A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos

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Submitted on October 4, 2011; resubmitted on February 29, 2012; accepted on March 28, 2012

TABLE OF CONTENTS
- Introduction
- Methods
- Principles and methodology of slow cooling and vitrification
- Outcomes from MII oocytes
- Outcomes from pronuclear stage oocytes
- Outcomes from cleavage stage embryos
- Outcomes from blastocysts
- Other considerations
- Overall conclusions

BACKGROUND: Vitrification is now a commonly applied technique for cryopreservation in assisted reproductive technology (ART) replacing, in many cases, conventional slow cooling methodology. This review examines evidence relevant to comparison of the two approaches applied to human oocytes and embryos at different developmental stages.

METHODS: Critical review of the published literature using PubMed with particular emphasis on studies which include data on survival and implantation rates, data from fresh control groups and evaluation of the two approaches in a single setting.

RESULTS: Slow cooling is associated with lower survival rates and compromised development relative to vitrification when applied to metaphase II (MII) oocytes, although the vitrification results have predominantly been obtained using direct contact with liquid nitrogen and there is some evidence that optimal protocols for slow cooling of MII oocytes are yet to be established. There are no prospective randomized controlled trials (RCTs) which support the use of either technique with pronuclear oocytes although vitrification has become the method of choice. Optimal slow cooling, using modifications of traditional methodology, and vitrification can result in high survival rates of early embryos, which implant at the same rate as equivalent fresh counterparts. Many studies report high survival and implantation rates following vitrification of blastocysts. Although slow cooling of blastocysts has been reported to be inferior in some studies, others comparing the two approaches in the same clinical setting have demonstrated comparable results. The variation in the extent of embryo selection applied in studies can lead to apparent differences in clinical efficiency, which may not be significant if expressed on a ‘per oocyte used’ basis.

CONCLUSIONS: Available evidence suggests that vitrification is the current method of choice when cryopreserving MII oocytes. Early cleavage stage embryos can be cryopreserved with equal success using slow cooling and vitrification. Successful blastocyst cryopreservation may be more consistently achieved with vitrification but optimal slow cooling can produce similar results. There are key limitations associated with the available evidence base, including a paucity of RCTs, limited reporting of live birth outcomes and limited reporting of detail which would allow assessment of the impact of differences in female age. While vitrification has a clear role in ART, we support continued research to establish optimal slow cooling methods which may assist in alleviating concerns over safety issues, such as storage, transport and the use of very high cryoprotectant concentrations.

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Slow cooling versus vitrification of oocytes and embryos

**Key words:** cryopreservation / human oocytes / human embryos / vitrification / slow cooling

**Introduction**

Cryopreservation is of crucial, and increasing, importance in reproductive medicine. Since the mid-1980's, cryopreservation and storage of in vitro-derived cleavage stage embryos has been employed to ensure that most of the fertility potential of an oocyte-collection cycle is made available to patients without the need for simultaneous transfer of multiple embryos. In the past decade, this has helped to drive clinical practice towards single embryo transfer and dramatically reduced the multiple pregnancy rate in many countries.

Two specific developments in assisted reproductive technology (ART) have raised new challenges for embryologists and cryobiologists. First, in order to improve embryo selection for single embryo transfer, the use of culture systems which support development to the blastocyst stage in vitro became more common, establishing a need for cryopreservation methodology which would be appropriate for these more complex biological entities. Second, there has been a resurgence of interest in the cryopreservation of oocytes. This was driven by a desire to preserve fertility potential in young women undergoing gonadotoxic treatments and also by restrictive legislation which prevented embryo cryopreservation in some countries, but has found other applications in areas such as oocyte donation. Since cryopreservation of metaphase II (MII) oocytes was widely viewed as inefficient, methodology which would increase efficiency was a necessary prerequisite for progress in this area.

In the particular context of the above challenges, but also more generally, the technique of vitrification has been widely employed in recent years at the expense of the more traditional approach of cryopreservation by slow cooling. While the governing principles may remain relatively constant, the methodology used for vitrification may vary significantly between laboratories and this is also true for slow cooling. This point and the need for randomized controlled trials (RCTs) carried out under optimal conditions for both slow cooling and vitrification were made out in this review. The order of preferential inclusion was (i) comparative studies in a single setting, randomized when available, (ii) studies with adequate non-cryopreserved controls, (iii) studies with adequate non-cryopreserved controls, (iv) substantial studies with survival and implantation rates and (iv) substantial studies with survival data only.

**Principles and methodology of slow cooling and vitrification**

**Principles**

The aim of cryobiology is to shift the pendulum from cell death to immortality at low temperatures. To achieve this, it is necessary to eliminate the two main causes of cell death associated with cryopreservation, i.e. ice formation (Mazur, 1963) and lethal concentrations of solutes (Kleinman and Mazur, 2007) while maintaining the functional capacity of intracellular organelles. Expulsion of intracellular water and, consequently, reduction in intracellular ice formation is facilitated by exposure to cryoprotectants that either permeate the cell membrane displacing water via an osmotic gradient, e.g. propanediol (PROH), dimethyl sulphoxide (DMSO) or ethylene glycol (EG) and/or cryoprotectants which provide a continuous gradient but do not permeate the cell membrane, e.g. sucrose. Permeating cryoprotectants also aid in balancing other intracellular solutes which are lethal at high concentrations. These hyper-osmotic lethal conditions also occur in domains between ice crystals in the extracellular environment as the temperature is reduced and, as a consequence, further dehydration of the cell occurs. The extent of dehydration is dependent on the rate at which the temperature is decreased (Leibo et al., 1978) and the permeability of the cell(s) to water (Leibo, 1980). These principles are fundamental to cryopreservation but are applied in different ways in the methods used for slow cooling and vitrification in clinical ART.

**Slow cooling**

Prior to slow cooling of both oocytes and embryos, dehydration without excessive shrinkage is achieved by exposure to permeating cryoprotectants at concentrations ≤ 1.5 M and non-permeating cryoprotectants at concentrations ≤ 0.3 M. During slow cooling, the dehydration process is thought to continue until −30°C, after which any
remaining water is super cooled (Mazur et al., 2005). When an appropriate rate of cooling is applied, sufficient dehydration is achieved while limiting exposure to extracellular hyper-osmotic conditions. There is no definitive evidence to support the conclusion that the cooling rates currently applied to human embryos and oocytes are optimal. The rates currently employed were initially established by Whittingham et al. (1972) and have remained largely unaltered in clinical practice. The necessity to limit the duration of exposure to hyper-osmotic environments may not, however, be as critical as optimal dehydration, as suggested by the increased survival rates achieved using higher sucrose concentrations prior to slow cooling of oocytes (Fabbri et al., 2001; De Santis et al., 2007a) and, more recently, embryos (Edgar et al., 2009). In this respect, the zona pellucida may be the key, providing protection from the external environment and limiting ice seeding within the oocyte/embryo. This would be supported by the observation of reduced cryosurvival in embryos in which the zona has been breached to facilitate blastomere biopsy for PGD (Joris et al., 1999; Magli et al., 1999; Jericho et al., 2003). Dehydration is dependent, fundamentally, on the permeability of the cell membrane to water (referred to as the hydraulic permeability) and this property can vary by as much as 7-fold between individual human MII oocytes (Hunter et al., 1992; Van den Abbeel et al., 2007). This variation can have a major impact on the success of slow cooling of oocytes [review (Gook and Edgar, 2007)] but can be circumvented to some extent by increasing the osmotic gradient via exposure to a higher extracellular sucrose concentration (Fabbri et al., 2001) and/or increasing the rate of dehydration via a higher initial dehydration temperature (Yang et al., 2007; Gook and Edgar, 2011).

