Human oocyte cryopreservation

Debra A. Gook1,2,3 and David H. Edgar1,2

1Reproductive Services/Melbourne IVF, Royal Women’s Hospital, 132 Grattan Street, Carlton, Victoria 3053, Australia; 2Department of Obstetrics and Gynaecology, University of Melbourne, Melbourne, Australia

3Correspondence address. Tel: +61 3 9344 2354; Fax: +61 3 9349 1387; E-mail: debra.gook@rwh.org.au

The clinical role of oocyte cryopreservation in assisted reproduction, as an adjunct to sperm and embryo cryopreservation, has been comparatively slow to evolve as a consequence of theoretical concerns related to efficacy and safety. Basic biological studies in the 1990's alleviated many of these concerns leading to more widespread adoption of the technology. While a number of babies were born from the approach validated in the 1990's, its perceived clinical inefficiency led to the search for improved methods. Introduction of elevated dehydrating sucrose concentrations during cryopreservation increased survival and fertilization rates, but there is no well-controlled evidence of improved clinical outcome. Similarly, the use of sodium-depleted cryopreservation media has not been demonstrated to increase clinical efficiency. More recently, and in the absence of basic biological studies addressing safety issues, the application of vitrification techniques to human oocytes has resulted in reports of a number of live births. The small number of babies born from clinical oocyte cryopreservation and the paucity of well-controlled studies currently preclude valid comparisons between approaches. Legal restrictions on the ability to select embryos from cryopreserved oocytes in Italy, where many of the available reports originate, also obscure attempts to assess oocyte cryopreservation objectively.

Keywords: cryopreservation; human; oocyte

Introduction

The ability to cryopreserve and store the structure and function of biological cells and tissue plays a pivotal role in many areas of biology and clinical medicine. The role of cryopreservation in human assisted reproduction was recognized at an early stage in the development of the technology and has increased in importance as a result of a range of clinical, ethical and legal considerations.

The efficiency and safety of the treatment of human infertility/subfertility using IVF relies heavily on the ability to cryopreserve early stage embryos. Deferring conception and/or implantation in cases where embryo cryopreservation is not an option due to the absence of an immediately available male or female partner, however, is dependent on the ability to cryopreserve human gametes. Successful establishment of pregnancies and subsequent live births using human cryopreserved semen was first reported in the 1950’s and has been widely applied in a number of clinical situations. Although sporadic early successes were reported using human cryopreserved oocytes, this technology was widely perceived to be highly inefficient and subject to major concerns relating to safety until many of these concerns were alleviated by fundamental validation studies carried out in the 1990’s. This led to the adoption of oocyte cryopreservation as a clinical tool, particularly in Italy, where pending legislative change sought to outlaw embryo cryopreservation.

Although it had been recognized for some time that oocyte cryopreservation could potentially circumvent many of the ethical issues associated with embryo cryopreservation, the perceived inefficiency had limited its adoption to clinical situations in which no realistic alternative was available, e.g. prior to cytotoxic therapy in young single women. The imminent legal framework in Italy accelerated adoption of oocyte cryopreservation as an alternative to embryo cryopreservation in routine assisted reproductive technology (ART) and reports of pregnancies and births followed from 1997 onwards.

In the last decade, a number of reports have been published describing the clinical outcome from oocytes cryopreserved using the methodology, which was originally validated in the 1990’s, and, subsequently, using various modifications to this methodology designed to improve the clinical results. Additionally, there have been reports of attempts to cryopreserve oocytes at immature stages of development and contained within follicles in ovarian tissue.

