Review

Recent developments in human oocyte, embryo and blastocyst vitrification: where are we now?

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Abstract

The target of any cryopreservation procedure should be to ensure high survival rates of living cells after thawing. Two important parameters determine the success of any cryopreservation protocol: the manner in which cells regain equilibrium in response to cooling, and the speed of freezing (cooling rate). Slow-rate freezing protocols result in the formation of ice crystals during cooling and warming. Vitrification, in which high cooling rates in combination with a high concentration of cryoprotectant are used, does not produce any ice crystals during cooling and warming. However, there is a practical limit to the attainable cooling speed, and also a biological limit to the concentration of cryoprotectant tolerated by the cells during vitrification. Although post-warming survival depends on the species, the developmental stage and the quality of the embryos being vitrified, it seems clear that vitrification methods are increasingly successful and might be a better method than slow cooling procedures in the field of cryobiology. Many of the potential problems and benefits underlying vitrification as a method of choice for embryo cryopreservation in clinical embryology will be discussed in this review.

Keywords: *cooling rate, cryoprotectant, liquid nitrogen contamination, solidification, toxicity, vitrification*

Introduction

The main principle of cryopreservation for mammalian oocytes and embryos is to reduce damage caused by intracellular ice formation. To reach this goal, it is important to consider the behaviour of intracellular water at subzero temperatures as one of the fundamental cryobiological principles. Two basic techniques have been developed: controlled slow-rate freezing protocols, and rapid freezing protocols such as vitrification. The cryopreservation of biological material includes six steps: initial exposure to cryoprotectant, cooling (slow/rapid) to subzero temperature, storage, thawing/warming, dilution and removal of the cryoprotectant, and return of the biological material to a physiological environment.

Since the first report of human pregnancy and the first live birth following cryopreservation two decades ago, thawing and transfer of embryos today is mostly done using traditional slow-rate or equilibrium freezing and thaw protocols, combined with tailored patient preparation protocols, thawed embryo cycles have become relatively successful. The transfer of cryopreserved embryos represents around 20% of all transfers worldwide. The cryopreservation for example of pronuclear stage (PN) embryos or cleavage-stage embryos are integral components of the contemporary assisted reproductive technique (ART) centre and enhance significantly the cumulative pregnancy rates from oocyte retrieval (Schroder *et al*., 2003). So far, there are few indications that progeny resulting from cryopreserved pronuclear stage embryos (PN), cleavage-stage embryos or blastocysts are in any way adversely affected.

While human PN and cleavage-stage embryo cryopreservation has become a well-established technology in ART, slow cooling is potentially inappropriate for the cryopreservation of human

oocytes, due to their chilling sensitivity and low hydraulic conductivity (L_p). As a consequence, oocyte cryopreservation, regardless of some success with this approach, is still considered experimental, and is thought to be of limited clinical use. Therefore it is still a controversial issue, especially with regard to the low efficacy of the oocyte freezing technique, the potential risks associated with oocyte freezing, and also with reported pregnancy rates similarly between using frozen oocytes (17.2%) and frozen embryos (18.7%) (Marina and Marina, 2003). Furthermore, Yoon *et al*. (2003) reported live births after oocyte vitrification. It is of some concern that freezing human oocytes could cause irreparable harm to future generations (Winston and Hardy, 2002). However, it should be mentioned that in countries like Spain, the use of cryopreserved oocytes in an IVF procedure is currently specifically forbidden by the Spanish Law of Assisted Reproduction (Pellicer and Simón, 2003), because it has to be proven that oocyte cryopreservation is safe for any resulting children. Nevertheless, cryopreservation of human oocytes may solve the legal and ethical problems linked to cleavage-stage embryo freezing in certain countries, in particular with respect to 'shared ownership' of frozen embryos. Finally, oocyte cryopreservation, if consistent and successful, may provide a wonderful opportunity to help drive IVF programmes to consider cryostorage of excess oocytes and not embryos, inseminating post-thaw/warming (with ICSI) only a limited number of oocytes at any one time, in anticipation of the need for only one or two embryos at transfer (Tucker *et al*., 2003).

Slow cooling versus rapid cooling technique

Usually, in the absence of a cryoprotective agent (CPA) mammalian cells remain unfrozen above –10°C. Even as a result of the presence of extracellular ice, supercooling of the cell interior occurs, but this disrupts equilibrium between the interior and exterior of the cell. To prevent supercooling, the vial or straw containing the cells is manually or automatically seeded, which allows for large ice crystals to grow slowly and to maintain a very delicate balance between factors, which may result in damage mostly by ice crystallization. Therefore to achieve this balance the time to complete slow cooling procedures for human oocytes and embryos requires a minimum of 90 min (**Figure 1**). Under slow cooling conditions it is possible to prevent intracellular ice formation, because during dehydration the concentration of intracellular solutes is slightly higher than outside, thus lowering the freezing point within the cell. However, it is undesirable to remove too much intracellular water because dehydration can result in an increase in the intracellular solute concentration of solutes to toxic concentrations.

Embryos can be damaged during cryopreservation by six forms of cryoinjury (Kasai *et al*., 2002): intracellular ice, chemical toxicity of the cryoprotectant, osmotic swelling, osmotic shrinkage, fracture damage, and extracellular ice. Therefore, if it were possible to deduce the mechanism of injury in cryopreserved embryos by their appearance, it would help to optimize cryopreservation protocols.

Beside the extended time of these protocols, slow cooling rate cryopreservation also requires expensive programmable freezing equipment. But more seriously, as mentioned above, slow cooling protocols have limitations for several type of cells (e.g. pig embryos, in-vitro derived bovine embryos or human metaphase II oocytes, complex cell systems such as blastocysts with their fluid filled cavity) which are chilling sensitive, since unlike all stages of preimplantation human embryos, oocytes are more vulnerable to the cryopreservation procedures involving ice crystallization. This can be explained by the reduced permeability of the cytoplasmic membranes of oocytes. In general, clinical results on oocytes and blastocyst cryopreservation have not been consistent and so controlled slow cooling rate protocols may not be the optimal technique to cryopreserve these cells. Therefore to overcome these cryobiological factors it has been a motivation for cryobiologists to employ alternative freezing protocols. Exquisite chilling sensitivity can be compensated for by very rapid cooling, as is applied in vitrification techniques, which have been described for over 100 years. Furthermore, the strategy of vitrification achieves the total elimination of ice crystal formation, both within the cytoplasm being vitrified (intracellular) and in the surrounding solution (extracellular). Therefore, for chilling sensitive cells a feasible option appears to be ultra-rapid vitrification.