In practice, slow cooling routinely involves equilibration of oocytes or embryos in one or more dehydrating solutions for periods up to 10 min before loading (in a volume of ~200 μl) into plastic straws which are sealed at both ends. Straws are then placed in the chamber of a programmable freezing machine which slowly reduces the temperature (~0.3°C/min) to ~−30°C. During this cooling phase, ice nucleation (seeding) is induced manually at between −5 and −8°C. On reaching −30°C, the temperature is then reduced rapidly (at ~−50°C/min) to −150°C before storage in liquid nitrogen. Rapid thawing is followed by rehydration in decreasing concentrations of permeating cryoprotectant, usually in the initial presence of increased concentrations of non-permeating cryoprotectant.

Vitrification

The principle of dehydration also applies in vitrification but concepts such as hydraulic permeability play a less significant role. To achieve vitrification (i.e. a glass-like state) within a cell, both a reduction in water content and a highly viscous cytoplasm are necessary (Rall and Fahy, 1985). This is facilitated by exposure to high concentrations of permeating (>4 M) and non-permeating (≥0.5 M) cryoprotectants which result in extreme shrinkage. However, in order to minimize the impact of the hyper-osmotic conditions the exposure time is reduced to <1 min (Shaw et al., 1992). Since a high concentration of a single permeating cryoprotectant, however, may in itself be lethal (Ali and Shelton, 1993) or may result in impaired development (Wood et al., 1993), multiple cryoprotectants (Rall et al., 1987) have been used in combination to reduce the individual cryoprotectant toxicity while achieving a highly viscous solution (Ali and Shelton, 1993). It is possible to successfully vitrify using reduced cryoprotectant concentrations (<3 M) if a sufficiently rapid reduction in temperature is achieved by direct contact of the oocyte/embryo with liquid nitrogen, an approach which will also require a similarly rapid rate when warming (Vajta and Nagy, 2006).

In practice, vitrification is routinely achieved by exposure of oocytes/embryos initially to concentrations of permeating cryoprotectants (commonly combinations of EG, DMSO or PROH) similar to those employed prior to slow cooling, followed by a short (<1 min) exposure to the high concentrations described above before loading on to a variety of microtools (Cryotop: Kuwayama et al., 2005a, Cryoloop: Lane et al., 1999, Hemi straw: Vanderzwalmen et al., 2003 and Flexipet: Liebermann et al., 2002) in a very small (0.1–2 μl) volume to facilitate rapid cooling. Rapid reduction in temperature (>10,000 °C/min) is achieved by immediate exposure to liquid nitrogen in either an open or closed system. Importantly, equivalent rapid warming is required and is followed by stepwise rehydration using approaches similar to those employed after slow cooling.

Assessment of outcome

Whether cryopreservation is by slow cooling or vitrification, it must be emphasized that survival is necessary, but not sufficient, to ensure success. It is also essential that normal physiological function is retained. For example, while high survival rates have been reported following slow cooling of oocytes dehydrated in PROH with 0.3 M sucrose, there is also evidence of poor ultrastructure (Nottola et al., 2007), embryo development (Bianchi et al., 2007) and implantation (Levi Setti et al., 2006). Similar issues may also be true with vitrification. As mentioned above, ultrarapid cooling is a critical feature of vitrification and the need to accommodate safety considerations by using closed vitrification systems must be balanced against the likely impact in terms of reducing the cooling rate. Although similar survival rates can be achieved with open or closed systems, the observation of multiple vesicles throughout the cytoplasm of oocytes vitrified using a closed vitrification system (Bonetti et al., 2011), a likely consequence of minuscule ice crystal formation, suggests insufficiently rapid temperature reduction.

Ultimately, the success of cryopreservation will be defined by clinical efficiency. In assessing this we must also consider factors which may affect comparisons between methodologies, particularly in relation to pre- and post-cryopreservation selection of biological material (Edgar and Gook, 2007a; Gook and Edgar, 2009) and also compare outcomes with those from appropriate non-cryopreserved controls. In this appraisal of the relative efficacy of slow cooling and vitrification, we will address two key questions. We will assess the proportion of oocytes/embryos which survive cryopreservation using the various methods and we will ask whether a method results in impairment of developmental and/or implantation potential in surviving oocytes/embryos relative to non-frozen controls. In combination, the answers to these questions should allow us to compare the clinical efficiency of each technique.

Outcomes from MII oocytes

Slow cooling

The first reports of implantation of human embryos derived from oocytes cryopreserved by slow cooling, using DMSO as the
cryoprotectant, appeared in the 1980s (Chen, 1986; Al-Hasani et al., 1987; Van Uem et al., 1987; Siebzenruebl et al., 1989). However, failure to reproduce the initial success combined with concerns over the safety of oocyte cryopreservation ensured that this clinical activity was short lived. The current era of oocyte cryopreservation had its foundations in biological studies carried out in the early 1990s which alleviated many of the safety concerns (Gook et al., 1993, 1994, 1995a, b). These studies validated the feasibility of using a slow cooling method based on the cryoprotectants PROH (1.5 M) and sucrose (0.1 M), which had been developed for use with human embryos, for the cryopreservation of human MII oocytes. The subsequent clinical application of this method resulted in a live birth (Porcu et al., 1997) and the method was adopted by a number of groups, specifically in Italy where impending legislation was to prohibit embryo cryopreservation.

A number of pregnancies and births were subsequently reported following transfer of relatively small numbers of embryos derived from oocytes cryopreserved by this method (Antinori et al., 1998; Nawroth and Kissing, 1998; Polak de Fried et al., 1998; Tucker et al., 1998; Young et al., 1998; Wurfel et al., 1999; Chia et al., 2000; Porcu et al., 2000, 2002; Huttelova et al., 2003; Notrica et al., 2003; Allan, 2004; Borini et al., 2004; Miller et al., 2004; De Santis et al., 2007b) but the available data did not include reference to parallel non-frozen controls. The only published study, using this method, which allows assessment of the impact of cryopreservation on developmental and/or implantation potential was that of Borini et al. (2006a) in which the outcomes from 705 thawed oocytes were reported together with the outcomes from over 150 fresh oocytes generated in the same cycles. The implantation rate of transferred embryos derived from thawed oocytes was 12.3% compared with a rate of 22.6% for embryos derived from fresh oocytes. Although this is suggestive of impaired embryonic developmental competence in the thawed oocyte group, no definitive conclusion based on statistical significance can be claimed. What is unequivocal, however, in relation to slow cooling of human oocytes with PROH and 0.1 M sucrose, is that only ~50% of thawed oocytes survive cryopreservation (Tucker et al., 1996; Porcu et al., 1997; Antinori et al., 1998; Nawroth and Kissing, 1998; Polak de Fried et al., 1998; Tucker et al., 1998; Young et al., 1998; Wurfel et al., 1999; Chia et al., 2000; Porcu et al., 2000., 2002; Huttelova et al., 2003; Notrica et al., 2003; Allan, 2004; Borini et al., 2004; Miller et al., 2004; De Santis et al., 2007b; Gook and Edgar, 2007; Greco et al., 2008). This, together with fertilization rates which appeared to be reduced (just over 50% overall) relative to those normally observed with fresh oocytes, resulted in low overall efficiency (Gook and Edgar, 2007).

The relative inefficiency of these early attempts to apply oocyte cryopreservation clinically led to the introduction of modified techniques. Understandably, much of the focus was on development of methods which would increase the proportion of oocytes which would survive cryopreservation. Increasing the level of prefreeze dehydration by increasing the concentration of non-permeating cryoprotectant was introduced and higher survival rates were reported using 0.2 M sucrose (Fabbri et al., 2001; Chen et al., 2004) and 0.3 M sucrose (Fabbri et al., 2001). The overall fertilization rates of oocytes cryopreserved using elevated sucrose concentrations appeared to be similar to those of fresh oocytes (Fosas et al., 2003; Chen et al., 2005; Li et al., 2005; Tjer et al., 2005; Borini et al., 2006b; Chamayou et al., 2006; Levi Setti et al., 2006; Barritt et al., 2007; Edgar and Gook, 2007a; De Santis et al., 2007b; Gook and Edgar, 2007; Konc et al., 2008a; Parmegiani et al., 2008, 2009; Ferraretti et al., 2010) but, again, the data from many studies did not allow valid comparisons with non-frozen controls. In a comparison of oocytes slow cooled using 0.1 or 0.3 M sucrose (De Santis et al., 2007a, b), survival and fertilization rates were higher but the implantation rate was lower in the 0.3 M sucrose group. We will now consider studies in which elevated sucrose concentrations were used and which did include reference to fresh controls.