The aims of this review were to: (i) highlight the role of oocyte cryopreservation in the evolution of clinically applied technologies used for storage of reproductive potential; (ii) review the biological data available on the effects of cryopreservation on oocyte survival and function; (iii) survey the clinical outcomes reported from the use of cryopreserved oocytes and (iv) review the evidence for recent improvements associated with modified...
Cryopreservation of reproductive potential in assisted reproduction

Embryo cryopreservation

The demonstration that cryopreserved mammalian embryos could give rise to live offspring following thawing and intrauterine transfer (Whittingham et al., 1972) opened the door to the possible application of this technology in the context of animal breeding and clinical assisted reproduction. Soon after the introduction of IVF as a clinical tool in the treatment of human infertility, this methodology, based on the use of slow cooling and rapid thawing in the presence of the cryoprotectant dimethyl sulphoxide (DMSO), was applied to human early cleavage stage embryos and resulted in the first reports of pregnancy and live birth from human cryopreserved embryos (Trounson and Mohr, 1983; Zeilmaker et al., 1984). This approach was rapidly superseded by substitution of the cryoprotectants 1,2 propanediol (PROH) and sucrose (Lassalle et al., 1985; Testart et al., 1986), and embryo cryopreservation now plays a pivotal role in clinical assisted reproduction. Although blastomere loss and, to a lesser extent, total embryo loss can occur in a proportion of cryopreserved embryos with consequent loss of implantation potential (Van den Abbeel et al., 1997; Burns et al., 1999; Edgar et al., 2000; Guerif et al., 2002), the application of embryo cryopreservation within an assisted reproduction programme can have a profound impact on treatment strategy (Edgar et al., 2005).

Gamete cryopreservation

In cases where single men or women are at risk of losing their fertility as a result of exposure to cytotoxic therapies or where ethical/religious concerns preclude the cryopreservation of embryos, cryopreservation of gametes may be the only option for storage of reproductive potential. The potential advantages of gamete over embryo cryopreservation are clearly seen in circumstances where the fate of frozen embryos has to be decided following the death of one or both of the ‘parents’ (Robertson, 1991). The necessity for this approach may also be imposed in circumstances where ethical concerns lead to legislation, which permits IVF but outlaws embryo cryopreservation (Boggio, 2005).

The ability of glycerol to act as a cryoprotective agent during freezing and thawing of spermatozoa was first reported in 1949 (Polge et al., 1949) and thousands of offspring in a variety of species have subsequently arisen from the use of cryopreserved sperm each year. The first births following the use of human cryopreserved sperm were reported in the 1950’s (Bunge et al., 1954) and this approach is now in widespread use for storage and quarantine of donated sperm and storage of reproductive potential when loss of fertility is imminent. However, cryopreservation of human oocytes has posed a greater challenge.

Survival, fertilization and fetal development following cryopreservation of unfertilized mouse oocytes using DMSO was first reported in the 1970’s (Whittingham, 1977). However, although cryosurvival was similar to that observed for embryos, a number of subsequent reports (Glenister et al., 1987; Kola et al., 1988; Carroll et al., 1989, 1990; Trounson and Kirby, 1989; George et al., 1994) suggested that fertilization, embryo development and fetal development may be compromised in embryos derived from cryopreserved mouse oocytes. Around the same time, reports of pregnancies and births from human oocytes cryopreserved using DMSO appeared in the literature (Chen, 1986; Al-Hasani et al., 1987; Van Uem et al., 1987; Siebzehnruebl et al., 1989), but concerns over reproducibility, low rates of survival and fertilization and high rates of polyploidy (Al-Hasani et al., 1987; Van Uem et al., 1987; Mandelbaum et al., 1988a,b; Siebzehnruebl et al., 1989; Todorow et al., 1989b) resulted in a cessation of clinical activity in the area.

