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REVIEW ARTICLE

Cryopreservation in ART and concerns with contamination during cryobanking

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Abstract The cryopreservation of gametes and embryos is vital to numerous fields of reproductive biology, including assisted human reproduction. With improved culture conditions, there are an increasing number of embryos to cryopreserve for potential use in subsequent cycles. Many of the gametes and embryos in human IVF are cryopreserved in open systems. Because liquid nitrogen is not sterile, concerns have been raised with regard to contamination from the liquid nitrogen and also cross-contamination between patients' germplasm. Human gamete and embryo cryopreservation are discussed, with recommendations on how to minimize and eliminate contamination, emphasizing the benefits of closed vitrification devices.

Keywords Closed device · Contamination · Cryopreservation · Liquid nitrogen · Vitrification

Introduction

Cryopreservation in reproduction

Cryopreservation allows for the long-term storage of gametes and embryos, which is highly advantageous in a number of reproductive fields. For example, unique mouse strains (e.g., transgenic mice) can be cryo-stored to protect valuable genetics. It also provides a practical solution for facilities housing large numbers of research animals or those looking to transfer animals without the risk of introducing an animal-derived pathogen. Cryopreservation is vital for domestic animal genetic maintenance and propagation, with millions of doses of bull semen and cattle embryos being cryopreserved and shipped worldwide [1, 2]. Cryopreservation is also seen as a potential safeguard for endangered animals, with the creation of "frozen zoos" [3]. In human assisted reproductive technology (ART), cryopreservation has become an essential component for almost every single IVF cycle.

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Human gamete cryopreservation

Artificial insemination with frozen semen was first reported in 1954 [4]. A decade later, spermatozoa that had been cryopreserved in liquid nitrogen for 5 months were used for insemination [5]. These early successes paved the way for routine clinical application and the formation of human sperm banks. Despite its widespread application, a report by the World Health Organization indicates that around 50 % of the sperm are damaged by the cryopreservation process [6]. Freezing generally causes a decrease in the percentage of motile sperm, but the extent varies considerably among individuals. Although this variability might not be considered an issue with healthy males, it has been noted that men with malignant diseases can have significantly reduced quantity and quality of sperm [7]. With the ability to inject a single sperm into an oocyte, one might argue that efforts to improve the efficacy of the sperm cryopreservation protocol are not that relevant. However, it

is evident that cryopreservation protocols should be continually improved to ensure minimal damage to sperm in order to increase the chance of success and the creation of healthy offspring [8].

Following the first birth from a cryopreserved human oocyte in 1986 [9], very few births were reported during the subsequent decade. Frustratingly, the success of Chen's technique, which had been developed using the mouse oocyte as a model, could not be repeated. Furthermore, subsequent laboratory studies revealed the potential negative effects of cooling and exposure to cryoprotectants on oocyte physiology, which raised concerns regarding the safety of such a procedure [10–13]. Meiotic spindle disruption, chromosome abnormalities, zona hardening, and reduced fertilization all indicated that oocyte cryopreservation protocols were suboptimal.

The unique physiology and membrane composition of the metaphase II ovulated oocyte clearly provided greater challenges than that of the embryo. The report of the first pregnancy [14] and first birth [15] from a cryopreserved human embryo were around the same time as that of the oocyte [9]. In contrast to oocyte cryopreservation, the efficacy of embryo cryopreservation has meant that it has been considered a routine ART for a number of years. For example, frozen embryos were used in around 26 % of the total number of IVF embryo transfers performed in the USA in 2011 [16]. Almost 34 % of these frozen embryo transfers resulted in a live birth, which equates to approximately 10,000 live births in 2011 in the USA alone. This is also the case in Europe, where the number of live births derived from frozen embryo transfers is around 11,000 per year [17]. In contrast, until 2012, oocyte cryopreservation was still considered an experimental procedure by the American Society of Reproductive Medicine and has to date resulted in only ~1,000 live births worldwide over two decades [18, 19]. This number, however, is likely to significantly increase in a relatively short period of time given the recent success of oocyte cryopreservation with vitrification (discussed below).

Embryo cryopreservation

Embryo cryopreservation is a routine assisted reproductive technique that has many benefits. One example is the ability to store supernumerary embryos following the initial transfer. This eliminates the negative aspects associated with further ovarian stimulation, which includes the financial cost and ovarian hyperstimulation syndrome. Most importantly, cryopreservation maximizes the cumulative success of each and every IVF cycle [20]. A more recent and practical application of embryo cryopreservation has been to permit successful comprehensive chromosome screening at the blastocyst stage. Due to the time taken to perform genetic analysis on human blastocysts, in most cases cryopreservation is required to permit timely embryo transfer [21]. Furthermore, it is plausible that a proficient cryopreservation protocol will result in an improved implantation/pregnancy rate alone, since the cryopreserved embryos, in contrast to freshly transferred embryos, are not exposed to an artificially stimulated uterine environment, and are therefore returned to a more receptive endometrium [22–27].