First, we will summarize results using 0.3 M sucrose. A study of 40 fresh and 40 oocyte cryopreservation cycles by Chamayou et al. (2006) demonstrated equivalent fertilization rates and a suggestion of reduced cleavage in the cryopreservation group but the very low implantation rates in both groups make further conclusions impossible.

In a study of 303 fresh and 159 thawing cycles (Levi Setti et al., 2006), fertilization and cleavage rates were similar in both groups but the implantation rate was significantly reduced in the cryopreserved group (5.7 versus 23.2%). Slower development relative to fresh controls, both with respect to timing of the first cleavage division and the developmental stage reached on Day 2, was reported in oocytes slow cooled in 0.3 M sucrose (Bianchi et al., 2005), and the same group (Borini et al., 2006b) subsequently reported a high proportion of embryos (68.3%) which had only completed one cleavage division to the 2-cell stage on Day 2 of development using the same method. Decreased embryo quality in the cryopreserved oocyte group was also reported in an age-stratified study (Magli et al., 2010). Comparable fertilization rates but significantly slower development in the cryopreserved group were reported by Konc et al. (2008b), although implantation rates were similar in this small study, in which assisted hatching of embryos was only performed in the cryopreserved group.

Data from the Italian National Register (Scaravelli et al., 2010) suggests an odds ratio of ~2.0 when comparing implantation rates from fresh and cryopreserved oocytes over a period when cryopreservation was likely to involve predominantly slow cooling in 0.3 M sucrose.

Combined data from oocyte slow cooling carried out in 0.2 and 0.3 M sucrose (Borini et al., 2007) showed significantly reduced pregnancy and implantation rates in embryos derived from cryopreserved versus fresh oocytes.

Using 0.2 M sucrose for dehydration prior to slow cooling, Yang et al. (2002) reported a decreased implantation rate in embryos from cryopreserved oocytes compared with sibling fresh controls. Using a protocol involving dehydration in 0.2 M sucrose and rehydration in a differential sucrose concentration (0.3 M) after slow cooling (Bianchi et al., 2007), equivalent fertilization, cleavage, pregnancy and implantation rates to a fresh control group were reported in women under 38 years of age. A subsequent report (Borini et al., 2010) showed reduced implantation and pregnancy rates relative to fresh controls using this method but this report was based on a multicentre observational study and was not stratified according to female age. Our own recent results (Gook and Edgar, 2011), using dehydration in 0.2 M sucrose at 37°C prior to slow cooling, show equivalent implantation rates for embryos derived from cryopreserved and fresh oocytes in women under 38 years of age.
An alternative approach involving the use of sodium-depleted medium in conjunction with PROH and sucrose (the latter at concentrations of 0.1, 0.2 and 0.3 M) as cryoprotectants during slow cooling has been reported in studies involving relatively small numbers of oocytes (Quintans et al., 2002; Boldt et al., 2006; Petracco et al., 2006; Stachecki et al., 2006; Azambuja et al., 2011). However, the clinical results (reviewed by Gook and Edgar 2007) do not suggest improved outcomes relative to those obtained with more conventional basal media.

Although survival of slow cooled oocytes has improved with the introduction of sucrose concentrations >0.1 M during prefreeze dehydration, evidence from widespread practice suggests that a plateau has been reached at 70–80% (Gook and Edgar 2007) with currently available methodology.

In summary, the weight of available evidence suggests that embryos derived from oocytes cryopreserved by slow cooling have compromised development and implantation relative to fresh controls. This is with the caveat that there is a shortage of adequately controlled studies (one of which suggests equivalent outcomes) and also the suggestion that technical improvements, such as limiting the time from oocyte collection to cryopreservation (Parmegiani et al., 2008, 2009; Ferraretti et al., 2010), may improve outcomes. In addition to the apparent impact of slow cooling on oocyte developmental potential, the current expectation is that 20–30% of slow cooled oocytes will not survive after thawing. The current evidence in the literature does not suggest that there is a single available slow cooling method which can improve this outlook. Does cryopreservation of human MII oocytes by vitrification offer a better prospect?

Vitrification

The first successful clinical application of human oocyte vitrification (Kuleshova et al., 1999) involved the use of a very high concentration of a single permeating (7.1 M EG) and a single non-permeating (0.6 M sucrose) cryoprotectant and resulted in a single live birth. A modification of this approach (5.5 M EG, 1.0 M sucrose) was used successfully in other small studies (Yoon et al., 2000; Wu et al., 2001; Yoon et al., 2003; Kuwayama et al., 2005a; Kim et al., 2010) before a method based on a mixture of permeating cryoprotectants (2.7 M EG and 2.1 M DMSO) and 0.5 M sucrose was introduced (Katayama et al., 2003) and became the most widely adopted method (Kyono et al., 2005; Okimura et al., 2005; Lucena et al., 2006; Selman et al., 2006; Antinori et al., 2007).

In a study reporting outcomes from 251 fresh and 120 vitrified oocytes (99.4% survival), fertilization rates, embryo development to Day 3 and implantation rates were shown to be equivalent for oocytes vitrified by this method and fresh control oocytes (Antinori et al., 2007). These findings were confirmed independently in a study involving over 200 oocytes in each group (96.9% survival after vitrification and warming), which also demonstrated equivalent development to the blastocyst stage following vitrification (Cobo et al., 2008a). The latter group have, more recently, published their findings from an RCT of over 3000 fresh and 3000 vitrified oocytes (92.5% survival) in an oocyte donation programme (Cobo et al., 2010a), confirming no detrimental effects of vitrification on subsequent fertilization, development or implantation. Further evaluation of this form of vitrification in the context of oocyte donation programmes (Nagy et al., 2009; Garcia et al., 2011; Trokoudes et al., 2011) again demonstrated fertilization, embryo development and implantation rates using vitrified oocytes (ca. 90% survival in these studies) which were similar to those from the use of fresh oocytes.

The results reported from application of this vitrification technique in a standard infertility programme (Rienzi et al., 2010; Ubaldi et al., 2010; Paffoni et al., 2011) showed no significant differences in fertilization rates or proportion of top-quality Day 2 embryos between fresh and vitrified (80–90% survival) oocytes. Implantation rates in fresh and vitrification cycles were not significantly different for women ≤34 years (Ubaldi et al., 2010). Although there was a trend towards lower overall clinical outcomes from vitrified oocytes, it was emphasized that the outcomes from vitrified oocytes were only being assessed in patients who failed to become pregnant from the preceding fresh embryo transfer, introducing a bias which precludes valid conclusions. These results were essentially mirrored in a study reported by Almodin et al. (2010) who also applied oocyte vitrification (84.9% survival) in the context of an infertility programme.

In summary, there is now a substantial body of evidence, much of it generated from optimal biological material, i.e. oocytes donated by young women, to suggest that vitrified oocytes are functionally equivalent to their fresh counterparts in terms of fertilization, development and implantation potential. Importantly, in addition to this, most studies report survival rates of around 90% for vitrified oocytes, a value which has not been obtained with slow cooling.

Comparison of slow cooling and vitrification

The most valid way to compare outcomes from slow cooling and vitrification of MII oocytes would be to assess their relative efficiency in a single setting where the biological material and the handling and culture methods are identical. Few such studies have been reported. Two small studies (Grifo and Noyes, 2009; Noyes et al., 2010) comparing outcomes from the most commonly used vitrification solutions in conjunction with a closed vitrification system (Cryotip) and slow cooling in PROH with 0.3 M sucrose, suggested equivalent survival and a trend towards reduced development in the vitrification group but no conclusions relating to clinical outcomes could be made. While the slow cooling method used in these studies may not be optimal, it must also be noted that vitrification in these studies was not performed using the more commonly used open system protocol which facilitates higher cooling and warming rates. Both survival and development rates were higher following vitrification of oocytes donated for research relative to a slow cooled (0.3 M sucrose) group (Cao et al., 2009).