The chief difference of vitrification of the cells over conventional cryopreservation procedures is that it is an 'open system' lacking any thermo-insulating layer around the specimen. However, it is important to realize that similarities exist between slow cooling and vitrification, i.e. it is important to induce a glasslike solidification intracellularly as well as extracellularly, as with balanced freezing both inside and outside of a cell when using traditional protocols. Furthermore, in case of slow cooling protocol (equilibrium) theoretically an intracellular vitrification state is obtained during the equilibrium process of cooling, because the pure water is initially removed as ice, such that the remaining solution becomes more and more concentrated until it reaches its point of vitrification, at a temperature usually referred to as the glass transition temperature (T_g) at equilibrium freezing. In the case of vitrification, theoretically the amorphous state is already reached during the exposure of the cell to the cryoprotectant solution before plunging into liquid nitrogen (LN2).

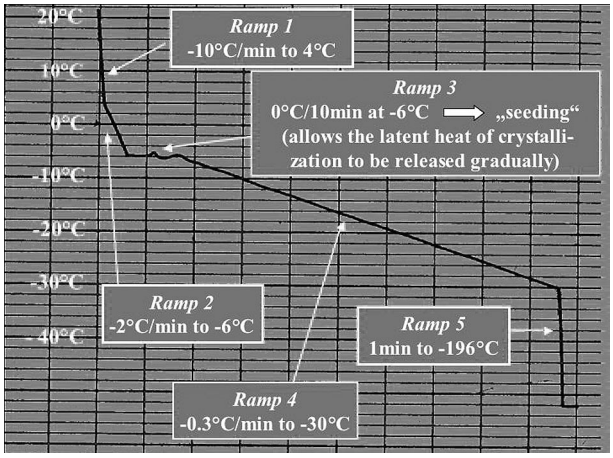

Figure 1. A graph of a typical slow freezing protocol with automatic seeding (induced ice crystal formation) at –6°C for 10 min used for human pronuclear stage embryos (PN). The freezing programme contains five temperature ramps with a total time of 98 min.

Physical background

A pioneer of cryobiology, Luyet (1937), wrote that crystallization is incompatible with living systems and should be avoided whenever possible. Thus, to preserve living organisms stably for a long period of time, it seemed to him essential to eliminate ice formation, and create instead a glass-like (vitreous) state, i.e. the organisms have to be placed into a solution or environment where the viscosity of either the intra- and extracellular water is raised to a level that results in the arrest of molecular diffusion and all chemical processes. This includes degradation and aging, which are effectively stopped. It is known that vitrification is used as a natural form of cryoprotection in some arctic plants. 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 temperature below the glass transition temperature of the solution (T_g, the temperature at which the transition to the vitreous conditions begins), not by ice crystallization (**Figure 2**), but by extreme elevation in viscosity using high cooling rates from 15,000 to 30,000°C/min.

Protocols for vitrification greatly simplify the cooling process for cells and tissue, which are placed into the cryoprotectant, and then plunged directly into LN2, and they eliminate injuries caused by extracellular ice. Otherwise the key aim of any vitrification technique and protocol is to achieve high rates of cooling in association with a small volume of high concentrations of cryoprotectant. Inevitably, this is biologically problematic and technically difficult. There is a practical limit to the attainable cooling speed, and a biological limit on the concentration of cryoprotectant tolerated by cells during vitrification. Furthermore, a critical concentration of cryoprotectant is required for vitrification itself to occur. When using the vitrification technique, it is important to achieve a balance between maximizing the cooling rate and minimizing the cryoprotective concentration, because a high concentration of cryoprotectant can lead to toxic effects of the cryoprotectant

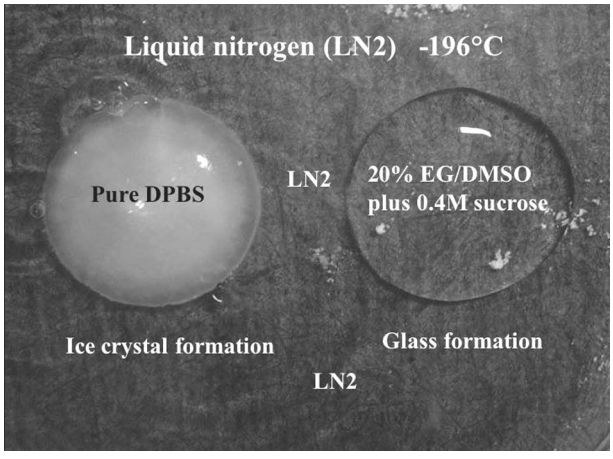

Figure 2. Vitrification is the solidification of a solution ('glass formation') without ice crystallization. Two droplets of different solutions plunged directly into liquid nitrogen: left droplet is pure Dulbecco's phosphate-buffered saline (DPBS) with ice crystallization, in contrast to the right droplet containing an equimolar combination of 20% ethylene glycol (EG) and dimethyl sulphoxide (DMSO) with 0.4 mol/l sucrose in DPBS without ice crystallization (glassy, vitrified state).

(the primary obstacle in vitrification) such as injured cells due to the toxicity of the concentrated electrolytes, fracture damage and osmotic swelling. In traditional slow cooling protocols more than 50% of embryos may be damaged physically, i.e. zona damage is relatively common feature of embryos. (Rall and Meyer, 1989; Tucker *et al*., 1995; Van den Abbeel and Van Streiteghem, 2000; Kasai *et al*., 2002). Yeoman and co-workers (2001) cryopreserved rhesus monkey blastocysts and reported that cracked zonae were occasionally seen with the slow freezing protocol but were not observed with vitrification procedure. In addition, with vitrification protocols, the incidence of physical damage is considerably lower despite ultra rapid cooling and warming. Kasai and colleagues (1992) vitrified with 40% (v/v) EG mouse and rabbit morulae, and observed an incidence of zona damage of 1.6% and 3.6% respectively, probably because of the absence of ice crystal formation. However, the direct and rapid contact with LN2 coupled with a minimal volume of the vitrification solution as much as is practical allows achieving higher cooling rates, because conductive heat transfer in liquid is much faster than in vapour. Furthermore, a positive effect of the high cooling rate is, to reduce the deleterious effects of the cryoprotectant concentration and the exposure time. In this way, the toxic effect of both is also decreased.

