametes and preimplantation stage embryos has become an essential part of assisted reproduction treatment procedures. The first success was achieved with spermatozoa,[1] then with embryos,[2] and finally with oocytes.[3] However, in contrast with the former two, cryopreservation of oocytes has remained inefficient for a long time and was regarded as the greatest challenge of human reproductive cryobiology in the first decade of the 21st century.

At the end of the decade, we may say that the goal to cryopreserve oocytes efficiently has been accomplished. With significant improvement of traditional freezing techniques[4] and especially with the introduction of ultrarapid vitrification methods,[5] we may say that cryopreservation (using vitrification) of mature human oocytes approaches the required overall efficiency (i.e., the outcome after intracytoplasmic sperm injection, embryo culture, and transfer) does not seem to be significantly different from that achievable with fresh oocytes. There are still some remaining challenges, including replacement of some constituents of vitrification media as well as biosafety issues, but these are technical details, and the solutions for these problems have been outlined recently. The future required step is the standardization of a safe and highly efficient vitrification protocol. This is a now a realistic prospect, and widespread application of a safe vitrification technique for human oocyte cryopreservation is foreseeable in the next 5 years.

The purpose of our work is to summarize briefly the road that has led to this success, discuss recent achievements worldwide, and deal with medical, social,

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and other indications of oocyte cryopreservation where the new techniques have resulted in breakthroughs or they are expected to do so in the near future. Finally, we outline the efforts to make ultrarapid vitrification and storage safe and free of potential contamination.

ESTABLISHMENT OF AN EFFICIENT VITRIFICATION TECHNIQUE FOR HUMAN OOCYTES

The first successful application of vitrification (glasslike transformation of the whole medium containing the sample) of mammalian embryology was reported by Rall and Fahy.[6] Subsequent efforts to improve the technology can be divided into four partially overlapping phases: (1) to find the optimal solutions for equilibration, cooling, warming, and rehydration; (2) to establish tools and techniques to ensure extremely high cooling and warming rates under simple conditions; (3) to fine-tune parameters (concentrations of cryoprotectants; times and temperatures for equilibration and dilution); and (4) to exclude possibilities for disease transmission during cooling, storage, and warming.

Media, Cryoprotectants, and Supplements

Based on the early experiments in mouse and domestic animal embryos, it has been revealed that media for cryopreservation should consist of at least one permeable and one nonpermeable cryoprotectant, dissolved in a phosphate or rather N-[2-hydroxyethyl] piperazine N′-[2-ethanesulfonic acid] (HEPES)-buffered holding medium, preferably supplemented with proteins (bovine serum albumin, serum, or egg yolk). Further research has indicated that the use of two permeable cryoprotectants instead of one is advisable to decrease the specific toxicity of each one. This is important because under the circumstances achievable in an average embryology laboratory, vitrification requires high concentrations of permeable cryoprotectants.

Due to its high permeability, low toxicity, and other only partially understood characteristics, ethylene glycol has become a standard constituent of vitrification media. Regarding the other permeable cryoprotectant, dimethylsulfoxide (DMSO) is the most common choice because the 1:1 mixture of DMSO and ethylene glycol is more permeable than any of the two components alone and excellent survival and developmental rates are achieved.

However, the general evaluation of DMSO is very controversial. It is a free radical scavenger and a very potent solvent, has topical anti-inflammatory and analgesic properties, and it is also used in the cosmetic industry. The Food and Drug Administration has approved it for local palliative treatment of interstitial cystitis. Its potential mutagenic effect is much disputed, and no definite conclusion has been obtained; some effects may be related to trace contaminations dissolved in DMSO. Very recent results have shown, however, that DMSO induces widespread apoptosis in the brain of adolescent mice. Damage was also observed in rat hippocampal preparations.[7] Although this new observation still requires confirmation, and, in spite of the widespread use, no report has supported the harmful effect of DMSO in mammalian embryology, efforts seem to be justified to find an appropriate substitute for DMSO. It will be a difficult task because a recent study has demonstrated the unique membrane-protective effect of DMSO during cryopreservation.[8] Previous efforts to use propylene glycol together with ethylene glycol for the vitrification of mouse oocytes found that the successful application may also require modification of the holding media.[9]

As a nonpermeable cryoprotectant, trehalose was demonstrated in many publications as superior to sucrose. However, the difference was usually minimal, and in practical use, sucrose is the most common component.