A prospective RCT of slow freezing and vitrification of MII oocytes (Smith et al., 2010) showed increased survival, fertilization, development and clinical pregnancy rates in the vitrification group. Vitrification was also associated with increased survival, fertilization and implantation rates relative to slow cooling in a retrospective study reported by Fadini et al. (2009). Both these studies used the PROH + 0.3 M sucrose method in the slow cooling arm and it should be emphasized (see slow cooling component earlier in this section) that this may not be the optimal approach for slow cooling of oocytes.
Conclusions
There is now reproducible evidence to support the conclusion that vitrification as first described by Katayama et al. (2003) is capable of preserving normal developmental potential in human MII oocytes and that around 90% of vitrified oocytes will survive cryopreservation by this approach. In contrast, and accepting that improved methods may still be developed, the currently available methods for slow cooling of human MII oocytes appear to impact negatively on subsequent developmental potential and also result in survival rates which are lower than those reported for vitrification. Despite the fact that there is a paucity of controlled studies comparing the two approaches, vitrification would now appear to be the methodology of choice for human oocyte cryopreservation in terms of clinical efficiency. It should, however, be borne in mind that the results obtained with human oocyte vitrification have been achieved predominantly using open systems which involve direct contact with liquid nitrogen. The issues associated with this will be discussed later in this review.

Outcomes from pronucleate stage oocytes

Slow cooling
In a clinical IVF context, cryopreservation of human fertilized oocytes and embryos can offer distinct advantages over cryopreservation of MII oocytes. Importantly, it eliminates oocytes which are not capable of fertilization and allows selection of the ‘best’ embryos for fresh transfer on the basis of developmental and morphological markers. For these reasons, most clinics would prefer to cryopreserve surplus embryos at early cleavage stages (2–8 cells) or later when evidence of developmental potential is available. However, cryopreservation involving slow cooling of oocytes at the unicellular pronucleate stage following fertilization has been performed for a number of reasons.

Although there were no conclusive data published, early experience suggested that success rates may be improved by slow cooling at the pronuclear stage relative to early cleavage stages. In a study using PROH and sucrose as cryoprotectants including almost 500 embryos in each group (Demoulin et al., 1991), survival rates of 54% for pronucleate stages and 47% for cleavage stages were reported with significantly higher implantation rates in the pronuclear group (10.7 versus 4.7%). In a large series of over 2000 thawed pronucleate stage oocytes (Veeck et al., 1993), a survival rate of 68% (using PROH only as a cryoprotectant) was reported and pregnancy rates were reported to be similar to those from fresh embryo transfer when corrected for the number of embryos transferred. Using stricter criteria for timing of cryopreservation and PROH + 0.1 M sucrose as cryoprotectants (Damario et al., 1999), a survival rate of ~90% and an implantation rate of 19.5% was reported.

Cryopreservation of all embryos is often performed when fresh transfer is contraindicated, usually because of risk of ovarian hyperstimulation syndrome (OHSS). Since fresh embryo selection is not required in these cases, cryopreservation (slow cooling with PROH and 0.1 M sucrose) has often been performed at the pronucleate oocyte stage. Under such circumstances, survival rates of 74% (Queenan et al., 1997) and over 90% (Hoover et al., 1997; Tatpati et al., 2010) have been reported together with pregnancy rates (from the transfer of a mean of approximately three embryos) of 16% to over 50%. However, in order to more accurately assess the impact of slow cooling on pronucleate oocytes, it is necessary to evaluate studies in which fresh controls have been reported.

In a prospective randomized study of cases involving slow cooling of pronucleate stage oocytes (PROH + 0.1 M sucrose) to avoid risk of OHSS, Ferraretti et al. (1999) reported 78% survival in association with an implantation rate of 15% compared with an implantation rate of 19% in the control group when fresh transfers were performed (not significantly different). Using the same method (Damario et al., 2000), a survival rate of 90% and an implantation rate of 21.5% was reported from cryopreserved pronucleate oocytes compared with an implantation rate of 25.3% in fresh controls (not significantly different). Using an approach based on post-thaw extended culture (to the blastocyst stage) of slow cooled pronucleate oocytes, Shapiro et al. (2009) reported a survival rate of 85% and implantation rate of 64.2% in women under 35 years of age and subsequently reported (Shapiro et al., 2010) that this strategy resulted in increased implantation rates when compared with fresh blastocyst transfer in cycles with elevated pre-ovulatory progesterone (premature luteinization). This improved clinical outcome was observed despite a significantly reduced blastulation rate (19.7%) in thawed versus fresh (36.8%) pronucleate oocytes suggesting that it may be due to a more appropriate endometrial environment which overrides the decreased blastulation rate in these cases. However, this study does suggest that slow cooling has an inhibitory effect on post-thaw development to the blastocyst stage.

Other than in cases where risk of OHSS is anticipated, cryopreservation of pronucleate oocytes was largely abandoned in favour of cryopreservation of cleavage stages, which offered the advantage of embryo selection as discussed earlier in this section. This was supported by a study of Kattera et al. (1999) who reported a significantly increased survival rate (73.9%) in cleavage stage embryos compared with pronucleate oocytes (64.4%). However, in some countries, including Germany and Switzerland, legislation precludes the cryopreservation of cleavage stage embryos and leaves storage at pronucleate stages as the preferred option. A survival rate of 77%, a pregnancy rate of 18% and an implantation rate of 6–7% was reported (Al-Hasani et al., 1996) using slow cooling of pronucleate oocytes with PROH and 0.1 M sucrose. Unfortunately, no control fresh data were presented in this study. In a subsequent study from the same centre (Schroder et al., 2003), however, the pregnancy rate of 10.2% in the frozen-thawed group was lower than that in the fresh transfer group (28.0%).

Vitrification
Case reports of pregnancies from pronucleate oocytes vitrified using EG and sucrose (Jelinkova et al., 2002; Selman and El-Danasouri, 2002) demonstrated the feasibility of this approach. Using a similar approach, Iachenko et al. (2003) reported a survival rate of 71.1% from 59 vitrified/warmed pronuclear oocytes with 4 clinical pregnancies established from the transfer of 26 embryos. Results from application of vitrification with EG, DMSO and sucrose using the Cryotop in the context of German legislation (Al-Hasani et al., 2007) showed a survival rate of 89%, an implantation rate of 15.6% and a pregnancy...
rate of 36.9%. Although no parallel control group was included, the authors compared these outcomes to their previously reported pregnancy rate of 10.2% using slow cooling (Schroder et al., 2003). In cycles where all pronuclear oocytes were vitrified owing to risk of OHSS, the same group also reported a survival rate of 77.8% and a pregnancy rate of 29.2% in subsequent warming cycles (Griesinger et al., 2007).

**Comparison of slow cooling and vitrification**

In a large series of 5881 vitrified and 1944 slow cooled pronuclear oocytes, support for the reproducibility and effectiveness of vitrification was provided by Kuwayama et al. (2005b), using the Cryotop and EG with DMSO and sucrose as cryoprotectants, as described earlier for unfertilized oocytes. In this study, survival, cleavage and blastulation rates were higher for vitrified (100, 93 and 56%, respectively) than that for slow cooled (89, 90 and 51%, respectively) pronucleate oocytes. The authors stated that pregnancy and live birth rates did not differ between the two methods, although no data were included in the report.

**Conclusions**

Although there is some evidence to support the conclusion that implantation and pregnancy rates from slow cooled pronuclear oocytes are equivalent to fresh rates (Ferraretti et al., 1999; Damario et al., 2000), there is also evidence that embryo development may be compromised by slow cooling (Shapiro et al., 2010). Developmental rates following vitrification may be slightly but significantly improved relative to following slow cooling (Kuwayama et al., 2005b). A group which had long clinical experience with slow cooling of pronucleate stage oocytes found improved outcomes when adopting vitrification (Al-Hasani et al., 2007). Overall survival rates appear to be somewhat higher with vitrification but rates of over 90% survival have also been achieved with slow cooling.