Sterilization of LN2 to prevent possible cross-contamination of embryos

The vitrification technique is based on direct contact between the vitrification solution containing the cryoprotectant agents and the LN2. However, this technique is still open to debate in the scientific literature concerning LN2 as a potential source of contamination. Several questions arise as to how IVF laboratories might overcome this technical difficulty. In addition, this issue becomes increasingly important because in some countries potential changes of legislation or regulations on safe storage must be complied with (e.g. in the USA increasing oversight by the Food and Drug Administration).

First, several potential routes of exposure are discussed: from a clinical point of view is there a need to sterilize LN2, and if so, then how do IVF laboratories obtain sterile LN2? Should the LN2 in Dewar flasks assumed to be contaminated? If the LN2 storage tanks are contaminated, is there a way to sterilize or decontaminate these tanks? Would a source of sterile LN2 be at all useful for filling storage tanks? How is it possible to maintain its sterility? Is there a biocompatible way to decontaminate the outside of the straws or vials or a need? What will IVF laboratories need to do to satisfy requirements of potential changes in legislation on safe storage in certain countries, or the FDA requirements for validation of cryopreservation protocols? Finally, is there a need for the validation of cryopreservation protocols, pathogen contamination of samples and assays at every step of the cryopreservation and thawing process, and if so, how practical is it?

Today there are standards of purity for LN2 that are met but the specifications for purity are chemical (e.g. labelling the product as 'ultra-high purity' or 'semiconductor grade'), not biological. Potential routes for biological contaminants may be introduced to the LN2 either at the production facility or in the laboratory via airborne mechanisms, or from contaminated materials in

leaky containers placed into the LN2 for storage. LN2 in storage vessels may become contaminated with bacteria and/or fungi. Anyhow, usually these biological contaminants are easily assayable by evaporating the LN2, and then the evaporate can be reconstituted with culture medium to grow out and identify whatever contaminants are present. In conclusion, commercially practical systems have been developed to avoid contamination, i.e. some manufactures have the technology in place to do large particulate filtration to purify LN2, but to date the technology in place for sterilization of LN2 may have less practical application, as it exists, to IVF laboratories with a cryopreservation programme.

A more serious issue is the cross-contamination with viruses as described by Bielanski *et al*. (2000). In this study, the potential for viral transmission in experimentally contaminated LN2 to embryos vitrified and stored in open freezing containers was evaluated. From a pool of 83 batches, 21% tested positive for viral association. In contrast, all embryos vitrified in sealed plastic cryovials and modified open pulled straws were free from viral contamination. The data from this study have shown that sealing of freezing containers might prevent exposure to contaminants. It should be mentioned that the seal is not guaranteed, i.e. it can be broken.

Finally it may be necessary in the future to consider only working with sterile LN2 for cryopreservation within human ART programmes. So how can cryopreservation methods like vitrification that use direct exposure to LN2, be reconciled and adapted for use in IVF laboratories? It has to be stated that in no publication concerning vitrification of human or animal embryonic developmental stages (since 1985 about 400 publications), has contamination after cryostorage and further in-vitro culture been observed or mentioned. That said, unless it is expressly screened for, then viral contamination for example would not necessarily be picked up on. One recommendation to avoid any possibility of contamination is to store the cells in the vapour phase of LN2. First, similarly to common cryovials or French mini straws special carrier systems such as open pulled straws (OPS), flexipet-denuding pipettes (FDP), electron microscope grids (EM), hemi-straw systems (HSS) or cryoloops used for vitrification are practical for safe vapour-stage storage. However, vapour storage may be not compatible with maintenance of a stable vitrified droplet. Until today the issue of a 'safe' storage vessel for these carrier systems has yet to be fully tested, but a better vessel seal on the cryovial would be an easy place to start. Secondly, as alternative storage of vitrified cells in a double-straw system (high security like CBS straws) appear to be safe and does not compromise subsequent potential for survival and development of post-warming cells (Jelinkova *et al*., 2002).

Is the vitrification technique easily adaptable to IVF laboratories?

Although oocytes, pronuclear stage embryos, cleavage-stage embryos, and blastocysts have been successfully vitrified, many problems still remain to be solved. This novel cryopreservation technique presents the cryobiologist with new and challenging problems, and IVF laboratories must face up to additional technical difficulties and variables of the vitrification process that can profoundly influence its effectiveness. These may be modified with a view to improve the survival rates of vitrified cells, and include: (i) type and concentration of cryoprotectant (a high concentration of cryoprotectants is necessary to achieve vitrification and almost every kind of cryoprotectant is toxic); (ii) the addition or not of a macromolecule such as Ficoll or polyethylene glycol (PEG) to facilitate vitrification and/or a sugar like sucrose or trehalose; (iii) the temperature of the vitrification solution at exposure; (iv) the duration of exposure to the final cryoprotectant before plunging into LN2; (v) the type of device that is used for vitrification (which influences the size of the vapour coat and cooling rate); (vi) the technical proficiency of the embryologist; (vii) the quality as well as the developmental stage of the tested cells (since every developmental stage such as MII oocytes, PN-stage or cleavage-stage embryos has different osmometric behaviour and hydraulic conductivity); (viii) a possible contamination of cells by LN2; and (ix) the dilution procedure after thawing to minimize osmotic and toxic stress.

Although the advantages of vitrification (simple, low costs, very rapid) have been acknowledged, and this procedure has been used successfully to preserve mammalian oocytes and embryos ranging from the pronuclear to blastocyst stages of development and several pregnancies after vitrification and ultra-rapid freezing of human embryos have been reported, nevertheless this has not yet led to a widespread application of vitrification in ART programmes. In general, the inconsistent survival rates that have been reported and the wide variety of different carriers, vessels and vitrification solutions that have been used and formulated for vitrification have created some confusion on how vitrification should done correctly and has not helped to focus efforts on perfecting a single approach.

