EXPERT REVIEWS

Embryo cryopreservation: is vitrification ready to replace slow freezing?

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Cryopreservation of human gametes, embryos and blastocysts has become an integral part of assisted reproduction. This approach may have several advantages for infertile patients. It provides an opportunity to limit the numbers of embryos transferred while supernumerary embryos will be used in subsequent treatment cycles, thereby increasing the cumulative pregnancy rate. Furthermore, the potential risk of hyperstimulation syndrome can be reduced. In addition, storage of embryos from a cycle allows the patient to space the timing of sibling pregnancies and improve their potential of achieving a pregnancy at an advanced maternal age. Therefore, a reliable procedure for the cryopreservation of supernumerary embryos is needed. Since the early 1980s, two common techniques have been used in cryopreservation: the conventional slow cooling method and the more recent rapid procedure, known as vitrification. The latter needs a much higher concentration of cryoprotectants and faster cooling rates. Vitrification is qualified to be the future of cryopreservation procedure owing to increased survival and success rates over the last years. The aim of this review is to assess the outcomes of different methods of cryopreservation performed on human embryos and to evaluate whether vitrification nowadays is ready to replace slow freezing.

KEYWORDS: cryopreservation • cryoprotectant • embryo • slow cooling • vitrification

The first human embryos were frozen in the late 1970s with the first pregnancy reported in 1983 in Australia by Trounson and Mohr [1], with multicellular embryos that had been slow-cooled using dimethylsulphoxide (DMSO), the first successful pregnancy resulting in a delivery was reported in 1984 by Zeilmaker *et al.* [2]. Since then, simplifications and improvements in slow-freezing protocols have resulted in a wide application of the method for human embryos at early-cleavage stages, as well as for blastocysts.

On the other hand, successful vitrification of mammalian embryos was first reported by Rall and Fahy in 1985 [3]. Since then, a number of cryoprotectant solutions have been investigated for human use, including the use of DMSO, glycerol, ethylene glycol, propanediol and sugars in various combinations [4,5]. In addition, numerous carrier systems have been tried, including electron microscope grids, open-pulled straws, denuding pipettes, open hemistraws and cryoloops [6–10].

In cryopreservation, either by the traditional slow-cooling method (equilibrium cooling) or the more recent rapid procedure of vitrification (nonequilibrium cooling), subzero temperatures and the conditions those human gametes and embryos encountered during cryopreservation are not physiologic situations [11].

The characteristics of cryopreservation methods, such as the exposure time of cells to different cryoprotectant solutions and to their different concentrations, have critical roles in the survival and viability of embryos. The rate of formation of intracellular ice crystals and requirements of different development stages of cells are also heavily involved in the outcome of cryopreservation [11,12].

One of the most significant aspects regarding the success of cryopreservation is post-thaw embryo survival due to ice crystal formation during the freezing and thawing processes, which may damage cell membranes and lead to blastomere lysis [13]. Although successful pregnancies can be achieved following transfer of

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embryos with less than 50% of the blastomeres intact post-thaw, pregnancy rates are higher when all blastomeres survive. Indeed, if embryos survive the freeze–thaw process with all the blastomere intact, then the pregnancy rate is comparable with that of fresh IVF cycles. However, overall cryopreservation leads to a 30–40% reduction in the implantation potential [14].

There is much debate as to the developmental stage at which human embryos are best cryopreserved [15,16]. The disadvantage of two-pronucleate stage embryos is that nothing is known regarding their developmental competence. A major complication of cleavage-stage embryos on the other hand is that, after thawing, damaged blastomeres often coexist with intact ones and it has been demonstrated convincingly that the implantation potential of such embryos is much lower than that of fully intact ones [14].

Selection of fresh embryos for transfer on the basis of observations, such as growth rate, morphology and early cleavage, can enhance the outcome of embryo transfer, but usually relies on multiple embryos from which the 'best' can be selected. Similarly, selection on the basis of survival and resumption of mitosis can enhance the outcome from cryopreserved embryo transfer [17]. Clinical success with cryopreservation depends on many factors, including patient age and stimulation protocol, quality of embryos selected for freezing, developmental stage at freezing, media formulation (including type of cryoprotectants used) and parameters of cooling and warming.