On balance, vitrification may seem to be the method of choice for cryopreservation of pronuclear oocytes but it is important to stress that no prospective randomized trials have been conducted. In addition, and unlike the situation with unfertilized oocytes and cleavage stage embryos (see later), there has been no attempt to improve the slow cooling methodology which may invalidate any absolute conclusion relating to the relative effectiveness of the two approaches in this context.

**Outcomes from cleavage stage embryos**

When considering outcomes from the cryopreservation of human embryos, there are a number of additional factors to consider, and these factors will impact significantly on the analysis of the efficiency of cryopreservation, whether by slow cooling or vitrification (Edgar et al., 2005). First, in most cases embryo cryopreservation will be carried out following selection of the ‘best’ embryo(s) for fresh transfer. Thus, comparisons of outcomes from fresh and cryopreserved embryos will require correction for any disparity in embryo quality. In addition, criteria for freezing human embryos may vary between clinics and this will impact on the clinical outcome. For example, a clinic which only cryopreserves the very top-quality embryos would be expected to retain high implantation rates but may be discarding some reproductive potential which other clinics may wish to harness.

Furthermore, unlike the situation with cryopreserved oocytes and zygotes, post-thaw survival of cryopreserved embryos is not an ‘all or nothing’ phenomenon and an embryo may survive with a proportion of its pre-freeze cells intact while other cells in the same embryo may have lysed. As such, cryosurvival should be defined in terms of both complete and partial survival. Clinical outcomes from the transfer of thawed embryos must also take into account the extent of survival in the transferred embryos and the threshold which has been used to define survival. Unfortunately, in many publications which report clinical outcomes from the transfer of cryopreserved embryos, cryosurvival and post-thaw selection details are not included. In this section, we will attempt to concentrate on reports which allow adequate analysis of key factors.

**Slow cooling**

Although the first reported clinical successes with slow cooled cleavage stage embryos were achieved using the cryoprotectant DMSO (Trounson and Mohr, 1983; Zeilmaker et al., 1984), the overwhelming majority of thawed embryos which have been transferred over the past 25 years were cryopreserved using PROH and sucrose as cryoprotectants (Lassalle et al., 1985). Historically, embryo survival was defined as survival of at least 50% of the pre-freeze blastomeres. The earliest reported experience based on outcomes from only 48 thawed cleavage stage embryos using PROH as a cryoprotectant (Testart et al., 1986) suggested that a survival rate of 87.5% was achievable and that survival was inversely related to the number of cells in the frozen embryo. Results from more extensive experience, based on a total of 405 thawed cleavage stage embryos (Hartshorne et al., 1990), showed that 322 (80%) embryos survived and that 197 (49%) had all cells surviving. This group also reported that moderate loss of cells did not significantly influence implantation. In a large multicentre series of over 14 000 cleavage stage embryos thawed over a 10 year period (Mandelbaum et al., 1998), 73% had at least half of their initial blastomeres still intact with clinical pregnancy and implantation rates of 16 and 8.4%, respectively, after transfer.

Although there were suggestions, from early experience, that the pregnancy rate from the transfer of surviving frozen-thawed embryos was similar to that from fresh transfer (Mandelbaum et al., 1987) and that this may be particularly true in the case of embryos which were of high quality prior to freezing (Selick et al., 1995), these studies were based on relatively small numbers and did not adequately control for many of the complicating factors discussed above. What is unequivocal, however, is that loss of blastomeres, as a result of cryopreservation, can decrease the developmental (Archer et al., 2003) and implantation (Van den Abbeel et al., 1997; Burns et al., 1999; Edgar et al., 2000; Guerif et al., 2002; El-Toukhy et al., 2003) potential of cleavage stage embryos. Importantly, in two of these studies (Edgar et al., 2000; Guerif et al., 2002), the implantation rate of fully intact embryos was reported to be similar to that of equivalent fresh embryos.

Increased application of single embryo transfer is dependent on effective embryo cryopreservation and has been advocated under circumstances in which 76.6% of cleavage stage embryos survived freezing and thawing (Tiitinen et al., 2001). The same group (Hyden-Granskog et al., 2005) has also shown that single embryo
transfer is a feasible option in frozen embryo transfer and this approach has allowed a more detailed investigation of the relationship between blastomere loss and implantation potential. In a study of over 3000 single cryopreserved embryo transfers (Edgar et al., 2007b), Day 2 embryos at the 4-cell stage which only lost a single blastomere during freezing and thawing had similar implantation potential to equivalent fully intact embryos and also to equivalent fresh embryos. In a further study of factors associated with implantation of thawed cleavage stage embryos (Gabrielsen et al., 2006), embryo survival of 76.5% was reported and, although the overall implantation rate of thawed embryos was lower than that of fresh embryos, those which survived intact and resumed development had rates comparable to fresh.

It has been suggested (Rienzi et al., 2002, 2005; Nagy et al., 2005) that removal of lysed blastomeres may improve the implantation rate of partially intact embryos, although the similar implantation rates observed when three out of four and four out of four blastomeres survive thawing (Edgar et al., 2007b) suggest that one lysed blastomere has no significant impact on implantation potential. Irrespective of this, the consensus from experience of slow cooling in 1.5 M PROH plus 0.1 M sucrose is that ≏70–80% of all cryopreserved cleavage stage embryos will survive after thawing and that ≏50% of all thawed embryos will be fully intact. The consistent observation that fully intact embryos (and, in some cases at least, those in which only a single blastomere is lost) exhibit implantation potential similar to equivalent fresh embryos suggests that the efficiency of cleavage stage embryo slow cooling will be largely determined by the precise survival rates achieved. In this respect, any improvement in cryosurvival over that reported for conventional methodology may be expected to yield improvements in clinical outcome.

The demonstration of reduced cryosurvival after slow cooling of cleavage stage embryos which had been biopsied for preimplantation genetic testing (Joris et al., 1999; Magli et al., 1999) led to a number of studies specifically designed to establish an improved method for this subset of embryos. Some of these studies employed vitrification approaches which will be covered in the following section of this review (Zheng et al., 2005, 2009; Kahraman and Candan, 2010). Improved survival of biopsied cleavage stage embryos was shown to be achievable by modification of the traditional slow freezing protocol, most notably by increasing the concentration of the non-permeating cryoprotectant, sucrose, prior to freezing (Jericho et al., 2003; Stachekci et al., 2005). However, the methodology for slow cooling of non-biopsied cleavage stage embryos (Lassalle et al., 1985) had remained essentially static for 20 years. A comparative study in which dehydration was performed in a single step using 1.5 M PROH as the permeating cryoprotectant (Edgar et al., 2009) demonstrated that doubling the concentration of non-permeating cryoprotectant (sucrose) from 0.1 to 0.2 M resulted in a highly significant increase in cryosurvival. The proportion of thawed embryos (n > 470 in each arm) which survived with at least half of the original blastomeres intact increased from 78.5 to 92.6%, the proportion of fully intact embryos increased from 54.6 to 80.5% and the proportion of surviving blastomeres increased from 74.1 to 91.1%. The implantation rate per embryo thawed was increased by 26.3% when 0.2 M sucrose was used for dehydration (22.1% versus 17.5%); this increase was not statistically significant but the primary end-point of the study was cryosurvival and significantly higher embryo/patient numbers would have been required to confirm the impact on clinical outcome. A pragmatic RCT (≏100 patients in each arm) comparing two-step dehydration with either 0.3 or 0.1 M sucrose in the final step prior to slow cooling (Wood et al., 2011) also demonstrated a significant increase in cryosurvival associated with the elevated sucrose concentration but did not demonstrate a significant improvement in clinical outcome (cumulative birth rate). Whether this is related to the use of 0.3 M sucrose, which has been suggested to be detrimental to developmental potential when used for slow cooling of MII oocytes (Gook and Edgar, 2007), or whether larger numbers of patients are required for a study of adequate power at the clinical level remains to be established.