Methods of cryopreservation

Two cryopreservation methods are currently available; slow freezing and vitrification. The physical and practical differences, as well as the merits of each technique have been discussed in detail elsewhere [28–30]. The term vitrification is derived from the Latin *vitrum*, meaning glass. Thus, vitrification of cells requires the transition of the cytosol into an amorphous, glass-like solid. The absence of crystalline structures means there is no formation of potentially lethal ice crystals, which damage the plasma membrane and intracellular organelles [31]. Vitrification of water is achieved if the molecules are cooled at a rate that is too rapid for them to organize themselves into ice crystals. In theory, vitrification of pure water can be achieved if the cooling rate is at least 10^8 °C/min.

Although direct contact with liquid nitrogen (−196 °C) results in a cooling rate of approximately 10^4 °C/min, current vitrification solutions can be vitrified with cooling rates that are an order of magnitude slower. The intracellular water content must be sufficiently reduced, so that a cooling rate of 10^3 °C/min is rapid enough to ensure that the remaining water molecules vitrify, rather than freeze. A reduction in water molecules is achieved through osmotic potential (using extracellular sugars), to draw water out of the cell. Permeable cryoprotectants (e.g., glycerol, dimethyl sulfoxide, ethylene glycol, and/or propylene glycol) are introduced to disrupt interactions between the remaining water molecules.

Warming rates are equally if not even more important (see below). If the cell is warmed too slowly, the water will undergo devitrification, forming ice crystals. The cooling and warming rates required can be significantly reduced by increasing the percentage of cryoprotectant, but unfortunately, depending on their concentration, exposure time, and temperature, these chemicals can be cytotoxic. Therefore, it is necessary to use appropriate conditions whereby the cryoprotectant concentration and exposure time are adequate to introduce sufficient cryoprotectant into the cell, but without affecting embryo viability. Equally, the vitrification solution must also remove

sufficient intracellular water, so that cooling and warming rates do not permit intracellular ice formation. As a visual accompaniment, Fig. 1 shows two droplets of cryopreserved medium in a culture dish. The droplet on the left is composed of a base medium with no cryoprotectant. The droplet on the right is the same base medium, but with 32 % v/v cryoprotectant. The culture dish was submerged into liquid nitrogen. Upon removal from liquid nitrogen, a photograph was immediately taken to demonstrate that without appropriate levels of cryoprotectants, the water in the base medium will freeze. The frozen droplet has an opaque appearance due to the ice crystals, whereas the vitrified droplet is amorphous and glass-like, and therefore translucent.

Vitrification of mammalian embryos was first reported by Rall and Fahy [32]. Since then, the technique has been further developed to minimize the concentration of cryoprotectants. This has been achieved principally through the introduction of miniature devices, with high levels of temperature conduction, which hold sub-microlitre volumes. Decreasing the volume of medium that is to be vitrified and the direct application into liquid nitrogen significantly increases the cooling rates (approximately 20,000 °C/min). Studies to date have employed the electron microscope grid, open pulled straw, cryoloop, solid surface vitrification, nylon mesh, cryotop, and cryotip devices [33–39]. These minimal volume devices have been successfully applied to the cryopreservation of domestic and laboratory animal gametes and embryos [33, 35, 40–42], and more recently to the clinical field of in vitro fertilization [43–45].