Current relevance of vitrification in ART

In the field of ART in 1999 and 2000, successful pregnancies and deliveries following vitrification techniques and protocols for human oocytes were reported (Kuleshova *et al*., 1999a; Yoon *et al*., 2000, 2003). Furthermore, the efficacy of cryopreservation by direct plunged into liquid nitrogen (vitrification) of human pronuclear stage embryos using the OPS or the FDP as well as the ultrastructural changes caused by vitrification were evaluated (Liebermann *et al*., 2002a; Isachenko *et al*., 2003a). The described protocol of vitrification of human pronuclear oocytes was shown to be effective in producing blastocyst stages and pregnancies (Jelinkova *et al*., 2002; Liebermann *et al*., 2002a; Isachenko *et al*., 2003a). The normality of ultrastructure after undergoing the described vitrification protocol was detected. Similarly results were obtained in vitrifying human oocytes and embryos at the cleavage and blastocyst stage (Reed *et al*., 2002; Mukaida *et al*., 2003a,b; Son *et al*., 2003, Vanderzwalmen *et al*., 2000). To this point there is no good opportunity to study the differences between slow freezing and vitrification to enable an effective comparison of their performance in the literature reported clinically.

Oocytes

Conventional cryopreservation protocols for human oocytes have resulted in various types of injuries (damage of intracellular lipid droplets, and the cytoskeleton), leading to low overall success rates of about 1% implantation per egg frozen (Mandelbaum *et al*., 1998). In addition, the developmental potential of 'slow-rate' frozen human oocytes following fertilization, up to cavitation and blastocyst formation is only 5.6% (Cobo *et al*., 2001). In contrast, a recent study showed the beneficial effect of microinjected trehalose on the survival of human oocytes after conventional cryopreservation (Eroglu *et al*., 2002). About 66% of oocytes in the intracellular trehalose group survived cooling to –60°C, while all oocytes in the control group degenerated when cooled to –60°C. More recently, an investigation of human oocyte cryopreservation utilizing a slow-rate protocol achieved a higher survival rate, when the sucrose concentration was 0.3 mol/l (82%) (Fabbri *et al*., 2001). However, it is important to note that similar survival rates (up to 70%) have been reported after vitrification of human oocytes using high concentrations of cryoprotectant agent (Hunter *et al*., 1995; Kuleshova *et al*., 1999a; Chen *et al*., 2000b; Yoon *et al*., 2003). So far, very high post-warming survival rates of 91% (Kuwayama and Kato, 2000) and even 96% (Chen *et al*., 2000b) have been reported in studies of more limited oocyte populations ($n < 65$). In a more recent study, using two vitrification protocols in more than 1000 human oocytes resulted in post-warming survival of 81–85% (Liebermann and Tucker, 2002a; Liebermann *et al*., 2003). The time taken to vitrify oocytes required 11.5 min, warming was conducted during 15 min (Liebermann *et al*., 2003). In contrast, the slow-cooling freezing protocol reported by Fabbri *et al*. (2001) requires about 108 min; the thawing was conducted in the time frame of 30 min. Liebermann *et al*. (2003) concluded that elevated rates of cooling are beneficial for oocyte vitrification. Also results with respect to increased cooling rates giving rise to more successful vitrification of animal oocytes have previously been documented (Arav *et al*., 2000; Hochi *et al*., 2001; Isachenko *et al*., 2001). Recently, Yoon *et al*. (2003) have confirmed the feasibility of the vitrification of human oocytes by clinical results in a stimulated IVF–embryo transfer programme. They achieved a pregnancy rate of 21.4% and an implantation rate of 6.4%. All pregnancies resulted in the delivery of healthy babies.

Pronuclear stage embryos

Conventional cryopreservation of multipronuclear zygotes (PN) is well established in countries such as Germany where freezing of later stage human embryos is by law or by ethical reasons not allowed. In Germany the total pregnancy of frozen–thawed PN is about 17%. Recently successful vitrification of PN with high survival (81–88%), cleavage rate on day 2 (77–85%) and blastocyst formation of 31% and pregnancies were reported (Park *et al*., 2000; Jelinkova *et al*., 2002; Liebermann *et al*., 2002a; Selman and El-Danasouri, 2002). The pronuclear stage is well able to withstand the vitrification and warming conditions, probably due to processes during and after the fertilization, such as the cortical reaction and subsequent zona hardening that may give the ooplasm membrane more stability to cope with the low temperature and osmotic changes.

Cleavage-stage embryos

Reports of human embryo vitrification are rare. Liebermann and Tucker (2002a) achieved with the cryoloop and the hemi-straw system (HSS) post-warming survival rates (after 2 h of culture) of day 3 embryos with more than half of intact blastomeres from 84 to 90%, that was dependent on the carrier system used. There was a reasonable further cleavage and compaction rate of 34%. This finding supports previous reports in which high survival rate of 8-cell human embryos using 40% EG were documented (Mukaida *et al*., 1998). In comparison, with a traditional cryopreservation, a survival rate of cleavage-stage embryos of 76% was reported (Jericho *et al*., 2003). Recently reported successful pregnancies and deliveries after vitrification of day 3 human embryos using the OPS were reported (El-Danasouri and Selman, 2001). Their results showed a negative correlation between stage of development and survival, 8-cell embryos showed a higher survival rate (79.2%; 62/78) than did embryos with fewer than six cells (21.1%; 11/53) after vitrification (El-Danasouri and Selman, 2001). Despite the fact, that Liebermann and Tucker (2002a) achieved a promising post-warming survival rate, overall only about 34% of the surviving embryos had the developmental potential to reach the stage of compaction. Therefore, this issue needs more attention in further studies. Furthermore, particularly in relation to human cleavage-stage embryos, since vitrification avoids ice crystallization, this may be have an impact on the quality of embryos that are able to be frozen, i.e. ice crystal formation during slow cooling of fragmented embryos may actually lead to an increased rate of failure-to-thaw in such low-grade (highly fragmented) embryos. Using vitrification, a greater percentage of supernumerary embryos following embryo transfer might successfully freeze and thaw. These are hypothetical thoughts only, as there is insufficient clinical data to confirm these speculations. Clearly, vitrification may have a positive impact on overall embryo utilization.

Human blastocysts

Clinical results with blastocyst cryopreservation have not necessarily been consistent. With the introduction of sequential culture media in ART, driven by the large increase in the rate of multiple pregnancies made extended culture to the stage of blastocyst on day 5 or later more common. Therefore, the need to cryopreserve human blastocysts is also increasing. Yeoman *et al*. (2001) achieved with conventional slow freezing method a post-thaw survival rate of rhesus monkey blastocysts from 36% with 5% hatching under similar culture conditions. In contrast, vitrification of rhesus monkey blastocysts with the cryoloop method resulted in a over two-fold increase in survival (85% survived, 77% expanded, and 71% hatched). The vitrification of blastocysts using the cryoloop reports survival rates of 72–90%, clinical pregnancy rates of 37–48%, and an implantation rate of 22–29%, (Mukaida *et al*., 2003a,b; Son *et al*., 2003; Vanderzwalmen *et al*., 2003). Clinical outcomes showed that vitrification using cryoloop, HSS or EM grid is effective and practical for the cryopreservation of human blastocysts. In general, these reports confirm the effectiveness of this vitrification procedure simply as a feasible option.