Vitrification has improved viability and survival rates of cells owing to the prevention of intracellular ice crystallization [18]. Nevertheless, this procedure requires much higher concentrations of cryoprotectants that may also cause possible toxic and osmotic effects when compared with slow freezing. More recently, it has been addressed as the future of cryopreservation of human gametes and embryos due to the highest survival and pregnancy rates [19].

One of the central issues is whether slow- or rapid-cooling protocols that have been employed in human IVF programs satisfy the fundamental principle of minimizing damage during cryostorage. Therefore, it is essential to establish a simple and reliable procedure to optimize human embryos cryopreservation, a prerequisite of which is to develop a better understanding of factors that influence their survival and implantation potential in order to have the utmost clinical outcome.

Is vitrification more favorable than slow cooling?

In slow cooling, embryos are usually pre-equilibrated at room temperature. The concentration of cryoprotectants is then gradually increased inside and outside the embryos during several steps over a 2-h period. When the concentration of cryoprotectant was high enough to support glass-like solidification of the cells and the outside solution, at temperatures approaching -33°C to -40°C, the cells could then be rapidly exposed to much lower temperatures such as -150°C or lower. Today, we can call the Rall and Fahy studies a bridge between vitrification and slow cooling. Using improved vitrification solutions and technology, human oocytes and embryos can be pre-equilibrated and introduced into vitrification solutions at room temperatures or even at physiological temperatures [20].

Kuleshova and Lopata highlighted the advantages and disadvantages of slow freezing compared with vitrification [21]. They asserted that there is no proper control of solute penetration or control of dehydration rate in slow freezing. In addition, vitrification only needs 10 min, which is nearly ten-times less than slow freezing. Furthermore, they also reported that slow cooling is a rather expensive method compared with vitrification regarding the running cost and equipment.

On the other hand, we must consider that, in vitrification, a skilled embryologist is required and only few embryos can be worked with at a time because of the need to move quickly through solutions. Therefore, a technician will have to be occupied solely with freezing until all embryos are placed in liquid nitrogen for storage, while slow freezing does not require direct attention by an embryologist.

Kuwayama *et al.* stated that the primary disadvantages to slow cooling for human embryo cryopreservation are the requirement for an expensive programmable freezing machine and the time-consuming procedure [18]. The introduction of a technique that could be performed without the use of costly equipment and could be completed by one cryopreservation specialist within minutes would provide significant benefits for any busy IVF program. Vitrification of embryos and oocytes may offer a solution to this problem. The authors also stated that the considerable advantages of vitrification include elimination of ice crystal formation, which may increase their chances for survival, it is a simple technique involving direct plunging into liquid nitrogen and the time required for equilibration and cooling is considerably reduced. On the other hand, disadvantages of vitrification are the required high cryoprotectant concentration and, consequently, the increased risk of toxic and osmotic damage, and the need to use special tools permitting high cooling rate by reducing radically the volume of solutions containing the embryos.

Impact of human embryo cryopreservation on clinical outcome of assisted reproduction

Embryos have been successfully cryopreserved at zygote, cleavage and blastocyst stages, using various freezing protocols with either DMSO, 1,2-propanediol or glycerol as cryoprotective agents.

Human zygote & cleavage embryos

Using the slow-freezing method, day 2 or 3 embryos were cryopreserved with DMSO with a 53% survival rate, an overall pregnancy rate of 21.8% per transfer and 12.9% clinical pregnancy rate [22]. Another group that had used propandiol concluded that pronuclear-stage (PN) oocytes resulting from intracytoplasmic sperm injection can be successively frozen–thawed. The pregnancy rates (17%) achieved were comparable with those for zygotes obtained after IVF (20%) [23]. Furthermore, it

was observed that PN embryos resulting from intracytoplasmic sperm injection could be cryopreserved successfully and thawed, with a clinical pregnancy rate (14%) comparable with conventional IVF (17.4%) [24].