In summary, slow cooled embryos which survive predominantly intact after thawing appear to retain their pre-freeze developmental potential and optimal dehydration prior to slow cooling can result in high (ca. 90%) survival rates.

### Vitrification

Although slow cooling was widely adopted for cryopreservation of cleavage stage embryos from the mid-1980s, the possibility of using vitrification was attractive to some groups. Using an EG-based method, 41 of 52 vitrified embryos (79%) survived with at least 50% of their original blastomeres intact and 4 out of 29 transferred (14%) implanted (Mukaida et al., 1998). In a larger study of over 1500 vitrified embryos, using a DMSO based method, only 62.5% survived with at least half of the original blastomeres intact and a pregnancy rate of 11.4% was reported (Hsieh et al., 1999). In a study of 215 surplus Day 3 embryos vitrified using EG, only 49.3% survived (El-Danassouri and Selman, 2001). These early results did not suggest that vitrification could replace established slow cooling approaches, although a survival rate (at least 50% of original blastomeres) of 86.8% was reported in a study of 266 embryos derived from abnormally fertilized oocytes (Liebemann and Tucker, 2002).

The demonstration of a high survival (not defined in the publication) rate (95.3%) in 127 warmed day 3 embryos, vitrified using an EG-based method (Rama Raju et al., 2005), encouraged further interest in vitrification of cleavage stage embryos. The same group (Rama Raju et al., 2009) went on to report survival (≧50% intact blastomeres) of 90.4% in a larger series of 907 warmed embryos, with an implantation rate of 18.1% which was comparable to that of fresh embryos (23.5%) given that the outcomes were not controlled for original embryo quality. A survival rate (≧50% intact blastomeres) of 85% and an implantation rate of 20% were also reported using a vitrification method based on DMSO, EG and sucrose (Desai et al., 2007). Using similar cryoprotectants, high rates of survival (≧90%) have also been reported using a nylon mesh container (Nakashima et al., 2010) and cut standard straws (Gvakharia and Adamson, 2011).

Using small numbers of discarded embryos and embryos derived from abnormally fertilized oocytes, which were biopsied and vitrified at cleavage stages, survival rates of 94% [n = 49, Zheng et al. (2005)] and 64% [n = 25, Zhang et al. (2009)] have been reported using EG/sucrose and EG/DMSO/sucrose, respectively. In a study of embryos vitrified (EG/DMSO/sucrose) on Day 4 with or without biopsy on Day 3 (Kahraman and Candan, 2010), similar survival rates were observed in biopsied (89.8%, 53/59) and non-biopsied embryos (85.9%, 55/64).
Comparison of slow cooling and vitrification

Studies in which slow cooling and vitrification have been employed in the same laboratory are obviously of most importance in comparisons because other confounding variables are constant. Comparing vitrification using DMSO/sucrose to slow cooling, Mauri et al. (2001) reported higher survival (at least one surviving blastomere) and implantation rates in the slow cooling group, whereas Vutyavanich et al. (2008) reported higher survival with vitrification, although implantation rates were almost identical.

Using an EG/sucrose-based vitrification method, Rama Raju et al. (2005) reported increased survival (95.3%) and implantation (14.9%) rates compared to slow cooling (60 and 4.2%, respectively), although the outcomes from slow freezing were significantly poorer than those reported elsewhere in this review.

Vitrification of cleavage stage embryos with EG/DMSO/sucrose (Kuwayama et al., 2005b) resulted in a small but significant increase in survival (98% versus 91%) but no difference in pregnancy rate relative to slow cooling, and Wilding et al. (2010) found no significant difference in survival or implantation rate in a similar comparative study. Rezzadah Valojerdi et al. (2009) reported increased survival and implantation rates using this vitrification method compared with that in the slow cooling, although the slow cooling method described involved dehydration in PROH with a very high (0.5 M) concentration of sucrose, a hitherto unreported method. Debrock et al. (2011) observed similar but low (40–45%) survival rates after vitrification and slow cooling of Day 3 embryos.

The importance of RCTs when attempting to evaluate the comparative efficiency of alternative approaches is widely recognized. In a well-designed RCT, Balaban et al. (2008) reported a higher survival rate (94.8% versus 88.7%) and a higher rate of fully intact embryos (73.9% versus 45.7%) in vitrified (PROH/EG/sucrose) compared with slow cooled day 3 embryos which had been dehydrated in PROH plus 0.1 M sucrose.

Conclusions

From the available evidence, it would appear that cleavage stage embryos which survive cryopreservation have similar implantation potential to fresh embryos when allowances are made for differences in embryo quality between the respective populations. As such, survival becomes the predominant consideration when comparing the efficiency of alternative cryopreservation methodologies. It is clear from work quoted in this review that overall survival rates of 90% or more, with fully intact rates of over 70%, can be achieved using vitrification, although a number of other studies report less success. In some comparative studies vitrification appears to outperform slow cooling. However, in other comparative studies, including some with very high survival rates, slow cooling produces very similar results. Importantly, all comparative studies have used a longstanding traditional method for slow cooling which has recently been shown to be significantly inferior to a modified method. This modified method, involving single-step dehydration in PROH with a higher (0.2 M) concentration of sucrose, has produced results which are equivalent to the best results obtained with vitrification. As such, there is no evidence to support the superiority of either approach when cryopreserving cleavage stage embryos.

Outcomes from blastocysts

Slow cooling

Although pregnancies from cryopreserved blastocysts were reported in the early years of IVF (Cohen et al., 1985), the trend towards transfer at the early cleavage stages restricted further progress in this area. These early pregnancies were achieved following slow cooling using the slower permeating cryoprotectant, glycerol. Interest in extended culture to the blastocyst stage and the corresponding requirement for blastocyst cryopreservation resurfaced a number of years later with the introduction of techniques for co-culture of embryos on Vero cells (Menezo et al., 1992a, b). Using a modified slow cooling method with glycerol and sucrose as cryoprotectants, Kaufman et al. (1995) reported cryosurvival of 83% with co-cultured blastocysts and implantation potential which the authors claimed to approach that of fresh transfers. This interest was relatively short lived, however, owing to a number of issues surrounding the use of heterologous somatic cells in association with culture of human embryos in clinical ART.

The advent of sequential media designed to support blastocyst development in the absence of somatic cells (Gardner and Lane, 1997) heralded a new era of application of extended culture in clinical ART towards the end of the millennium. Slow cooling of blastocysts using glycerol and sucrose as cryoprotectants was adopted by most groups and a number of reports detailing survival and implantation of thawed blastocysts have been published. Behr et al. (2002) reported that 98% of slow cooled blastocysts had at least 50% survival of the inner cell mass and tropheoblast cells after thawing and an implantation rate of 16%. Using a modified start temperature prior to slow cooling Gardner et al. (2003) reported a survival rate of 69% and an implantation rate of 30%. Higher immediate survival of early Day 5 blastocysts (82.6%) was in contrast to reduced rates in more advanced and slower growing blastocysts (Van den Abbeel et al., 2005). In a single thawed blastocyst study (Desai and Goldfarb, 2005), 85% of slow cooled blastocysts survived with an overall implantation rate of 27%. Similar outcomes were reported by Anderson et al. (2005)—79.5% survival and 32.7% implantation rate, and Surrey et al. (2010)—80.9% survival and 27.3% implantation rate.

A survival rate of 76.3% and an implantation (fetal sac) rate of 38.6% were reported (Veeck et al., 2004) for thawed blastocysts in comparison to a fresh blastocyst implantation rate of 56.5%. It is possible that this difference may reflect the fact that the embryos of the highest quality were transferred in the fresh cycle because equivalent implantation rates have been reported from the transfer of fresh (35.2%) and slow cooled (37.4%) blastocysts which had expanded on Day 5 of culture (Shapiro et al., 2008) in a study which also reported a 98% survival rate for the slow cooled blastocysts. A survival rate of 88% was reported for slow cooled blastocysts, whether or not they had been biopsied for PGD on Day 3 of development (El-Toukhly et al., 2009), in a study which also demonstrated similar implantation rates for fresh (27–34%) and thawed (35%) PGD blastocysts.