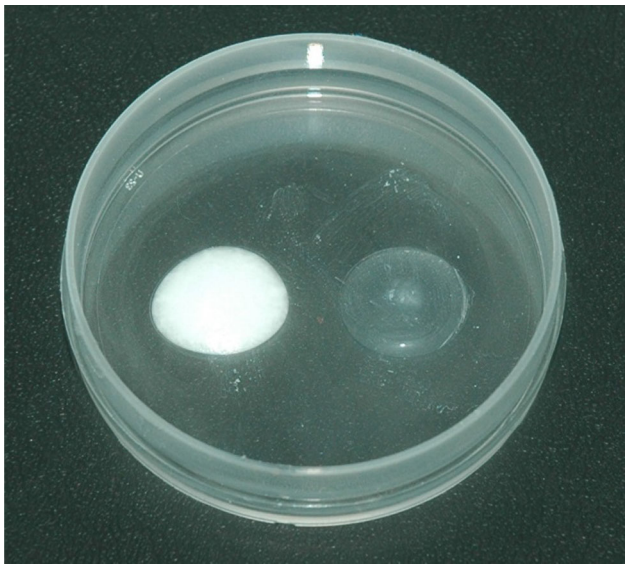

Fig. 1 The difference between freezing and vitrification. To indicate the difference between freezing and vitrification, two different droplets of medium were placed on a culture dish and submerged into liquid nitrogen. The droplet on the left is composed of a base medium with no cryoprotectant. The droplet on the right is composed of the base medium containing 16 % (v/v) ethylene glycol and 16 % (v/v) DMSO. Upon removal from liquid nitrogen, a photograph was immediately taken. The droplet on the *left* appears *white* from the ice crystals that have formed during the freezing process. The droplet on the *right* contains sufficient cryoprotectant, which prevents the water molecules from organizing themselves into a crystal lattice. Thus, the droplet containing cryoprotectant permits light to pass through and appears translucent

Recent publications in laboratory and domestic animals, as well as in humans, have demonstrated that vitrification is superior to conventional slow freezing, with greater survival rates and more viable embryos [46–48]. Mouse oocytes cryopreserved using slow freezing produce blastocysts with significantly fewer cells and reduced viability, as compared to those undergoing vitrification [49]. Furthermore, oocyte and embryo metabolism, plasma membrane integrity, and protein expression are significantly altered by slow freezing compared to vitrification [49–51]. For example, Fig. 2 shows the difference in protein expression of mouse oocytes following slow freezing and vitrification. From these profiles, it can be seen that there are protein expression levels not affected by either cryopreservation technique. There are, however, proteins that are both up- and downregulated by slow freezing.

One of the main concerns with oocyte cryopreservation has been depolymerization of the meiotic spindle and chromosome disruption. The meiotic spindle is not a static structure and is under constant flux. Microtubule dimers (composed of α and β-tubulin) are preferentially lost at the microtubule organizing center (centrosome) and added at the end that is associated with the chromosome kinetochore. The rate at which the microtubule dimers are lost/added determines the state of the meiotic spindle. The meiotic spindle will rapidly depolymerise if oocytes are cooled below 37 °C [52–55]. The spindle in mouse oocytes can recover from cooling and take around 1 h to repolymerize [56]. The human oocyte, however, appears to be more sensitive to temperature than that of the mouse [55]. In the 5 min it took to cool human oocytes from 37 to 27 °C, spindle disassembly had occurred. The spindle could repolymerize within 20 min if the oocytes were immediately returned to 37 °C. However, if oocytes were maintained at a cooled temperature and then returned to 37 °C, the spindle failed to reform in the same time period. The effect of cooling may even be permanent, since the majority of human oocytes cooled to room temperature for 10 min failed to repolymerize their spindles within 4 h of being returned to 37 °C [12, 13]. It seems that the cryopreservation method can also impact the meiotic spindle. Performing vitrification at 37 °C compared to slow freezing at room temperature maintained the meiotic spindle in both human and mouse oocytes [45].

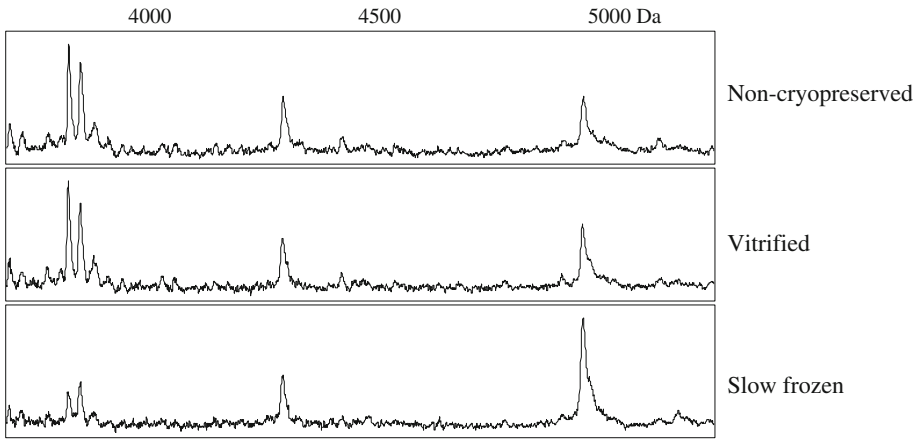

Fig. 2 Profiling of proteins following slow freezing and vitrification of mouse oocytes. *Line plots* of MII mouse oocyte protein expression profiles generated through SELDI-TOF–MS. The *top* profile is from one sample of MII in vivo (control) oocytes, the *middle* profile is one sample of MII vitrified oocytes and the *bottom* profile is one sample of slow frozen oocytes (groups of five oocytes, replicated eight times) (modified from [91])