Moreover, many IVF teams performing cryopreservation report clinical pregnancy rates of 20–30% per transfer of frozen–thawed cleaved embryos [25,26]. In a recent study carried out by Schroder *et al.* using the slow-freezing method for cryopreservation of human zygotes, a 10.2% pregnancy rate per embryo transfer was reported [27].

The effectiveness of zygote and cleavage-stage embryo cryopreservation in terms of embryo survival has been the subject of several studies. However, few studies have directly compared the results of different freezing strategies utilizing either zygote or cleavage-stage embryo cryopreservation, and these studies have yielded highly controversial results, either similar results for zygotes (74.4%) and day 2 embryos (77.4%) [28] or better results for day 2 embryos (73.9%) than for zygotes (64.4%) [29].

A retrospective study, conducted by Salumets *et al.,* was devised to evaluate the impact of developmental stage of embryos on the pregnancy outcome of frozen embryo transfer [30]. A total of 4006 embryos were analyzed, the highest survival rate was observed for zygotes (86.5%), followed by day 2 (61.7%) and day 3 (43.1%) embryos, with overall clinical pregnancy and implantation rates of 20.7 and 14.2%, respectively. There were no significant differences in clinical pregnancy, implantation, delivery and birth rates between frozen zygote, day 2 and 3 embryo transfers. The findings of this study support the conclusions of a group that reported a better survival rate for zygotes (80.4%) than for day 2 embryos (71.8%) [31].

On the other hand, interest in vitrification was reported for the first time as an efficient method in mouse embryo cryopreservation [3]. Afterwards, successful vitrification of human four- to eight-cell embryos was documented [32]. Thereafter, vitrification of human embryos, especially at early stages, increased due to reported comparable clinical and laboratory outcomes [8,18,33,34].

However, the majority of studies on vitrification of human embryos reported high survival rates (>85%) and pregnancy rates of approximately 22–30%, which were completely in acceptable ranges and much higher than the rates of slow freezing [8,18,34,35]. Recently, after 3 years experience Al-Hasani *et al.* reported that 849 PN zygotes were vitrified, 339 PN zygotes were thawed resulting in an 89% survival rate [36]. The mean number of embryos per transfer was 2.2. The pregnancy rate obtained was three-times higher (36.9%) than that obtained with the slow-rate freezing method (10.2%) used previously in the same centre.

El-Danasouri and Selman reported that survival rates following vitrification are positively correlated with the number of blastomeres in the cleavage-stage embryos [33]. In addition, higher pregnancy and slightly higher survival rates were commonly attributed to the further stages of human embryos, such as eight cells [37] and blastocyst stage [38]. More notable is the observation that vitrification either at blastocyst stage or at cleavage and PN stage, along with subsequent embryo transfer either at day 3 or 5, was shown to result in almost similar pregnancy rates as fresh cycles [18,39,40]. Furthermore, the rate of blastocyst formation after vitrification, either at PN or cleavage stage, was similar to fresh cycles and commonly above 40–50%. These findings suggest the advantage of early-stage vitrification based on the similar survival rates of vitrification at different stages and high blastulation rates, comparable to fresh cycles.

Another concern regarding vitrification is the risk of exposure of embryos and gametes to contaminants through direct contact with liquid nitrogen. To tackle this concern, different and numerous types of cryocarrier and vessels have been described in the literature, eliminating potential contamination by isolating cells [18,41]. While no studies have demonstrated unintentional uptake by a human or mammalian embryo of any pathogen during vitrification or storage, under experimental conditions such contamination may occur. To date, the largest reported survey is for the Cryotop method [18].

Comparing slow freezing versus vitrification, Kuwayama *et al.* published that 100% of vitrified human PN embryos survived and 52% developed to blastocysts compared with 89% survival and 41% blastocyst development after slow cooling [18]. Similar survival rates were seen with vitrification of four-cell embryos (98%) compared with slow cooling (91%). They stated that vitrification is a simple, efficient and cost-effective way to improve cumulative pregnancy rates per cycle (Table 1).