Vitrification

The first pregnancy and birth from the transfer of vitrified blastocysts (Yokota et al., 2000, 2001) were achieved using EG and DMSO as cryoprotectants. This approach was widely adopted in conjunction
with a number of carrier systems which allowed ultrarapid cooling in very small volumes of cryoprotectant solution. Vanderzwalmen et al. (2003) reported overall survival of 67% but higher survival (79%) in blastocysts from good quality embryos. The implantation rate was higher (23%) in the good quality group after assisted hatching relative to a non-hatched control group (16%). In a study of excess embryos (Cremades et al., 2004), 82% of early blastocysts and 73% of morulae survived. Mukaida et al. (2003) reported 87% survival of 569 Day 5 blastocysts and an implantation rate of 21%, but later reported improved survival (97%) and implantation (47%) rates when artificial shrinkage was induced by micro-needle or laser pulse in a study of 502 warmed blastocysts (Mukaida et al., 2006).

Using a closed vitrification system, survival and implantation rates of 74 and 39%, respectively (Ebner et al., 2009) have been reported. Using another closed system (Van Landuyt et al., 2011a), the overall survival was 78%, with 56% of blastocysts fully intact after warming. The implantation rate of the fully intact blastocysts was 16.2% compared with 6.4% in those with moderate damage. Vanderzwalmen et al. (2009) reported an 86% survival rate and an implantation rate of 30% using an aspetic vitrification system.

Recent studies based on large numbers of vitrified/warmed blastocysts have shown high survival rates of 99% (Goto et al., 2011) and 96% in both closed and open systems (Liebertamnn, 2009). In the former study (single embryo transfers), pregnancy rates were related to both blastocyst quality and female age and were highest (67%) in young (22–33 years of age) patients with top-quality blastocysts, whereas an overall implantation rate of 29.4% was reported in the latter study.

An alternative method using a high concentration of EG (40%) resulted in a survival rate of 84% and an implantation rate of 21% (Cho et al., 2002), although the survival and implantation rates may be improved using this method when artificial shrinkage is applied to blastocysts before vitrification (Son et al., 2003). One group (Stachecki et al., 2008) has described a closed system using 0.25 ml sterile straws and EG and glycerol as cryoprotectants which resulted in 81.4% of vitrified blastocysts surviving and an implantation rate of over 40%. Unfortunately, this publication appeared without inclusion of details of the methodology.

In comparison to fresh controls, vitrified/warmed blastocysts have been shown to have equivalent implantation rates (Takahashi et al., 2005) or higher (Zhu et al., 2011) than that of slow cooled blastocysts (Shibl et al., 2009). In blastocysts vitrified with an open zona pellucida (Zech et al., 2005), the subsequent implantation rate (26%) was similar to that of fresh blastocysts (25%). Comparable implantation rates from the transfer of vitrified blastocysts were also described by Van Landuyt et al. (2011b).

**Comparison of slow cooling and vitrification**

In a study comparing blastocyst vitrification to slow cooling within the same clinic, Stehlik et al. (2005) reported increased survival (100% versus 83.1%) and implantation rates (19.4% versus 7.4%) in the vitrification group, although the implantation rate in the slow cooling group appeared to be significantly lower than reported by other groups (see slow cooling section). Huang et al. (2005) reported increased survival with vitrification (84%) relative to slow cooling (56.9%) in blastocysts donated for research. In a study of cryopreserved blastocysts from biopsied (Day 3) and non-biopsied embryos (Keskinetpe et al., 2009), higher survival rates were observed with vitrification relative to slow cooling in both groups but the pregnancy rate was only higher in the biopsied group. Significantly higher pregnancy rates were also associated with vitrification relative to slow cooling in a retrospective analysis involving 189 slow cooled and 58 vitrified blastocysts (Kuc et al., 2010).

For comparative purposes, the most important studies will always be those in which both techniques are applied to significant numbers within the same unit and performed to equivalent high standards. In one such study (Kuwayama et al., 2005), the survival of vitrified blastocysts was slightly, but significantly, higher (90%) than that of slow cooled blastocysts (84%). However, pregnancy rates (53 versus 51%) and live birth rates (45 versus 41%) per transfer were not significantly different. Survival rates of 96.5% for vitrification and 92.1% for slow cooling (Liebertamnn and Tucker, 2006) were not significantly different in a study with over 500 blastocysts in each group and, again, pregnancy rates per transfer (46.1 versus 42.9%, respectively) and implantation rates (30.6 versus 28.9%, respectively) were not significantly different.

**Conclusions**

As with cleavage stage embryos, it appears that blastocysts which survive cryopreservation by vitrification or slow cooling can implant at the same rate as equivalent fresh blastocysts. Vitrification is now associated with more consistent reports of high cryosurvival, although laboratories which perform slow cooling to optimal standards can achieve comparable results.

**Other considerations**

This review has focused on survival and developmental potential following cryopreservation by vitrification or slow cooling. However, theoretical risks relating to the safety of vitrification have been raised elsewhere.

Direct contact with liquid nitrogen (open systems) may be necessary to achieve the required high rates of cooling during vitrification (Vajta and Nagy, 2006), although the use of sterile liquid nitrogen (Parmegiani et al., 2010) may circumvent contamination problems (Belanski and Vajta, 2009). This may not be as important for blastocyst vitrification, where closed systems have been shown to be equally effective (Kuwayama et al., 2005b; Liebertamnn, 2009) but optimal oocyte vitrification may require open systems (Cobo et al., 2008b; Paffoni et al., 2011).

Post-vitrification storage may also pose contamination risks (Tedder et al., 1995; Bielanski et al., 2000) but sealed systems (Vanderzwalmen et al., 2009; Van Landuyt et al., 2011a) or the use of liquid nitrogen vapour for storage (Cobo et al., 2010b) may overcome this. Transport of material vitrified in very small volumes may also raise questions related to impact on survival (McDonald et al., 2011).

The birth outcomes from slow cooled oocytes (Noyes et al., 2009) and embryos (Wang et al., 2005; Shih et al., 2008; Wennerholm et al., 2009) where 1.5 M PROH was used as the permeating cryoprotectant
are reassuring. However, the same information is not yet available for the more potentially toxic, permeating cryoprotectants DMSO (Kola et al., 1988) and EG (Klug et al., 2001), which are used at significantly higher concentrations during vitrification.

**Overall conclusions**

Vitrification has now been adopted by a large number of clinics worldwide for the cryopreservation of oocytes and embryos. In contrast to historical experience with slow cooling, consistently high survival rates have been reported following vitrification of MII oocytes in association with clinical outcomes similar to those from fresh oocytes. There is, however, some evidence that optimal protocols for slow cooling of MII oocytes are yet to be established and more recent results suggest that improvements are possible. Although no RCTs have been carried out to compare vitrification with optimal slow cooling, the available evidence suggests that vitrification is the current method of choice for cryopreservation of MII oocytes. This

### Table I Key publications relating to cryopreservation of human oocytes.

<table>
<thead>
<tr>
<th>Cryoprotectants</th>
<th>Survival rate (%)</th>
<th>Cleavage stage ET</th>
<th>Blastocyst ET</th>
<th>Fresh IR (%)</th>
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<tr>
<td><strong>Optimal slow cooling</strong></td>
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<tr>
<td>Gook and Edgar (2011)</td>
<td>1.5 M PROH, 0.2 M sucrose</td>
<td>75.8</td>
<td>30.0 (&lt;38 years)</td>
<td>26.0 (&lt;38 years)</td>
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<td>15.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Ferraretti et al. (2010)</td>
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<td>71.8</td>
<td>18.9 (&lt;35 years)</td>
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<td><strong>Optimal vitrification</strong></td>
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<td>Antinori et al. (2007)</td>
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<td>10.3</td>
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<td>Rienzi et al. (2010)</td>
<td>15% EG, 15% DMSO, 0.5 M sucrose</td>
<td>96.7–89.7</td>
<td>27.3 (&lt;34 years)</td>
<td>30.0 (&lt;34 years)</td>
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<td>Ubaldi et al. (2010)</td>
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<td>65</td>
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<td>75</td>
<td>38&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td>85</td>
<td>NR (mixed transfers)</td>
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<td>Fadini et al. (2009), vit</td>
<td>EG, PROH, sucrose (Medicult)</td>
<td>78.9</td>
<td>9.3</td>
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PROH, propanediol; EG, ethylene glycol; DMSO, dimethyl sulphoxide; IR, implantation rate; NR, not reported; ET, embryo transfer; Donor, donor oocyte programme.