Additional evidence that vitrification imparts less overall cellular stress than slow freezing was elegantly demonstrated through the repeated cryopreservation of mouse embryos at successive stages of development (1-cell, 2-cell, 8-cell, and blastocyst) [57]. Using vitrification, it was possible to re-cryopreserve mouse embryos at four success stages without loss of development in culture or implantation potential. In contrast, mouse embryos could not survive three rounds of slow-freezing. These data confirm that the cumulative stress of slow freezing significantly compromises oocyte and embryo physiology, and ultimately, viability [47].

Despite data demonstrating that slow freezing has greater detrimental effects on the physiology of oocytes and embryos, it is difficult to draw clinical conclusions because there are very few randomized control trials with human embryos comparing slow freezing and vitrification. In such studies, it appears that the survival rate is higher with vitrification, but there is no difference in overall pregnancy data [58–60]. One could also argue that slow freezing can still be optimized, given that many of the protocols have remained largely unchanged since their implementation [61–63].

Liquid nitrogen and concerns of contamination

The risk of contamination through liquid nitrogen has been of concern for several years [64]. Over 40 years ago, virus transmission was reported following topical cryotherapy [65, 66]. Liquid nitrogen was found to be contaminated with infectious vesicular stomatitis when glass ampules of the virus became compromised [67]. The risk of cross-contamination during cryostorage of biological material was highlighted by the transmission of hepatitis B from cryostored bone marrow [68]. Leakage from the cryopreservation bag resulted in four other patients being infected with hepatitis B following the transfusion of blood components.

Viral contamination is not the only consideration. Microbial analysis of ice sediments from liquid nitrogen tanks found both bacterial and fungal contaminations that are capable of causing illness [69]. Bacterial and fungal species were also found in liquid nitrogen used to store bovine embryos and semen [70]. Some of these microbial contaminations were found in the embryos and semen, but it was unclear if they had been introduced during preparation for cryostorage. Because of the potential to contaminate the germplasm during cryostorage, it is not too surprising that viral and microbial transmission have been investigated and are a topic of discussion.

Cross-contamination with bacteria between samples was first demonstrated with semen pellets [71]. Within 2 h of cryostorage, practically all the sterile samples had become contaminated with *Escherichia coli* and *Staphylococcus aureus*. By spiking liquid nitrogen with different viruses, it has been shown that embryos can also be contaminated. Bovine embryos cryopreserved in open containers were exposed to liquid nitrogen contaminated with bovine viral diarrhea virus (BVDV), bovine herpes virus-1 (BHV), and bovine immunodeficiency virus (BIV) [72]. BVDV and BHV were found in 21 % of the samples, whereas BIV transmission did not occur. As expected, storing semen and embryos in sealed containers prevents viral contamination [70, 72]. In one study with relatively limited numbers, viral screening was performed on spent culture media and liquid nitrogen used to vitrify oocytes and embryos from infected women [73]. No viral sequences were detected, suggesting that the risk of cross-contamination is low, although it was suggested that safer cryopreservation methods should be developed to avoid any possible contamination.

Minimizing and avoiding contamination

Germplasm quarantine

Storage of the germplasm should always be initially quarantined until the donor has been tested for seroconversion and/or the samples have been tested for infectious entities. This will minimize any risk of cross-contamination. Semen in particular is susceptible to a high microbial load, so it should always be stored separate from oocytes and embryos. Once the infectious nature of the sample has been determined, it is then possible to arrange appropriate storage.

Washing of gametes and embryos

Washing and performing a swim-up of semen samples has been shown to significantly reduce or remove viruses and bacteria [74]. Oocytes and embryos have a natural first line of defense in the zona pelluicda. It has been demonstrated in animal models that multiple washing is very effective in removing microbial and viral pathogens [75]. However, it is now very common for the zona pelluida to be breached because of intracytoplasmic sperm injection (ICSI), biopsy, and assisted hatching, which may make the oocytes and embryos more susceptible to contamination.

Decontamination of cryotanks

Because of current handling of liquid nitrogen and the difficulty in sterilizing the large quantities used in IVF clinics (see below), all storage tanks should be handled as if they contain potentially infectious contaminants. Cryotanks and dry shippers should undergo periodic decontamination with a solution that does not react with the lining, and then be rinsed with sterile water (discussed in [76]).