Biological studies of oocyte cryopreservation

Cryosurvival

While it is possible to cryopreserve most cell types even without the use of a cryoprotectant (Mazur, 1963, 1970), the critical parameters are the proportion which remain intact following thawing (survival) and the extent to which normal function has been preserved. The importance of survival is especially critical when a limited number of cells are available for cryopreservation. Fundamental to cryosurvival is intracellular water and its movement across the cell membrane to equalize the vapour pressure gradient, as a result of ice formation in the external solute with reduced temperature (Mazur et al., 1972, 1984). The rate of outward flow of water is dependent on the relative proportions of free water, which is associated with macromolecules, the membrane kinetics (hydraulic permeability and activation energy) and the surface area to volume ratio of the cell. The importance of these properties can be clearly demonstrated by the differential survival rates observed when oocytes at various developmental stages are exposed to identical cryopreservation conditions. Survival rates of mouse oocytes cryopreserved at the metaphase II (MII) stage and following fertilization are dramatically different [4 and 53%, respectively (Gook et al., 1993)], indicating that the water composition and/or membrane kinetics have altered in the process of fertilization. Measurements of the hydraulic permeability and activation energy of such oocytes confirm that fertilized mouse oocytes are more permeable to water (Otrico et al., 1988). Differences in kinetic parameters between species (Bernard et al., 1988; Shabana and McGrath, 1988) also highlight the difficulties inherent in translating successful oocyte cryopreservation protocols from animal to human oocytes. In human, these parameters differ between fresh and aged MII oocytes (Bernard et al., 1989; Newton et al., 1999) suggesting that the latter are also an inadequate model for developing appropriate methodology. Another critical observation with respect to human oocyte cryopreservation was made by Hunter et al. (1992b), who demonstrated that the above properties also differed between individual MII oocytes.

Movement of water out of the oocyte is facilitated by addition of a cryoprotectant (Mazur et al., 1984) providing an osmotic gradient and, in the case of permeating cryoprotectants, limiting shrinkage by replacing the lost water. The rate at which a permeable cryoprotectant enters the oocyte varies between cryoprotectants (Newton et al., 1999) and is temperature dependent (Paynter et al., 2001). Cryoprotectants also reduce the temperature at
which ice crystallization occurs (Leibo et al., 1978), thereby extending the dehydration time during cooling. However, some cryoprotectants are known to be toxic (Fahy et al., 1990) and, as the oocyte dehydrates further during cooling, the concentrations of both permeating and non-permeating cryoprotectants increase in a similar way to the solutes (Grout and Morris, 1987). Therefore, a balance is required between the beneficial and detrimental effects of the cryoprotectant.

Whittingham et al. (1972) first reported oocyte cryopreservation using DMSO as a cryoprotectant in conjunction with the slow rate of freezing developed for embryos. Cryopreservation of mouse mature oocytes resulted in a 65–75% survival rate and birth of live young following IVF and transfer to recipients (Whittingham, 1977). However, variable survival rates and poor fertilization have been reported in other species including rat (Kasai 1977). However, variable survival rates and poor fertilization have been reported in other species including rat (Kasai 1977). 

Although survival rates were promising for mouse oocytes, subsequent fertilization and embryo development were variable and reduced in comparison to controls (Whittingham, 1977; Glenister et al., 1987; Carroll et al., 1989; Trounson and Kirby, 1989; Hunter et al., 1991). The low rate of fetal development following transfer of 2-cell embryos or blastocysts derived from cryopreserved mouse oocytes suggested that embryo quality was poor (George et al., 1994). This is also suggested by the reported reduction in cell number within the inner cell mass of blastocysts derived from cryopreserved mouse oocytes (Van der Elst et al., 1998).

Initial reports of human oocyte cryosurvival with DMSO and slow cooling ranged from 20–30% (Al-Hasani et al., 1987; Van Uem et al., 1987; Mandelbaum et al., 1988a,b; Siebzehnruebl et al., 1989; Todorow et al., 1989b), to as low as zero survival (Trounson, 1986). However, three of these groups did achieve pregnancies (Al-Hasani et al., 1987; Van Uem et al., 1987; Siebzehnruebl et al., 1989), with two resulting in birth (Van Uem et al., 1987; Siebzehnruebl et al., 1989). In contrast, an 80% survival rate was reported using the same procedure and resulted in birth of a set of twins (Chen, 1986). Other groups attempted to improve the results with minor modifications but to no avail (Trounson, 1986; Todorow et al., 1989b). Higher survival rates (~60%) were achieved for human oocytes following slow cooling in the presence of PROH (Trounson, 1986; Todorow et al., 1989b), although only a small number of oocytes (~<10) were cryopreserved. The cryoprotectant PROH had been shown previously to be less toxic than DMSO (Renard et al., 1984) and suitable for cryopreservation of both mouse (Renard and Babinet, 1984) and human embryos (Lassalle et al., 1985; Testart et al., 1986). Exposure of human oocytes to PROH during dehydration and rehybridation, but without cryopreservation, was shown to have no impact on subsequent fertilization and embryo development (Bernard et al., 1985). However, other groups using PROH obtained similar low survival rates to those observed with DMSO (Mandelbaum et al., 1988b) and addition of sucrose, which had been shown to be beneficial for embryo cryopreservation (Mandelbaum et al., 1987), did not improve oocyte survival (Al-Hasani et al., 1987; Mandelbaum et al., 1988b).