To increase consistency and reliability of cryopreservation, and also to decrease the risk of contamination, defined components are the preferred substitutes of holding media. However, in spite of the widespread efforts in this field, results achieved with semidefined serum albumin or undefined sera are usually superior to polyvinyl alcohol or other synthetic macromolecules. Unfortunately, some widely applied and quite successful synthetic serum substitute components of vitrification media—in spite of the somewhat misleading name—also contain undefined elements.

Tools and Methods Developed for Ultrarapid Vitrification

Although increasing the cooling and warming rates to improve vitrification results was also a widely known possibility in the first decade of mammalian embryo vitrification, the first tools and techniques developed for the purpose only occurred in the second half of the 1990s. These included the application of electron microscopic grids, open pulled straws and its multiple derivatives, and the carrier tools developed for the minimum drop method (see review by Vajta and Nagy[5]). Widespread application of these tools for various purposes has demonstrated that the simplest technique ensuring consistently high cooling-warming rates and reliable results are the Cryotop (Kitazato Biopharma, Japan) and its analogues McGill Cryoleaf (Medicult, Denmark), Cryolock (Biodiseno, Columbia), etc. These devices consist of a thin film strip connected to a simple handle, and they are also equipped with a non-airtight cap to protect the filmstrip. Less than 1 μl of the final vitrification solution containing the sample is loaded on the filmstrip, and then most of the solution is removed, leaving only a thin layer of solution

to cover the sample, and eventually the filmstrip is quickly submerged in the liquid nitrogen. At warming, the device is removed from the liquid nitrogen and the filmstrip is immediately submerged in the rehydration medium containing an osmotic buffer, usually sucrose. The achievable cooling and warming rates are far over 20,000°C/minute and seem to be appropriate to minimize the chilling injury, a mostly undefined irreversible change to which human oocytes are especially prone.

Adjusting Parameters

The increased cooling and warming rates have also allowed the use of less concentrated cryoprotectant solutions without compromising the efficiency of vitrification. This stepwise adjustment procedure has eventually led to a 40% decrease (25 to 15%) of both DMSO and ethylene glycol and has contributed significantly to the increased survival and developmental rates. Equilibration times have also been changed. Whereas the earlier approach was to keep exposure to cryoprotectants at the lowest possible level, the new methods try to ensure proper protection of all part of the oocytes, exposing them for long periods (5 to 15 minutes) in a diluted solution of cryoprotectants. To further decrease toxicity, the temperature at both equilibration steps has been decreased from 37°C to 25 to 26°C. *Note:* We discuss biosafety issues later.

RECENT ACHIEVEMENTS IN HUMAN OOCYTE VITRIFICATION WORLDWIDE

Although infants were reported after both slow-rate freezing and vitrification in the last century,[4,10,11] the real breakthrough happened after the millennium. Traditional freezing has reached the level required for practical application and become widely used in Italy, where cryopreservation of embryos is illegal.[12,13] Vitrification, in contrast, has made a breakthrough with the publication of achievements using the Cryotop procedure. The number of infants born after oocyte vitrification shows a very strange distribution between countries (Table 1). Whereas no or very few births were reported in countries with long traditions and a reputation in assisted reproduction (including Denmark, Iceland, Israel, Australia, many western, northern, and middle European countries) and the numbers are also very low in the United States, in some Latin American countries, most of all Colombia and Mexico, the numbers are surprisingly high and growing rapidly. Venezuela, Brazil, and Chile seem ready to join the list very soon. In Japan, the restrictions regarding oocyte donation hamper rapid advancement. Spain is at present the only European country where oocyte vitrification is performed on a large scale and with excellent efficiency, but there are a growing number of pregnancies and infants in Italy as well.

Typically, the method spreads almost accidentally, by personal contact, and the application is restricted to a very few clinics. Apart from the legal restrictions, a conservative approach and/or frustrating experiences related to the selection of the wrong procedure or inappropriate application can be blamed for the fact that oocyte vitrification has become the privilege of a handful of groups. However, in the next few years, a radical change and an exponential growth in the number of infants born and the clinics applying oocyte vitrification are expected.