Liquid nitrogen sterilization

In most cases, liquid nitrogen is not provided sterile by the supplier, and it is most likely that any contamination is introduced during distribution to dewars/storage vessels and inappropriately stored/compromised contaminated samples. There are, however, steps that may be taken to minimize the risk of contamination during storage in liquid nitrogen.

Sterile filtration of liquid nitrogen at the outlet was described by McBurnie and Bardo [77] using a 0.22-μm filter. UV radiation has also been suggested as an alternative for liquid nitrogen sterilization. With the belief that high cooling rates are necessary for vitrification, most protocols involve direct contact with liquid nitrogen. Subsequently, microbial sterilization of small volumes of liquid nitrogen is possible with UV radiation [78]. The irradiation of 500 ml of liquid nitrogen was performed by exposure to a UV lamp (254 nm), 15 cm from the surface for 15 min. This treatment successfully decontaminated liquid nitrogen that had been spiked with high titers of bacteria and fungi. Filtration and ultraviolet irradiation of liquid nitrogen can offer protection against bacterial and fungal contamination. However, these alternatives do not afford complete viral elimination and may be impractical or prohibitively expensive, and most importantly, do not prevent subsequent cross-contamination during liquid nitrogen storage. It must also be noted that the IVF laboratory is not a sterile environment, so although liquid nitrogen may be sterilized through filtration or UV sterilization, the sterility of the liquid cannot be guaranteed over time.

There is no direct evidence of cryopreserved human oocytes/embryos becoming contaminated during cryostorage and subsequently transmitting disease or causing infection [79]. However, given the available information from bovine embryo studies [70, 72], it would seem prudent to utilize alternatives that do not require direct contact with liquid nitrogen during vitrification and storage, with the proviso that they perform equally as well as so-called open systems.

Closed systems

Criado et al. [80] demonstrated that a quartz capillary closed system (Ultravit) was not contaminated when stored in liquid nitrogen that had been contaminated with *Pseudomonas aeruginosa* and *Escherichia coli* for 10 s. This was in contrast to the 45 % contamination rate with an open system (Cryotop). The principal of using a closed system, which does not require direct contact with liquid nitrogen, is not new. Kuleshova and Shaw [81] sealed an open pulled straw in an outer straw to provide a closed storage system that would eliminate the risk of contamination. This device was successful at vitrifying mouse embryos [81, 82] and human pronuclear oocytes [83].

Chen et al. [84], used an open pulled straw, but introduced air and vitrification solutions at the end of the straw (once the mouse oocytes were loaded) to create a "closed" system. Kuwayama took this one step further with the Cryotip, which is essentially an open pulled straw that is then heat-sealed at both ends. When compared to the Cryotop (open system) the survival and pregnancy rates with human blastocysts were comparable between the two devices [85]. This demonstrates that a closed system is capable of vitrifying human blastocysts, although one study did report unacceptably low recovery with the Cryotip [86], and another showed more ultra-structural damage of human oocytes with the Cryotip compared to the Cryotop [87].

In a modification of the hemi-straw plug device (whereby the device is placed inside a straw, sealed, and then plunged into liquid nitrogen) human blastocysts have been vitrified despite much slower cooling rates than those achieved with open systems [87–90]. It became dogma, however, that vitrification of human oocytes requires the high cooling rates afforded by direct contact with liquid nitrogen [76].

Moving away from direct contact with liquid nitrogen

A proof of principle paper, published by Larman et al. [91], demonstrated that the procedure using the Cryoloop can be modified such that it does not require direct liquid nitrogen contact. Cryovials were suspended in a polystyrene tray, so that liquid nitrogen surrounded the body of the vial, but did not enter, creating a super-cooled air temperature of around −190 °C inside the vial. This non-contact method of vitrification in super-cooled air was compared to the standard direct contact protocol using mouse pronuclear oocytes. Survival was comparable between the two techniques. Furthermore, there was no difference in subsequent embryo development including blastocyst cell number or cellular differentiation into the trophectoderm and inner cell mass. This method demonstrated that direct contact with liquid nitrogen is not necessary for embryo vitrification, and an alternative is super-cooled air. In principal, this would avoid contamination from the liquid nitrogen during the actual process of vitrification. Cryovials, however, are not leak-proof during long-term storage in liquid nitrogen. This is emphasized by the fact that the manufactures of cryovials only recommend storage in the vapor phase. Thus, cryovials are not suitable for a closed system.