Importance of cooling rates

During cryopreservation, the cooling rate seems to be the most important parameter to consider, because results with respect to increased cooling rates giving rise to more successful vitrification of animal oocytes have previously been documented (Arav *et al*., 2000; Hochi *et al*., 2001; Isachenko *et al*., 2001).

As cells are immersed into LN2, the LN2 itself is warmed, and this induces extensive boiling, evaporation, and a vaporous coat forms around the cells. As a result, the vapour surrounding the cells creates insulation that cuts down temperature transfer, and this result in a decreased cooling rate. To improve chances that the sample is surrounded with liquid and not vapour, the sample size should be minimized so that the duration of any vaporous coat is reduced and the cooling rate is consequently increased. In addition, to facilitate the vitrification by even higher cooling rates, it is also necessary to minimize the volume of the vitrification solution to be as small as possible. The toxic and osmotic effects are also decreased. Furthermore, high cooling rates require the use of high concentrations of the cryoprotectant solution, which depresses ice crystal formation; however, the toxicity of some cryoprotectants can cause further problems. Nevertheless, there is a practical limit of attainable cooling speed, and a biological limit on the concentration of cryoprotectant tolerated by cells during vitrification. Therefore, a balance between maximizing the cooling rate and minimizing the cryoprotective concentration is a key objective. In general, the rate of cooling/warming and the concentration of CPA required to achieve vitrification are inversely related.

Container (carrier system) for vitrifying procedures

To minimize the volume of the vitrification solution, special carriers are used during the vitrification process of human oocytes, cleavage-stage embryos and blastocysts (**Figure 3**). The following carriers or vessels have been used to achieve high cooling rates: (i) open pulled straws (OPS) (Kuleshova *et al*., 1999a; Chen *et al*., 2000a,b); (ii) French ministraws (Chen *et al*., 2000b; Yokota *et al*., 2000, 2001; Vanderzwalmen *et al*., 2002); (iii) flexipet-denuding pipette (FDP) (Liebermann *et al*., 2002a,b); (iv) electron microscopic copper grids (EM) (Hong *et al*., 1999; Choi *et al*., 2000; Chung *et al*., 2000; Park *et al*., 2000; Wu *et al*., 2001; Son *et al*., 2003; Yoon *et al*., 2003); (v) hemi-straw system (Kuwayama and Kato, 2000; Vanderzwalmen *et al*., 2000; Vandervorst *et al*., 2001; Liebermann and Tucker, 2002a; Vanderzwalmen *et al*., 2003); (vi) cryoloop (Lane *et al*., 1999; Mukaida *et al*., 2001; Liebermann and Tucker, 2002a; Liebermann *et al*., 2002b, 2003; Reed *et al*., 2002; Mukaida *et al*., 2003a,b); (vii) a new vitrification device called the VitMaster® (IMT Ltd, PO Box 2044, Ness Ziona, Israel) in combination with OPS in LN2 slush (Arav *et al*., 2000). This device is able to decrease the temperature of LN2 to –205 to –210°C (compared to –196°C). This is achieved by creating a partial vacuum, and thereby increasing significantly the cooling rate by using LN2 slush. The VitMaster® has been successfully used for human oocyte vitrification (Liebermann *et al*., 2002b).

Which human tissues can be vitrified?

The vitrification technique has been successfully applied for cryopreservation of: oocytes, pronuclear stage (PN) embryos, day 3 embryos and blastocysts (Kuleshova *et al*., 1999a; Lane *et al*, 1999; Chen *et al*., 2000a,b; Park *et al*., 2000; Saito *et al*., 2000; Yokota *et al*., 2000, 2001; Yoon *et al*., 2000, 2003; Vanderzwalmen *et al*., 2000, 2002, 2003; El-Danasouri and Selman, 2001; Mukaida *et al*., 2001; Tripodi *et al*., 2001; Jelinkova *et al*., 2002; Liebermann and Tucker, 2002a; Liebermann *et al*., 2002a, 2003; Reed *et al*., 2002; Isachenko *et al*., 2003a; Mukaida *et al*., 2003a,b; Son *et al*., 2003); spermatozoa (Arav *et al*., 2002; Nawroth *et al*., 2002; Isachenko *et al*., 2003b); embryonic stem cells (Reubinoff *et al*., 2001); female nuclear material (He *et al*., 2003); ovarian tissue (Shaw *et al*., 2000; Lee *et al*., 2000; Rahimi *et al*., 2001; Isachenko *et al*., 2002); human cornea (Armitage *et al*., 2002; Meltendorf *et al*., 2002); and human skin (Fujita *et al*., 2000).

Furthermore, the vitrification technique has also become a very useful scientific tool in the food and nutrition industries. Many studies tested this novel cryopreservation procedure very successfully on embryonic axes, internodes, and apices as well as parts of the meristematic regions such as shoot tips, nodal segments of different kind of plants of commercial value such as tobacco, plum, sugar beet, citrus, sweet potato, apple, banana, olives, and rice.

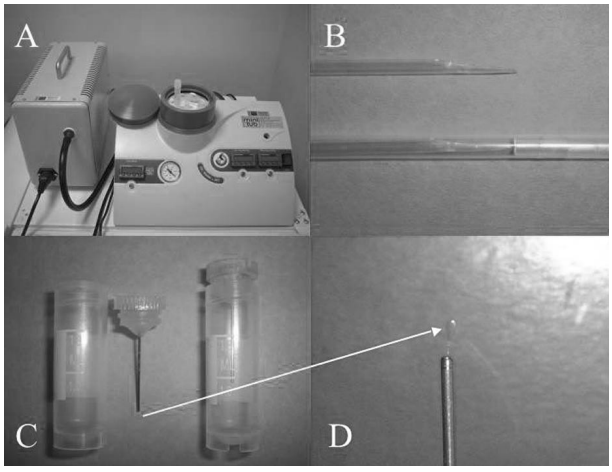

Figure 3. (**A**) VitMaster® with vacuum pump and Dewar with slush placed inside and filled with liquid nitrogen. By creating a partial vacuum in the Dewar, the temperature is decreased and thereby the cooling rate is increased significantly. (**B**) Hemi-straw system as carrier used for vitrification: one end of the 0.25 ml straw is cut with a sharp scalpel, so that the end is easily loaded with a small droplet on the open edge of the straw using a pipette. After loading with oocytes or embryos and plunging into liquid nitrogen, the hemi-straw is placed in a precooled 0.5 ml straw and plugged. (**C**) Cryoloop system: cryovial and nylon loop mounted on a stainless steel rod is loaded with the specimen, vitrified and inserted into the lid of the cryovial, and sealed to hold the oocytes or embryos securely. (**D**) Nylon loop.