Table 1 Estimated Number of Infants Born after Oocyte Vitrification

Country	Approximate Number of Births
Canada	35–40
Colombia	200
Italy	10–20
Japan	40–60
Mexico	100
Spain	40–50
United States	35–40

REASONS FOR HUMAN OOCYTE CRYOPRESERVATION

Oocyte cryopreservation may be required for medical reasons to maintain fertility in young patients if their disease requires systemic anticancer or other gonadotoxic treatment.[14–17] In the United States alone, more than half a million women each year are diagnosed with invasive cancer. About 50,000 of these women are of reproductive age, and many of them have not yet established families or had children.[18] For all these reasons, fertility preservation is clearly indicated.[19] Bilateral ovariectomy or irradiation of the region for any other reason also may necessitate oocyte cryopreservation.[20] An alternative approach is the low-temperature storage of ovarian tissue. Because both methods can be categorized as innovative techniques, the actual situation including the disease, the treatment, and the available preservation methods determines the choice. In the optimal case, both oocyte and ovary tissue cryopreservation are suggested. Other medical reasons include assisted reproduction of patients with polycystic ovarian and ovary hyperstimulation syndromes.[20–22] Poor responders to ovarian stimulation as well as patients with the risk of ovarian function loss through premature menopause are also candidates for oocyte cryopreservation.[20] Most recently, a young woman with severe and symptomatic endometriosis emerged as a new indication for fertility preservation.[23] Problems with the availability of sperm may also require oocyte vitrification, for example in cases of male factor infertility, difficulty

with sperm collection, inadequate seminal samples, or nonviable spermatozoa at the time of oocyte retrieval.[20] Very special medical situations may also necessitate oocyte storage (e.g., preservation of a mother's oocytes for her daughter with Turner syndrome).[24]

Nonmedical conditions include the ethical concerns and legal restrictions associated with embryo cryopreservation in several countries (e.g., Italy or Germany[25]). Another reason that has created contention both among professionals and the general population is the wish to delay motherhood for various reasons, including career demands or the lack of an appropriate partner.[26,27] Oocyte cryopreservation can also be used for regular in vitro fertilization (IVF) patients requiring infertility treatment, if couples have a larger number of oocytes retrieved and if they have moral or ethical problems with embryo freezing. In this case, only a limited number of oocytes may be inseminated, and all other oocytes can be cryopreserved for future use. Oocyte cryopreservation today may also be used in connection with in vitro maturation when immature oocytes are obtained from IVF patients (purposely or as an addition to in vivo matured oocytes[28,29]). In some clinics, the overall efficiency of oocyte cryopreservation has reached a such a level that it is considered a routine replacement for embryo cryopreservation.[30]

However, by far the most important reason for oocyte cryopreservation today is the oocyte donation because storage offers unique flexibility in time and location. In the foreseeable future, human oocyte banking will compete with sperm banking in this area with all the social and possible financial consequences.[30]

SAFETY PROBLEMS

Most microorganisms survive low temperature including deep cooling to −196°C. The addition of cryoprotectants, sera, and other components to the vitrification medium can support this survival. Open systems or damaged containers are constant potential sources of such infections. The other, frequently disregarded mechanism that infects liquid nitrogen is the surface of these containers because none of the existing cryopreservation methods in reproductive biology maintain all parts of the cryotool completely sterile. Although microorganisms do not proliferate in liquid nitrogen, their number and concentration slowly increases. Periodic sterilization of liquid nitrogen dewars is not part of the usual safety measures in an embryo laboratory, so accordingly the danger of an accumulation of pathogenic agents increases with the passing years. Fortunately, most microbes found in old containers are ubiquitous environmental organisms with low or no pathogenic effect.

The possibility of transferring pathogenic agents via liquid nitrogen was first demonstrated experimentally by Piasecka-Serafin[31] between infected and sterile semen samples. In 1995, leaky containers of bone marrow transplants caused transmission of hepatitis B virus infection.[32] Eventually Bielanski et al[33] demonstrated the transmission of pathogenic viruses experimentally through liquid nitrogen between two open vitrification containers containing embryos. Although no infection in human or domestic animal embryology attributed to liquid nitrogen–mediated transmission has yet been reported, the potential danger exists and should be eliminated.

Unfortunately, there is no practical alternative to the immersion of minimal volume solutions containing the sample into liquid nitrogen to achieve the extremely high cooling rates required for efficient and reliable oocyte vitrification. The unavoidable direct contact between liquid nitrogen and the solution may create concerns regarding contamination during cooling and especially during storage. Attempts to perform vitrification in a hermetically closed system, including sealed thin straws[34] or wrapping the open system into a sealed straw before immersion in liquid nitrogen,[35–37] were found to be successful in the case of pronuclear and cleavage-stage embryos; however, to our knowledge there is no report about a completely closed vitrification technique that approaches the efficiency of the minimum volume open methods including the Cryotop technique. No mechanical protection of the sample (e.g., those on Cryotop or Cryolock) can be regarded as leakproof, considering the very rapid temperature changes causing deformations in devices and the extreme expansion or shrinkage of the entrapped gas volumes. Alternative approaches, including solid surface vitrification and its commercially available version, the Cryohook technique, were not reported to be efficient for human oocyte vitrification.