Sealed straws have been used for a number of years with slow freezing and are regarded as a sterile cryopreservation

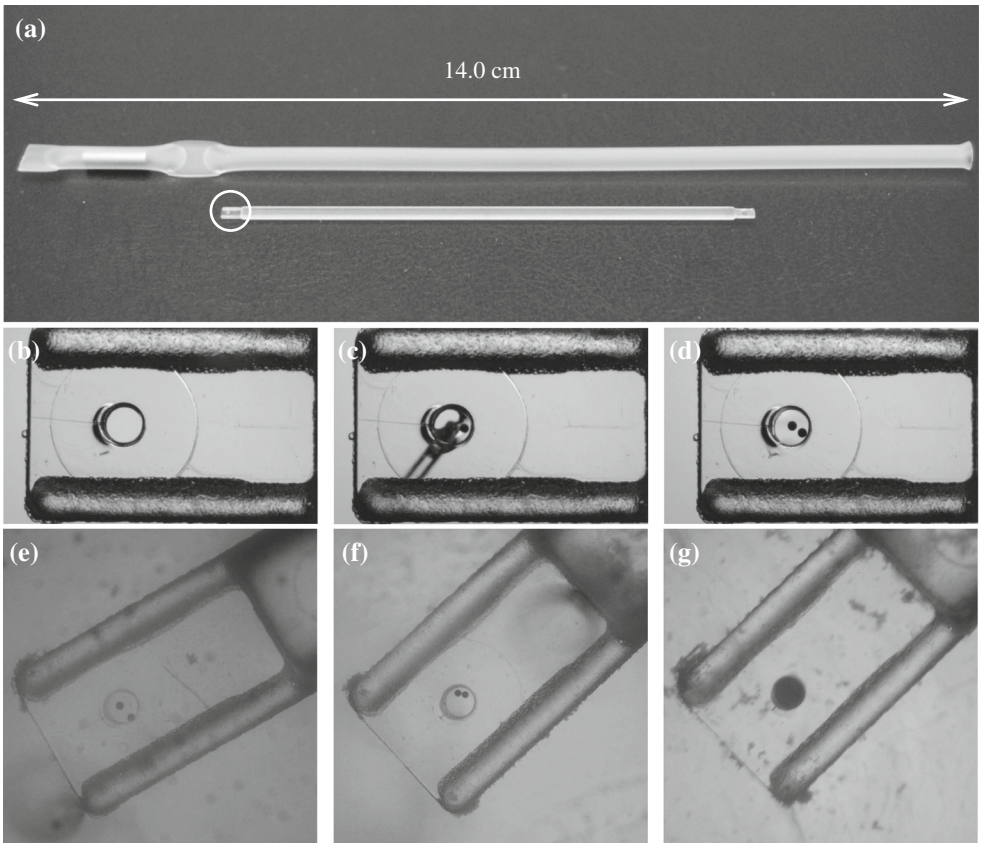

Fig. 3 Rapid-i: a closed-vitrification device. The Rapid-i is a non-contact vitrification device composed of a weighted storage straw that is sealed at one end and a plastic rod, which holds the embryos (*circled*). (**b**, **d**) The Rapid-i is loaded with the embryos and vitrification medium by pipetting the embryos (or in this case, 100-μm beads) into the 50-nl hole. The fact that the embryos sit in a hole, which is flanked by the flange, means that the embryos are very well-protected. The sub-microlitre volume and high viscosity of the vitrification solution also means that the embryos remain steadfast on the device. (**e–f**) demonstrate that cooling rate is sufficient to vitrify the vitrification solution; the Rapid-i was removed from the straw under liquid nitrogen in a large petri dish, so that images could then be taken while the Rapid-i remained submerged in liquid nitrogen. **g** Filling the hole with the holding solution (no cryoprotectant) results in it freezing, becoming opaque (replicated from [91])

device for storing human gametes and embryos in liquid nitrogen. As mentioned above, straws have been modified to be used with vitrification methods and can be used aseptically by placing them within a larger outer straw that is sealed before plunging into liquid nitrogen [81, 82, 87–89]. Using this principal and the results from Larman et al. [91] with super-cooled air, an in-straw device was developed; the Rapid-i^{TM} (Fig. 3a–d). This device is unique in that it is a fully-sealed system that uses super-cooled air to vitrify the sample. The Rapid-i is the most tested closed-vitrification device. It was developed using mouse embryos [92] and is capable of vitrifying mouse pronuclear oocytes with a 100 % survival rate. The subsequent embryo development, cell number, and embryo viability are not affected when compared to sibling non-vitrified embryos.