Type of cryoprotectants, saccharides and macromolecules

The buffered media base most commonly used for vitrification is either phosphate-buffered saline (PBS) or HEPES-buffered culture medium such as HTF.

Cryoprotective agents

Cryoprotective agents (CPA) are essential for the successful cryopreservation of living cells. Basically two groups of cryoprotectants exist: permeating (glycerol, ethylene glycol, dimethyl sulphoxide), and non-permeating (saccharides, protein, polymers) agents. The effect of the non-penetrating cryoprotectants is dehydration of cells by osmosis. The essential component of a vitrification solution is the permeating agent. These compounds are hydrophilic non-electrolytes with a strong dehydrating effect (**Figures 4 and 5**). Furthermore, these CPA are able to decrease the 'freezing point' of the solution. The result is that the spontaneous ice crystal formation, which usually appears at –10°C, is delayed depending on the concentration of the cryoprotectant as well as the cooling rate, and appears at –35 to –45°C. Therefore, in slow cooling the cell has more time for dehydration. In addition, permeating agents are able to compound with intracellular water and therefore water is very slowly removed from the cell. Therefore, the critical intracellular salt concentration is reached at a lower temperature.

It is important to mention that the permeating CPA should be chosen firstly by their toxicity and secondly by their permeating property. Because the permeating CPA is responsible for the toxicity (the key limiting factor in cryobiology), different cryoprotectants have been tested for their relative toxicity, and the results have shown, that ethylene glycol (EG; mol. wt 62.02) is the least toxic, followed by glycerol. Therefore, the most common and accepted CPA for vitrification procedures is EG. It appears to have a low toxic effect on mouse oocytes, embryos and blastocysts without any observed compromise in morphology and developmental functions, a rapid diffusion and a quick equilibration into the cell through the zona pellucida and the cellular membrane (Emiliani *et al*., 2000). In general, rapidly permeating agents are favoured, because the cells shrink the least and the exposure time before vitrification may be shortened. Furthermore, they are also more likely to diffuse out of the cells rapidly and the cells regained their original volume more quickly, thus preventing osmotic injury. Pregnancies and normal live births achieved with cryopreserved oocytes and embryos in animals suggest that the EG molecule is a good candidate for human embryo vitrification.

Saccharides

Additives with large molecular weights such as disaccharides like sucrose or trehalose do not penetrate the cell membrane, but can significantly reduce the amount of intracellular cryoprotectant required as well as the toxicity of EG by decreasing the concentration required to achieve a successful cryopreservation of human oocytes and embryos. It is well understood that solutions of non-permeating saccharides can serve as an osmotic buffer for the successful recovery of cryopreserved cells. Furthermore, the incorporation of non-permeable compounds in the vitrification solution and the incubation of the cells in this solution before any vitrifying helps to draw out more water from the cells, and lessens the exposure time of the cells to the toxic effects of the cryoprotectants. In addition, removal of the cryoprotectant agent during warming presents a very real problem in term of reducing toxicity to the cells. Firstly, because of the toxicity of the vitrification solutions, quick dilution of them after warming is necessary; and secondly, during dilution water permeates more rapidly into the cell than the cryoprotective additive diffuses out. As a consequence of the excess water inflow, the cells are threatened by injury from osmotic swelling. In this case the

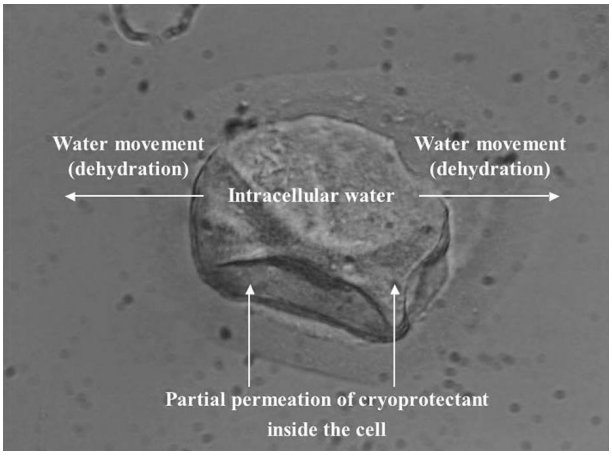

Figure 4. Human oocyte precooled for 60 s in a equimolar mixture of 10% ethylene glycol (EG) and 10% dimethyl sulphoxide (DMSO) and 1mg/ml polyethylene glycol (PEG). A supraphysiological volumetric contraction of the oocyte is caused by replacement of intracellular water by the EG/DMSO/PEG mixture.

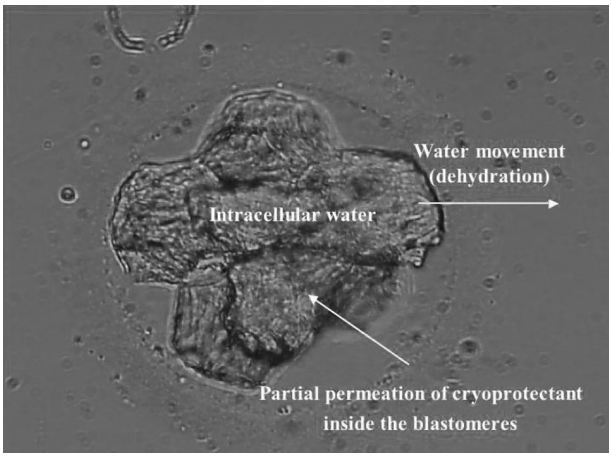

Figure 5. Human embryo (8-cell stage) precooled for 4 min in an equimolar mixture of 10% ethylene glycol (EG) and 10% dimethyl sulphoxide (DMSO). A supraphysiological volumetric contraction of each of the eight blastomeres is caused by replacement of intracellular water by the EG/DMSO mixture.

non-permeating sucrose acts as an osmotic buffer to reduce the osmotic shock. During warming using a high extracellular concentration of sucrose (e.g. 1 mol/l) counterbalances the high concentration of the cryoprotectant agents in the cell, as it reduces the difference in osmolarity between the intra- and extracellular compartments. It has shown that such high concentrations of sucrose are virtually non-toxic to embryos and oocytes (Kuleshova *et al.*, 1999b). The high sucrose concentration cannot totally prevent the cell from swelling, but it can reduce the speed and magnitude of swelling (Liebermann and Tucker, 2002a; Liebermann *et al.*, 2003).