<sup>a</sup>Sustained hatching.

<sup>b</sup>Pregnancy rate.
conclusion may also be true for pronuclear stage oocytes, although the evidence is less clear.

High survival rates have been reported for both vitrified and optimally slow cooled cleavage stage embryos with clinical outcomes similar to those obtained from equivalent fresh embryos. There is no basis for supporting the preferential use of either technique in this context.

In the case of blastocysts, more consistent reporting of high survival rates has been associated with vitrification but, as with cleavage stage embryos, optimal slow cooling appears to result in comparable survival and implantation rates under controlled conditions.

In all categories except cleavage stage embryos, and given the ascertainment bias when only dealing with published data, it would appear that vitrification may result in more consistent results. Whether this will translate to universal experience and whether optimal slow cooling protocols can be developed to achieve the same results will be established in the future, hopefully by carefully designed RCTs. The key publications relating to cryopreservation of oocytes (Table I), cleavage stage embryos (Table II) and blastocysts (Table III) demonstrate the paucity of evidence from RCTs and also the absence of data on critical end-points, such as embryo survival/implantation rate, in many published studies.

One additional point which must be emphasized in any review of the clinical results associated with cryopreservation is the crucial role that embryo selection plays in the outcomes from the eventual embryo transfer. For example, the number of stored oocytes thawed/warmed and the length of the subsequent culture period will have an inevitable impact on the implantation rate associated with oocyte cryopreservation. Similarly, the increased selection applied to cryopreserved embryos relative to oocytes, and to blastocysts relative to cleavage stage embryos, will also influence the apparent clinical outcome. The most valid way to compare approaches or techniques is to express implantation rates on a ‘per oocyte used’ basis but, unfortunately, the numbers required to establish this appropriate denominator are not commonly available from publications. As such, caution must be exercised when attempting to draw conclusions relating to the relative clinical efficiency of cryopreservation at different developmental stages. The limitations of the available evidence base have been referred to throughout this review. Notably, there is a lack of valid RCTs which allow comparisons of slow cooling and
<table>
<thead>
<tr>
<th>Table III</th>
<th>Key publications relating to cryopreservation of human blastocysts.</th>
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<tbody>
<tr>
<td><strong>Cryoprotectants</strong></td>
<td><strong>Survival rate (%)</strong></td>
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<td></td>
<td><strong>Day 5</strong></td>
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<tr>
<td>Optimal slow cooling</td>
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<tr>
<td>Shapiro et al. (2008)</td>
<td>Glycerol</td>
</tr>
<tr>
<td>Veeck et al. (2004)</td>
<td>9% glycerol, 0.2 M sucrose</td>
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<tr>
<td>El-Toukhy et al. (2009)</td>
<td>9% glycerol, 0.2 M sucrose</td>
</tr>
<tr>
<td>Anderson et al. (2005)</td>
<td>9% glycerol, 0.2 M sucrose</td>
</tr>
<tr>
<td>Surrey et al. (2010)</td>
<td>10% glycerol</td>
</tr>
<tr>
<td>Optimal vitrification</td>
<td></td>
</tr>
<tr>
<td>Takahashi et al. (2005)</td>
<td>15% EG, 15% DMSO, 0.65 M sucrose, 10 mg/ml Ficoll</td>
</tr>
<tr>
<td>Zhu et al. (2011)</td>
<td>15% EG, 15% DMSO, 0.5 M sucrose</td>
</tr>
<tr>
<td>Mukaida et al. (2006)</td>
<td>15%EG, 15% DMSO, 0.65 M sucrose, 10 mg/ml Ficoll</td>
</tr>
<tr>
<td>Lieberman and Tucker (2009)</td>
<td>15% EG, 15% DMSO, 0.5 M sucrose</td>
</tr>
<tr>
<td>Vanderzwalmen et al. (2009)</td>
<td>20% EG, 20% DMSO, 0.75 M sucrose, 10 mg/ml Ficoll</td>
</tr>
<tr>
<td>Comparison of slow cooled and vitrified</td>
<td></td>
</tr>
<tr>
<td>Lieberman et al. (2006), slow</td>
<td>9% glycerol, 0.2 M sucrose</td>
</tr>
<tr>
<td>Lieberman et al. (2006), vit</td>
<td>15% EG, 15% DMSO, 0.5 M sucrose</td>
</tr>
<tr>
<td>Kuwayama et al. (2005b), slow</td>
<td>9% glycerol, 0.2 M sucrose</td>
</tr>
<tr>
<td>Kuwayama et al. (2005b), vit</td>
<td>15% EG, 15% DMSO, 0.5 M sucrose</td>
</tr>
</tbody>
</table>

aassisted hatching.
bArtificially collapsed.
cpregnancy rate (no IR reported).
vitrification (only one with oocytes and one with cleavage stage embryos identified). The same limitation also applies to studies comparing fresh and cryopreserved oocytes with only one RCT identified. In addition, most studies do not report live birth outcomes and rely on surrogate end-points, such as survival and early embryo development. A further major complication is the number of studies in which there is inadequate detail of, or control for, female age, the most significant determinant of outcome in ART.

On current evidence, we would support the continued use of optimal slow cooling of cleavage stage embryos and the cautious adoption of vitrification in other circumstances. We would also support continued research to establish optimal slow cooling methods as they may assist in alleviating concerns over safety issues relating to storage, transport and the potential long-term consequences of exposure to the very high concentrations of cryoprotectants associated with vitrification.

**Authors’ roles**

D.G. and D.E. conceived and designed the review article. D.G. conducted the literature searches. D.G. and D.E. assessed the articles and data from the literature searches for suitability for inclusion in the review. D.G. drafted the first version of the ‘Principles and methodology of slow cooling and vitrification’ section and D.E. drafted the first version of the other sections. D.G and D.E. both reviewed and revised the entire draft and approved the final submitted version.

**Funding**

No external funding was sought or obtained for this study.

**Conflict of interest**

None declared.

**References**


Cohen J, Simons RF, Edwards RG, Fehilly CB, Fishel SB. Pregnancies following the
Desai N, Blackmon H, Szeptycki J, Goldfarb J. Cryoloop vitrification of human day 3
Desai N, Goldfarb J. Examination of frozen cycles with replacement of a single
Damario MA, Hammitt DG, Galanits TM, Session DR, Dumesic DA. Pronuclear
De Santis L, Cino I, Rabellotti E, Papaleo E, Calzi F, Fusi FM, Brigante C, Ferrari A.
De Santis L, Cino I, Coticchio G, Fusi FM, Papaleo E, Rabellotti E, Brigante C,
Cobo A, Romero JL, Perez S, de Los Santos MJ, Meseguer M, Remohi J. Storage of
Demoulin A, Jouan C, Gerday C, Dubois M. Pregnancy rates after transfer of
Cobo A, Kuwayama M, Perez S, Ruiz A, Pellicer A, Remohi J. Successful pregnancies and deliveries after a simple
El-Danasouri I, Selman H. An improved protocol for dilution of
El-Toukhy T, Khalaf Y, Al-Darazi K, Andritsos V, Taylor A, Braude P. Effect of
Edgar DH, Karani J, Gook DA. Increasing dehydration of human cleavage-stage embryos prior to slow cooling significantly increases cryosurvival.


Kleinheins FW, Mazur P. Comparison of actual vs. synthesized ternary phase diagrams for solutes of cryobiological interest. Cryobiology 2007; 54:212–222.