Using super-cooled air rather than direct contact with liquid nitrogen does mean that the cooling rate is much slower (1,200 °C/min), but it is still sufficient to vitrify the vitrification medium (Fig. 3e–g). Despite the slower cooling rates, the Rapid-i is apparently as effective as open systems in the vitrification of human oocytes and embryos (discussed below). The reason for its efficacy is, most likely, because it still maintains a warming rate similar to other open devices. Often, the focus on the development of minimal volume devices and direct contact with liquid nitrogen was to increase cooling rates, but it appears that the warming rate is actually more critical. Seki and Mazur [93] determined the functional relationship between cooling and warming rates and survival of mouse oocytes. It was found that mouse oocyte survival was more negatively affected by slower warming rather than cooling rates. The rates used in this initial publication were much lower than those observed with open systems and direct liquid nitrogen contact. To demonstrate that the same principle held true at high cooling and warming rates, a second study was performed [94]. Using a Cryotop, they examined a matrix of different cooling (range 95–70,000 °C/min) and warming (range 610–118,000 °C/min) rates. The same trend was observed in this study, i.e., that the warming rate is more critical than the cooling rate. The rationale behind the critical importance of the warming rate may be that although small ice nucleation events might occur with slow cooling rates, the warming rate must be fast enough to prevent them from aggregating and forming the larger, damaging ice crystals.

The pronuclear oocyte is a sensitive stage of early preimplantation embryo development. To investigate if the Rapid-i was capable of vitrifying and maintaining subsequent post-warming embryo development and viability, day 4 blastocyst synchronized embryo transfers were performed in the mouse [92]. The implantation percentage for transferred control and vitrified pronuclear oocytes was 66.7 and 75.0 %, respectively. The fetal development percentage for control and vitrified pronuclear oocytes was 50.0 and 53.8 %, respectively. Figure 4a shows the mean fetus crown-to-rump (C-R) length, weight, and placenta weight derived from transfers of non-vitrified (control) and Rapid-i vitrified embryos. Figure 4b shows that the Rapid-i could support embryo development and cell number even after long-term (12 months) storage in liquid nitrogen.

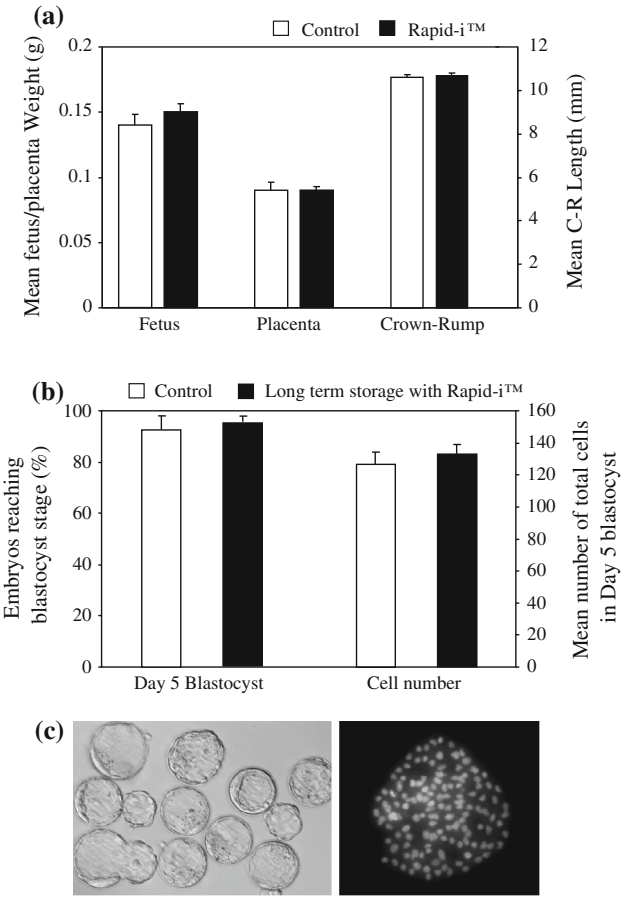

Fig. 4 Embryo transfer to determine embryo viability. **a** F1 pronuclear oocytes were vitrified and immediately warmed. Embryos were then cultured until day 4 alongside non-vitrified controls. Four-Five blastocysts were transferred on day 4 from the two groups into separate uterine horns of pseudopregnant recipients. On day 15 of the pregnancy, fetus and placenta weight and crown–rump (*C–R*) length were determined. Fetal development parameters including ear, eye, and limb were not different between control and vitrified groups. This data represents embryos transferred to 10 mice over eight replications. **b** F1 pronuclear oocytes were vitrified with the Rapid-i and stored for 12 months. Following warming, the embryos were cultured until day 5 (96 h of in vitro culture) alongside non-vitrified controls. Blastocyst development was monitored on the afternoon of day 4 and the morning of day 5. Embryo development was not affected by long-term storage. Following the day 5 score, embryos were fixed and stained to enable the number of cells in each blastocyst to be determined. Cell number was not affected by long-term storage. **c** Shows an image of day 5 blastocysts following long-term cryostorage on the Rapid-i and a representative nuclei staining image from which total cell numbers were determined. Thirty embryos were used per treatment over three replications (replicated from [91])