Poor cryosurvival and reports of abnormalities arising from organelle damage following cryopreservation (see later) halted clinical oocyte cryopreservation for a number of years.

A resurgence of interest came with the publication of the outcome from a large series of oocytes (171) cryopreserved using PROH and sucrose in a slow freeze/rapid thaw protocol which resulted in 64% immediate post-thaw survival (Gook et al., 1993). Exposure to these cryoprotectants without cryopreservation was shown to have no toxic effect, but the survival of oocyte-cumulus complexes was reduced relative to that of denuded oocytes. The study also identified that survival was overestimated when assessed immediately post thaw since ~10% of oocytes with leaky plasma membranes deteriorated with time in culture and subsequently lysed. Survival rates of only 34 and 25%, respectively, were reported using this procedure in two subsequent studies (Kazem et al., 1995; Tucker et al., 1996), one with two pregnancies but no births (Tucker et al., 1996). The first confirmation of a live birth (Porcu et al., 1997) heralded a number of subsequent reports from groups applying the procedure clinically, often for only small numbers of oocytes. In reports involving >50 oocytes (Antinori et al., 1998; Borini et al., 1998, 2004, 2006a; Porcu et al., 1998, 2000, 2001, 2002), survival was similar to that reported by Gook et al. (1993), i.e. ~50%, with one (lower) exception (De Santis et al., 2007).

Increasing the concentration of the non-permeating sucrose from 0.1–0.2 or 0.3 M during dehydration was reported to increase survival to 60 and 82%, respectively (Fabbri et al., 2001). These modifications have continued to maintain higher survival rates of around 70% when data is pooled from clinical studies using 0.2 M sucrose (Yang et al., 1998, 2002; Porcu et al., 1999; Winslow et al., 2001; Bianchi et al., 2007; Gook et al., 2007) and slightly higher (74%) using 0.3 M sucrose (Chen et al., 2002, 2005; Fosas et al., 2003; Li et al., 2005; Tjur et al., 2005; Borini et al., 2006b; Chamayou et al., 2006; La Sala et al., 2006; Levi Setti et al., 2006; Barratt et al., 2007; De Santis et al., 2007; Konc et al., 2007).

In mouse oocytes, the presence of sodium in the solute during cryopreservation was shown to be detrimental when compared with replacement with choline (Stachecki et al., 1998). However, clinical results for human oocyte cryopreservation in sodium-depleted media show no further improvement over the increase already observed with higher sucrose with 52% of oocytes surviving cryopreservation in sodium-depleted medium containing 0.1 M sucrose (Quintans et al., 2002; Stachecki et al., 2006), 62% in similar medium with 0.2 M sucrose (Quintans et al., 2002; Boldt et al., 2003; Azambuja et al., 2005; Pet racco et al., 2006; Stachecki et al., 2006) and 59% with 0.3 M sucrose (Boldt et al., 2006).