Macromolecules

Furthermore, the macromolecules polyethylene glycol (PEG; mol. wt 8000), polyvinyl pyrrolidone (PVP; mol. wt 360,000) and Ficoll (mol. wt 70,000 or 400,000) modify the vitrification properties of the vitrification solutions. Some studies have evaluated the potential beneficial effects of adding such macromolecular solutes to the vitrification solution to facilitate vitrification of embryos. The viability of oocytes following cryopreservation seems greatly improved, and the variability of cell survival is vastly reduced than with vitrification solution alone (Kuleshova *et al.*, 2001). These polymers are generally less toxic and can protect embryos against cryoinjury by mitigating the mechanical stress occurring during cryopreservation. They do this by modifying the vitrification properties of these solutions by significantly reducing the concentration of cryoprotectant required to achieve vitrification itself, but they have little or no effect on the glass transition of the solutions. They also influence the viscosity of the vitrification solution and reduce the toxicity of the cryoprotectant through lower concentration (Liebermann and Tucker, 2002a; Liebermann *et al.*, 2003). Furthermore, the polymers may be able to build a viscous matrix for encapsulation of these embryos, and also prevent crystallization during cooling and warming (Kuleshova *et al.*, 2001). Very recently, a study designed and tested cryoprotectant solutions that combined high polymer concentration with low penetrating cryoprotectant. These combinations yielded high rates of development of 2-cell mouse embryos after rapid cooling and warming (Kuleshova *et al.*, 2001).

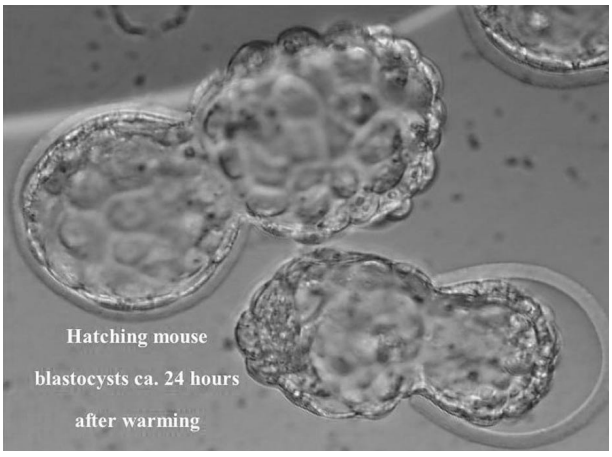

Figure 6. Vitrified hatching mouse blastocysts ca. 24 h after warming in sucrose.

Exposure to cryoprotectant agent

Time

A common practice to reduce the toxicity of the cryoprotectant but not its effectiveness is to shorten the exposure time of cells to the solution as much as possible. However, for an optimal exposure time a balance between preventing toxic injury and preventing intracellular ice formation is important. To improve survival by reducing the toxicity, a two-step procedure is recommended. The cells are placed first in a cryoprotective solution of lower-strength to load them partially with this cryoprotectant. Secondly there are transferred to the full-strength solution for a brief exposure. In addition, the vitrification solution may often contain an almost equimolar combination of EG and dimethyl sulphoxide (DMSO), thus reducing the concentration of CPA required to cryopreserved human oocytes and embryos efficiently and so the toxic and osmotic effect of them is also decreased. Furthermore, it is possible by further increased cooling and warming rates to reduce the cryoprotectant concentration and thus toxicity (Liebermann *et al.*, 2002b).

Temperature

The speed of penetration and also the grade of toxic effects of the cryoprotectants are influenced by temperature. One way to reduce the toxic effect is to lower the temperature. In pre-trials, the influence of the temperature of the vitrification solution was tested during the loading process (25 versus 37°C) on the survival potential of human failed-matured (uninseminated) or failed-fertilized (inseminated but without 2PN development) oocytes (J Liebermann and MJ Tucker, unpublished). The exposure time at 37°C did not allow any re-expansion of cells after warming at all. Therefore, it was decided for all protocols to use a loading temperature between 22 and 25°C. A total of 273 expanded mouse blastocysts were vitrified (J Liebermann and MJ Tucker, unpublished data), of which 96.7% (264/273) survived (**Figure 6**). The blastocysts were vitrified in 40% EG with 0.4 mol/l sucrose for 20 s at 25°C; however, survival decreased clearly with a longer exposure time.

Practical aspects in using the carriers

Cryoloop

The vitrification method using the cryoloop is based on the method described by Lane and colleagues (Lane *et al.*, 1999). In brief, about five cells are exposed in a vitrification solution of lower-strength (VS1) for 60 s, and finally transferred into full-strength solution (VS2) for 30 s. During this time the nylon loop is dipped into VS2 to create a thin, filmy layer of the VS2, by surface tension, on the nylon loop. After a short exposure to VS2 the five cells are placed onto the nylon loop pre-loaded with the thin film of VS2 using a small pipette (e.g. the FDP with an end hole of 140-micron) powered by the forces of surface tension

(**Figure 7**). The loaded nylon loop is plunged into LN2, and then screwed tight using the stainless rod into the cryovial that had previously been submerged under LN2. Standard canes are used for storage in LN2.

Flexipet-denuding pipette (FDP)

The FDP vitrification procedure is undertaken in a similar fashion to the OPS vitrification method, which was reported by Vajta *et al*. (1998) as follows. Briefly, four to six cells are pretreated with VS1 for 5 min. After precooling the cells are then placed in VS2, after which they are transferred with a minimum amount of VS2 into a 20 µl-droplet for 30 seconds, and are loaded into the FDP in approximately 1–2 µl of cryoprotectant by placing at a 30° angle, and allowing the cells to be drawn up by capillary action. The tip of the pipette is then plunged at a 10° angle directly into LN2, and placed into a prelabelled 0.25 ml cryo-straw held under LN2 (Liebermann *et al*., 2002a). This is then plugged with a plastic colour print coded plug prior to cryostorage.