The Rapid-i has been compared to two open systems for human embryo vitrification. The results of vitrifying day 3 embryos and blastocysts using the Rapid-i were compared to an open system (Cryoloop), which had been the clinic's device of choice for a number of years [95]. For day 3 embryos, the survival rates for the Rapid-i and Cryoloop were 99 %. The implantation rates were 37 and 35 %, respectively. The clinical pregnancy rates were 47 and 49 %, respectively. For blastocysts, the survival rate for the Rapid-i and Cryoloop were 97 and 91 %, respectively. The Rapid-i supported higher implantation (49 %) and clinical pregnancy (59 %) rates than the Cryoloop (38 and 46 %, respectively), although it did not reach significance.

The Rapid-i was compared to the Cryotop by Hashimoto et al [96]. The first comparison in this study used zygotes previously cryopreserved at the pronuclear stage. After re-warming, embryo development was assessed. There were no differences between the Rapid-i and Cryotop in survival, blastulation, good blastocyst rates, or mean cell number (Table 1; modified from [96]). To investigate the influence of the vitrification method on apoptosis, blastocysts were vitrified–warmed and compared to non-vitrified blastocysts. There was no difference in the proportion of dead cells between the three treatments. Lastly, the two devices were compared clinically. Two hundred and sixty-three high-grade blastocysts were randomly assigned to vitrification using either the Rapid-i or Cryotop. The survival rate for both devices was the same (97 %). Single blastocyst transfer was performed after warming. The implantation and ongoing pregnancy rates were similar for the Rapid-i and Cryotop (Table 2).

The results from these two studies demonstrate that a closed system such as the Rapid-i can vitrify human embryos with the same efficacy as open systems. As mentioned above, it was thought that vitrification of human oocytes required the high cooling rates only provided by direct contact with liquid nitrogen. As with embryos, it appears that human oocytes can also be vitrified with the Rapid-i. Sibling in vitro, matured human oocytes were vitrified using either the Rapid-i or Cryotop [97]. The survival rates were 92 and 90 %, respectively. At the time of writing this manuscript, the Rapid-i is being evaluated in a clinical trial for donor oocyte vitrification. Over 500 oocytes have been vitrified and warmed with a survival rate of 94 %. Following ICSI, the fertilization rate was 76 %. Blastocyst transfer resulted in a 49 % ongoing pregnancy rate, with five healthy live births reported so far [98].

Table 1 Survival rate of 2–4 cell embryos vitrified with the Rapid-i or Cryotop. Subsequent blastulation rate and percentage of good quality blastocysts are shown with the mean cell number in each blastocyst (modified from [96])

	Rapid-i ($n = 34$)	Cryotop ($n = 32$)
Survival rate (%)	100	97
Blastulation rate at 120 h (%)	68	56
Good blastocysts (%)	47	41
Mean cell number	137 ± 14	138 ± 18

Table 2 Survival and clinical results following blastocyst vitrification using either the Rapid-i or Cryotop (modified from [96])

	Rapid-i ($n = 100$)	Cryotop ($n = 163$)
Survival rate (%)	97	97
Implantation rate (%)	54	53
Ongoing pregnancy rate (%)	45	47

Conclusions

Cryopreservation of gametes/embryos offers numerous advantages, and consequently, has become a routine technique in assisted human reproduction. Cryobanking of biological material has resulted in cross-contamination between patients, and although there have been no reports of contamination following cryostorage in human IVF, there are real concerns with using open systems that use direct contact with liquid nitrogen during vitrification and subsequent storage. There are methods to minimize the risk of viral and microbial contamination, but the easiest solution is to prevent all contact with liquid nitrogen during the procedure by using a closed system. Having no direct contact with liquid nitrogen means the cooling rate with a closed system is significantly lower, but it appears that they can be used for human oocyte and embryo vitrification if the warming rate is sufficiently high. The Rapid-i is the most tested closed device and has been shown to be capable of vitrifying human oocytes and embryos with apparently the same efficacy as open systems.

Conflict of interest Mark G. Larman is an employee of Vitrolife AB. David K. Gardner receives a research grant from Vitrolife AB. Shu Hashimoto and Yoshiharu Morimoto have no conflicts of interest.

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