Raju *et al.* reported a post-thaw survival rate of vitrified human day 3 embryos of 95.3%, which was significantly higher than that of the conventional slow-freezing protocol (60.0%) [37]. The implantation and pregnancy rates were also higher (14.9 and 35.0%) than that in the slow-freezing protocol (4.2 and 17.4%). On average, three embryos were transferred per patient. Of the 40 transfer cycles, five deliveries and nine ongoing pregnancies were achieved. Four ended with delivery of a singleton and one in a miscarriage.

Ultimately, human embryos at two-PN and cleavage stage can be successfully cryopreserved by the use of vitrification, providing high survival rates and improved outcomes. It is a rapid and simple technique that favors the biological integrity of human embryos.

Blastocyst stage embryos

Recent advances in culture systems with sequential media have made it possible to develop human IVF embryos into blastocysts quite easily. In particular, prolonged culture of embryos to day 5 gives the chance of much detailed assessment of the embryonic development parameters [42]. Blastocysts are preimplantation embryos that have successfully passed the critical step of genomic activation and therefore have a high developmental potential [43].

One group cryopreserved human blastocysts, which were developed in a co-culture system using the slow-freezing method, and obtained reasonable clinical results. Ménézo *et al.* published a 21% pregnancy rate per transfer (15 ongoing) of

Table 1. Outcome of recent studies on vitrification of human zygote and early-stage embryos.

Study	Embryo stage	Cryoprotectants	Cryocarrier	Vitrified embryos (n)	SR (%)	PR (%)	Notes	Ref.
Al-Hasani *et al.* (2007)	Zygote	EG/DMSO/S	Cryotop	339	89	36.8	Abortion rate: 17.42%	[36]
Kuwayama *et al.* (2005)	PN stage Cleavage stage	EG/DMSO	Cryotip Cryotop	1300	100 98 90	 27 53	Delivery rate: 48–51%	[18]
Zhu *et al.* (2005)	Embryos	EG-based	Open-pulled staws	957	72.2	19–22		[59]
Rama Raju *et al.* (2005)	Embryos (8 cells)	EG-based	Open-pulled staws	40	95	35		[37]
Hredzak *et al.* (2005)	Cleavage stage	EG/S	100 µl pipetting tip	215	69	27		[35]
Isachenko *et al.* (2003)	PN stage	EG-based	Open-pulled staws	59	71			[60]
Liebermann *et al.* (2002)	Embryos (8 cells)	EG-based	Flexipet denuding pipette	266	83.8			[35]

DMSO: Dimethylsulphoxide; EG: Ethylene glycol; PN: Pronuclear stage; PR: Pregnancy rate; S: Sucrose; SR: Survival rate.

101 transfers (106 thawing) [11]. In a retrospective study by Kaufman *et al.*, 1239 blastocysts were thawed, they reported an 83% survival rate, 13.4% implantation rate, 21.7% pregnancy per transfer with a 19% ongoing pregnancy rate [44]. In addition, there was a higher pregnancy rate in the programmed cycle (26.2%) compared with the natural cycle (13%). Other studies conducted by Martin *et al.* and Anderson *et al.* support the previous results [45,46]. Overall, these studies obtained reasonable results (Table 2).

However, results of slow freezing of human blastocysts reported by other authors have been inconsistent; Troup *et al.* reported that at the expanded blastocyst stage, only 38% of embryos survived when frozen–thawed [47]. No pregnancies were achieved. Therefore, the results of slow freezing of blastocysts are contradictory and not satisfactory for the ambitious goals of this issue toward the advancement of assisted reproductive technology.

Many human pregnancies that originate from an ultrarapid vitrification technique are achieved after cryopreservation of blastocysts using the cryoloop, the cryotop, the electron microscope grids or the straws. In the study by Mukaida *et al.*, human blastocysts were successfully vitrified and the survival rate after warming was 63% [48]. Six clinical pregnancies were achieved after 19 transfers. One healthy baby was born, four pregnancies were ongoing and one ended in miscarriage. This report documented the first successful pregnancy and delivery achieved by blastocyst vitrification using the cryoloop containerless technique.