Hemi-straw system (HSS)

The HSS vitrification procedure was reported first by Vanderzwalmen and colleagues (2000). Recently, it was reported that the HSS was easy to use and successful for vitrification of different stages of development (oocytes, day 3 and blastocysts) (Liebermann and Tucker, 2002a; Vanderzwalmen *et al*., 2003). The number of cells that can be loaded onto the HSS depends from the stage of development used: for oocytes and day 3 embryos about four to five, for blastocysts a maximum of two in anticipation of the need for only one or two blastocysts at transfer. The 0.25 ml straw is prepared in the following manner: cut one end of the 0.25 ml straw with a sharp scalpel, so that the end is open (about 1 cm), and it is now easy to pipette a small droplet (<1.0 µl) onto the open inner face. Briefly, the cells are pretreated with VS1 for 3 min. After precooling, the cells are then placed in VS2 for 30 s, after which they are transferred with a minimum amount of VS2 into the 20 µl-droplet, and are loaded in a extremely small volume (<1.0 µl) on the inner surface of the open edge of a shortened 0.25 ml straw (**Figure 8**). The HSS is then plunged directly into LN2 (vertically), and placed into a larger pre-cooled and prelabelled 0.5 ml cryo-straw (CBS, Cryo Bio System, France) or into a 5 ml cryovial held under LN2 (Liebermann and Tucker, 2002a; Vanderzwalmen *et al*., 2003). The straw is then plugged with a plastic colour print coded plug, or the labelled 5 ml cryovial capped prior to cryostorage.

In general, none of the vitrification carrier systems listed above required any additional storage capacity in the laboratory.

Conclusions

In conclusion, increasing the thermal conduction speed and decreasing the concentration of cryoprotectants is an ideal strategy for cryostoring cells with vitrification methods. There are two main ways to achieve the vitrification of water inside cells efficiently. One is to increase the temperature difference between the samples and vitrification medium, and secondly to find materials with fast thermal conduction. However, the actual rate during vitrification procedures may vary widely, depending on the device used, technical proficiency, and also the specific movement during immersion into the LN2. Furthermore, low toxicity vitrification solutions must be designed, that may be composed using three different categories of agents: EG as cryoprotective agent; macromolecule such as the polymer Ficoll; and saccharides such as sucrose, trehalose or raffinose.

The toxic effect of EG can be overcome by combining two-step exposure at room temperature (EG as sole CPA or in equimolar combination with DMSO), low concentration of the CPA, and by minimizing the volume of the vitrification solution as small as possible. The design of such solutions allows the vitrification of cells at room temperature. A partial dehydration and permeation prior to cooling must be studied to get optimal exposure.

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 as other developmental stages, such as cleavage-stage embryos or blastocysts. This must be considered by finding the optimal time of exposure.

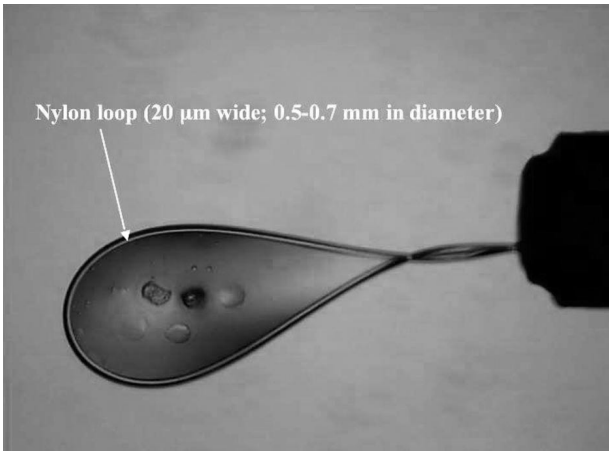

Figure 7. The nylon loop with a thin film of vitrification solution loaded with oocytes held by surface tension.

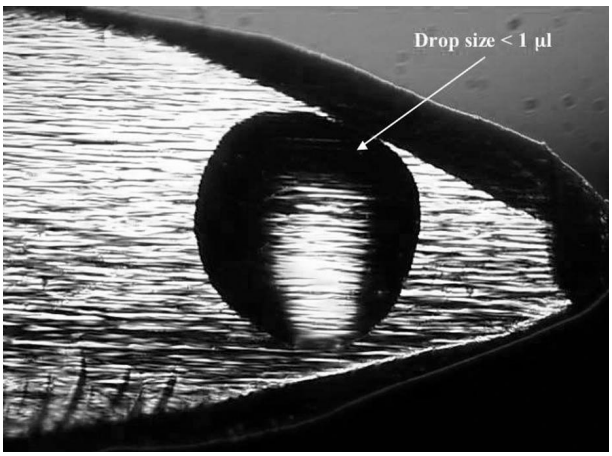

Figure 8. A small droplet of vitrification solution (<1 µl) loaded on the open edge of the hemi-straw system (0.25 ml straw).

The 'universal' vitrification protocol has yet to be defined. Besides the inconsistent survival rates that have been reported, another explanation for this lack of widespread use could be that such a variety of different carriers or vessels have been described for use in vitrification. Furthermore so many different vitrification solutions have been formulated that this has not helped to focus efforts on perfecting a single approach as yet. On the other hand, the reports of successfully completed pregnancies following vitrification are encouraging for further research, but clearly improvements must be made in the inconsistent survival rates following vitrification. Nevertheless, when considering all the literature to date, this does highlight the need for an improved understanding of the effects of vitrification on oocytes, embryos and blastocysts. Despite this, the convenience of vitrification is likely to push the development of this technique to higher levels of clinical efficiency and utilization (Kuleshova and Lopata, 2002; Liebermann and Tucker, 2002b; Liebermann *et al*., 2002c).

It seems plausible, therefore, that especially for cold-sensitive oocytes, and also for the more complex cell system as exists in the human blastocyst with over 100 cells shared between the inner cell mass, trophectoderm, not to mention the problematic blastocoelic cavity, that vitrification might be a more favourable technique than slow-cooling cryopreservation procedures ultimately providing higher efficacy and utilization rates.

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Paper based on contribution presented at the 'TWIN–Meeting Alpha–Andrology 2003' in Antwerp, Belgium, September 2003.

Received 29 April 2003; refereed 2 June 2003; accepted 16 June 2003.