Unfortunately, this simple technical problem has developed into a major issue. It has been the subject of numerous verbal and written debates on various scientific forums, and it hampers successful application of vitrification of oocytes in many countries worldwide. However, with a little modification of the technology, the problem can be resolved without requiring any compromise in the cooling and warming rates and in the overall efficiency of the cryopreservation. The principles of this approach have been described by Vajta et al[38] and can be used with slight modifications for the latest and most efficient cryotools as well.

The principle of the solution is to separate the cooling, storage, and warming phases. Cooling can be performed in factory-derived pathogen-free liquid nitrogen by using the common methods of ultrarapid vitrification. Once the carrier tool and the sample reaches the required low temperature, it can be wrapped in a precooled sterile container (e.g., a large CBS [IMV, France] straw), heat sealed, and stored in nonsterile

liquid nitrogen in dewars, together with other samples. At warming, the end of the CBS straw can be cut while the rest of the straw containing the vitrification tool and the sample is still immersed in the liquid nitrogen. Then the tool can be removed with a forceps and quickly immersed in the warming solution. By applying this method, the possibility of cross-contamination between samples can be completely eliminated. The safety of this procedure was confirmed in 2007 by an independent investigation performed by the same group who warned embryologists about the possible danger of disease transmission when using open vitrification methods.[39] The method has been successfully applied recently for human embryos producing pregnancies after vitrification in the eight-cell, or blastocyst, stages (Conceicao et al, unpublished data).

A source of pathogen-free liquid nitrogen used for cooling is a typical concern among embryologists. Airborne contaminants may occur in factory-derived liquid nitrogen; however, human pathogen viruses (hepatitis, herpes, and human immunodeficiency virus) are not airborne. Bacteria and fungi can be eliminated by a simple sterile filtration with disposable filtration units precooled in a large foam box and using the pressure caused by the slow evaporation of liquid nitrogen to force the rest to pass through the filter to the lower chamber, as described earlier.[38] This simple procedure is suitable to produce liters of completely sterile liquid nitrogen in several minutes, generously covering the daily needs of a common laboratory. The applicability of sterile filtration to decontaminate liquid nitrogen has also been confirmed by McBurnie and Bardo.[40] Sterilization of the containers used for pathogen-free liquid nitrogen can be performed by using a diluted solution of common bleach, and the cooling and warming should be performed under a laminar hood.

These simple and inexpensive precautions provide better safety than simple sterile filtration of water-based media and with an order of magnitude higher than common surgical masks in an operation theater. However, to eliminate the possibility of infection created by direct contact with liquid nitrogen entirely, further possibilities include finding a source for sterile liquid nitrogen or producing sterile liquid nitrogen in the laboratory by purchasing commercially available units for the purpose, for example from Veriseq SLG (Linde Gases Division, Pullach, Germany).

An alternative solution for safe vitrification was also suggested by using the vapor of liquid nitrogen for both cooling and storage.[41] However, the applicability of the method for human oocytes requires further studies because the cooling rate may be compromised without direct contact to liquid nitrogen, even by using the Cryoloop technique. For the long-term storage in the vapor phase, a safe solution without the danger of temperature fluctuation and accidental warming may require a considerable investment. From a disease transmission point, alerting data have been published by Fountain et al,[42] proving the presence of waterborne bacteria and fungi in the vapor phase above liquid nitrogen. Considering these data and other, still anecdotal information, caution and future investigations are required to investigate the safety of the vapor phase of recently introduced dry containers from the point of cross-contamination.

CONCLUSION

Recently introduced ultrarapid vitrification techniques have been found to be extremely efficient for the cryopreservation of human oocytes. Survival rates are close to 100%, and in vitro/in vivo developmental rates are similar to or identical with those achieved with fresh oocytes. This efficiency opens the way to the widespread application of the technique in various medical, legal, and social situations, even to replace embryo cryopreservation with the storage of oocytes. Disease transmission problems commonly associated with ultrarapid vitrification can be eliminated by using the proper techniques, without compromising the cooling and warming rates indispensable to maintain high efficiency. However, very recent data suggest caution in the selection of the appropriate cryoprotectant additives to avoid potential long-term consequences.

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