Subsequent to the improved practice of combined use of various cryoprotectacts as well as the use of different cryocarriers, the clinical outcomes of vitrification were improving, evidenced by a high survival rate of up to 100% and pregnancy rate up to 53%, as reported by several authors using cryoloop, electron microscopic grids or open-pulled straws [9,39,49,50–53].

Furthermore, several studies have been conducted to compare cryopreservation of day 5 and day 6 blastocysts with regard to survival, implantation and pregnancy rates. Two of these studies indicate that day 5 blastocysts have a higher survival rate [9], as well as pregnancy and implantation rates compared with day 6 blastocysts [38].

Blastocysts and further stages of human embryos have different physiological requirements than early-stage embryos, which affect the survival chance after unphysiological situations such as ultrarapid freezing. A major factor that affects the survival rate of blastocysts is the fluid-filled cavity called the blastocoele. Vanderzwalmen *et al.* analyzed the effectiveness of reducing the volume of the blastocoelic cavity before vitrification (i.e., induced collapse of the blastocoele) [6]. Initially, they encountered low survival rates of blastocyst after vitrification (20.3% with blastocysts or expanded blastocysts, 54.5% with morulae and 58.5% with early blastocysts). However, they were able to overcome this by reducing the blastocoelic cavity and puncturing it with a special pipette before the procedure. Thereafter, an increase in the survival rate of up to 70.6% was noted. The pregnancy rates were improved after the artificial shrinkage procedure (20.5%) compared with the control intact blastocyst group (4.5%; not significant). After reduction of the blastocoelic cavity, a significant increase in the implantation rate per vitrified blastocyst was observed. They also concluded that survival rates in cryopreserved expanded blastocysts could be improved by reducing the fluid content. This was presumably because mechanical damage caused by ice crystal formation was avoided (Table 2).

In another study conducted by Son *et al.*, artificial shrinkage was induced in expanded blastocysts using a needle [54]. Of 90 expanded blastocysts vitrified, 81 survived (90.0%) and 40 of them were hatched (49.4%) at the time of transfer. The implantation rate was 29.0% (20 of 69) and the pregnancy rate was 48.0% (12 of 25). Nine patients delivered 15 infants, two ongoing pregnancies and one ended in miscarriage. The results suggest that artificial shrinkage is a useful technique for vitrification of expanded blastocysts. Furthermore, the survival rate of expanded blastocysts after vitrification increases when – to reduce ice crystal formation – the blastocele is artificially shrunk with a glass microneedle [6] or the use of pipetting [55].

However, two retrospective studies were undertaken to compare a slow-freezing protocol with a vitrification protocol for cryopreservation of day 5 and 6 human blastocysts. In the first, published by Stehlik *et al.*, 71 day-5 slow-frozen blastocysts were thawed and 59 embryos survived the thawing (83.1%) [40]. An average of 2.5 slow-frozen blastocysts was replaced per embryo transfer, resulting in a pregnancy rate of 16.7% (4 of 24). On the other hand, 41 vitrified blastocysts were thawed and all 41 survived the thawing process (100%). An average of 2.0 vitrified blastocysts were replaced per embryo transfer, resulting in a pregnancy rate of 50% (10 of 20). Survival, implantation and pregnancy rates of day 5 vitrified blastocysts are significantly higher compared with day 5 slow-frozen blastocysts. A similar trend was observed with day 6 blastocysts (Table 2).

Another study was undertaken by Lieberman and Tucker [38], who documented that in 254 vitrified transfer cycles, survival, embryonic implantation and clinical pregnancy rates for day 5 blastocysts were 95.9, 33.4 and 48.7%, respectively, and for day 6 blastocysts 97.5, 25.9 and 42.8%. In 254 slow-frozen transfer cycles, survival, embryonic implantation, and clinical pregnancy rates for day 5 blastocysts were 91.4, 29.6 and 42.8%, respectively, and for day 6 blastocysts 94.8, 28.2 and 43.1%. Overall there was a slightly, but not significantly, higher outcome regarding implantation and clinical pregnancy with the use of day 5 blastocysts (31.3 and 45.4%, respectively) versus day 6 blastocysts (26.7 and 42.9%, respectively). They concluded that the vitrification technique yields the same implantation and pregnancy rate as slow-frozen blastocyst transfers. Slow growing, in addition to embryos, can be cryopreserved on day 6, because they yield satisfactory survival, implantation and pregnancy rates (Table 2).

Furthermore, the strongest data came from a large study by Kuwayama *et al.* [18]. In this study, the survival and development of human embryos were compared following slow cooling versus vitrification involving more than 6000 blastocysts. In this study, 5695 out of 6328 (90%) vitrified blastocysts survived and resulted in 53% (2516 out of 4745) pregnancy rate following transfer, as compared with 84% (131 out of 156) survival and 51% (50 out of 98) pregnancy rates following slow cooling. They stated that vitrification is a simple, efficient and cost-effective way to improve cumulative pregnancy rates per cycle.

Table 2. Outcome of recent studies on the vitrification of human blastocysts.

Study	Cryoprotectant	Cryocarrier	Vitrified embryos (n)	SR (%)	PR (%)	IR (%) or special notes	Ref.
Liebermann and Tucker (2006)	EG	Cryotop	254	D5: 95.9 D6: 97.5	48.7 42.8	33 25	[38]
Utsunomiya *et al.* (2006)		Straw	142 cycles	Protocol 1: 87 Protocol 2: 89.6 Protocol 3: 89.8	Protocol 1: 35.0 Protocol 2: 32.0 Protocol 3: 11.1	Protocol 1: 25.9 Protocol 2: 22.8 Protocol 3: 9.4	[58]
Zech *et al.* (2005)	DMSO/EG	Hemistraw	177	64–82	21–35	SR increases with intact ZP	[57]
Takahashi K *et al.* (2005)	DMSO/EG/S	Cryoloop	1129	85.7	44	Congenital defects 1.4%	[50]
Huang CC *et al.* (2005)	DMSO/S/EG/HSA	Cryoloop	249	77.1	53.8		[39]
Stehlik *et al.* (2005)	EG-based	Cryotop	41	100	50		[40]
Hiraoka *et al.* (2004)	DMSO/EG	Cryotop	49	98	50	33	[55]
Vanderzwalmen *et al.* (2003)	DMSO/EG	Hemistraw	281	60	27 (ongoing)	AH more favorable implantation rate	[7]
Mukaida *et al.* (2003)	EG-based	Cryoloop	444	79	36		[61]
Mukaida *et al.* (2003)	EG-based	Cryoloop	725	80.4	37	Day 5 survival rate is higher (87%)	[9]

AH: Assisted hatching; DMSO: Dimethylsulphoxide, EG: Ethylene glycol; IR: Implantation rate; PR: Pregnancy rate; S: Sucrose; SR: Survival rate; ZP: Zona pellucida.

As mentioned in vitrification of early-stage embryos, the zona pellucida can also be damaged and more hardened, which is presumed to cause a reduction in implantation, due to freezing and vitrification procedure [7,48,56]. Assisted hatching was added to the freezing and thawing procedure and performed prior to transfer of vitrified embryos. Adjunction of assisted hatching has been found to be beneficial in vitrification cycles by increasing pregnancy and implantation [7]. Furthermore, blastocysts with intact zona pellucidae demonstrated increased survival and resistance compared with those with partial or total loss of zona. However, successful cryopreservation of blastocysts, including those totally hatched, lost or escaped from their zona was also suggested in this study, with acceptable rates of survival and pregnancy [57].

Asynchrony between embryo development and endometrial differentiation is the limiting step of successful pregnancy in assisted reproduction. Utsunomiya *et al.* investigated whether or not post-thaw synchronization culture of day 5–6 vitrified embryos, prior to transfer, with endometrial differentiation resulted in pregnancy [58]. They concluded that developmentally retarded frozen embryos can be rescued with synchronizing culture prior to transfer by evading asynchrony.

Since blastocysts are much better suited to the uterine milieu, and because blastocyst formation is a form of selection for more viable embryos, blastocyst transfer has become a promising option to raise the pregnancy rate. Currently, the availability of sequential media has led to a dramatic increase in the practice of blastocyst freezing and much higher pregnancy rates. Reports have documented that the survival rates of human blastocysts using different vitrification protocols are approximately more than 90% with a pregnancy rate of over 50%.

Expert commentary

Cryopreservation of human embryos has progressed to become a useful tool in human IVF embryo-transfer programs. It is vital to avoid embryo wastage and to augment the pregnancy rate from single-oocyte retrieval. In addition, cryopreservation makes the postponement of embryo transfer in a future cycle possible, thus decreasing the incidence of ovarian hyperstimulation syndrome in high-risk patients.

Therefore, it is essential to establish a simple and reliable procedure to optimize human embryo cryopreservation, a prerequisite of which is developing a better understanding of the factors that influence their survival and implantation potential in order to have the utmost clinical outcome.

Human embryo cryopreservations, prove that vitrification is a simple, inexpensive and safe alternative of traditional slow cooling, resulting in higher survival and *in vitro* developmental rates for PN, multicellular and blastocyst-stage human embryos. With healthy live births following vitrification, the method can be accepted, without any doubt, as a safe procedure.

The main drawback of this method is the exposure of the embryos to a high concentration of cryoprotectant, which may have a detrimental effect. This can be minimized by allowing a very short exposure of the embryos to the cryoprotectants (i.e., 30–40 s), combination of one or more cryoprotectant and using a cryoprotectant thats has a less toxic effect on the embryos.

Today, we have to admit that vitrification is gradually replacing slow freezing for cryopreservation of human embryos at different development stages, as well as chill-sensitive cells such as oocytes. The possibility of easy and safe application of early preimplantation techniques and *in vivo* maturation procedure are also other advantages where professionals have benefitted from vitrification of embryos at all stages.

Recently achieved results using vitrification appear to convince more and more professionals of the advantages of this technique, reflected by the increasing number of publications and also by the number of commercial kits introduced for vitrification.

Five-year view

One of the central issues is whether slow-cooling or rapid-cooling protocols that have been employed in human IVF program satisfy the fundamental principle of minimizing damage during cryostorage. The extensive reports to date exhibit ongoing improvements in vitrification methods, and further support the overall efficacy of vitrification as a viable alternative to cryopreservation by slow-cooling methods. The early, long vitrification protocols have been replaced by very short and safer procedures, and it is envisaged that the time has come when vitrification of human oocytes and embryos will be used more widely.

The forthcoming challenge is that of oocyte vitrification. Reports published to date indicate that this procedure is significantly better than traditional slow-freezing methods. In comparing the principles, procedures and results of slow cooling and vitrification protocols, both methods resulted in the successful cryopreservation of human oocytes, although slow cooling gives much lower success rates. Although in the last few years, slow-freezing protocols have shown an increase in survival rates, even up to 90%, the pregnancy rate per thawed oocyte is up to 4.2%, which remains poor, appearing to be much lower in comparison with vitrification at 11.2%. Currently, prospective randomized studies are being conducted in some centers comparing these two methods. This will give evidence that vitrification will be the method of choice in future.

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Key issues

- Human gametes and embryos encounter subzero temperatures and other nonphysiological conditions in both the slow-cooling and vitrification methods of cryopreservation.
- Freezing and storing of surplus embryos also allow the number of replaced embryos in both fresh and frozen embryo transfers to be reduced, thereby diminishing the risk of multiple pregnancies.
- All embryos could be cryopreserved if the woman has a risk of developing ovarian hyperstimulation syndrome.
- The survival and pregnancy rates obtained from vitrification of human oocytes and embryos are at least equal to, or significantly better than, those obtained with traditional slow cooling.
- Vitrification appears to be a rapid and simple technique that favors the biological integrity of both human oocytes and embryos, resulting in higher survival and pregnancy rates.

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