**Fertilization**

Two critical functions, which provide evidence of successful cryopreservation, are fertilization and embryo development. For these events to proceed in a similar fashion to that observed in fresh oocytes, a number of critical organelles must withstand freezing and thawing without suffering impaired function. Low fertilization rates were observed following cryopreservation of mouse oocytes using the DMSO procedure (Whittingham, 1977; Glenister et al., 1987; Kola et al., 1988; Carroll et al., 1989, 1990; Todorow et al., 1989a; Trounson and Kirby, 1989). This was attributed to physical damage to the zona pellucida (Todorow et al., 1989a) and modification of its structure (Johnson et al., 1988; Johnson, 1989;
Carroll et al., 1990) and could be eliminated by removal of the zona prior to fertilization (Wood et al., 1992). The altered structure was attributed to cryoprotectant induced premature cortical granule (CG) discharge (Schalkoff et al., 1989; Vincent et al., 1990a) although stepwise removal of the cryoprotectant (Johnson, 1989), and cryopreservation in the presence of fetal calf serum eliminated this ‘zona hardening’ (Vincent et al., 1991; Carroll et al., 1993). Although cracks were observed in the zonae of human oocytes following cryopreservation in the presence of DMSO (Sathananthan et al., 1987), the CG population was conserved (Sathananthan et al., 1988). Similarly, using the PROH with sucrose cryopreservation procedure which included serum, Gook et al. (1993) observed a normal population of CG in human oocytes and normal fertilization rates similar to fresh oocyte controls following in vitro insemination (Gook et al., 1994), a finding confirmed by Jones et al. (2004).

In contrast to the low normal fertilization rates observed in cryopreserved mouse oocytes, higher normal fertilization rates (~50%) were observed following insemination of human oocytes cryopreserved using the DMSO (Al-Hasani et al., 1987; Siebzehnruebl et al., 1989; Hunter et al., 1991; Bernard et al., 1992) and the PROH procedures (Al-Hasani et al., 1987; Gook et al., 1994; Serafini et al., 1995). Further evidence that cryopreservation using the PROH procedure had no adverse affect on fertilization was demonstrated by the observation of equivalent fertilization rates (~50%) following insemination and ICSI (Gook et al., 1995b; Li et al., 2005). In contrast, Kazem et al. (1995) reported a lower rate with insemination (3%) relative to ICSI (43%). Despite the fact that there is no evidence from controlled comparisons of insemination techniques to suggest that ICSI is required to fertilize human cryopreserved oocytes, it has been adopted as the method of choice in subsequent clinical studies.

A high polyploidy rate was also observed following cryopreservation of both mouse (Glenister et al., 1987; Van der Elst et al., 1988) and human oocytes (Al-Hasani et al., 1987; Sathananthan et al., 1987; Mandelbaum et al., 1988b). Cracks in the zona were a potential cause and Sathananthan et al. (1987) identified multiple sperm within the ooplasm of these oocytes. However, Glenister et al. (1987) and Carroll et al. (1989) attributed this elevated rate of polyploidy to a high proportion of digynic oocytes resulting from retention of the second polar body. In the absence of specific information on the effect of ageing on human oocytes, oocytes in early studies were often cultured for 24 h prior to freezing (Al-Hasani et al., 1987). An elevated polyploidy rate following insemination of aged human oocytes (Gook et al., 1994) and an increased rate of second polar body retention following cryopreservation of aged human oocytes (Gook et al., 1995a) indicated that the use of aged oocytes may have been the reason for the observed increase in polyploidy rate in the above study. The clinical implications of this may include a delay in cryopreservation resulting in abnormal embryos and/or pregnancies (Chia et al., 2000).

Parthenogenetic activation in the absence of sperm
Resumption of meiosis in the absence of sperm (parthenogenetic activation, PA) has also been reported following exposure of rabbit and mouse oocytes to reduced temperature (Pincus and Shapiro, 1940; Graham, 1974) and to PROH (Cuthbertson, 1983; Shaw and Trounson, 1989; Van der Elst et al., 1992). The rate of PA increased with duration and temperature of exposure to PROH in mouse oocytes. In contrast, no PA was observed in freshly retrieved human oocytes following exposure to PROH, but 27% of such oocytes activated following cryopreservation (Gook et al., 1995a). In this study, two types of PA were observed: a single pronucleus with extrusion of the second polar body, which was the most frequent (18%), and regular cleavage into 2 cells prior to 18–20 h after thawing (9%).

Consequences of PA following fertilization
In other studies, regular cleavage into 2 cells was not seen 18–20 h following insemination and only once observed following ICSI (Gook et al., 1994, 1995b) of human oocytes cryopreserved using PROH. The occurrence of unipronuclear oocytes following insemination of human oocytes cryopreserved with PROH was between 5 and 13% (Gook et al., 1994, 1995b). Following ICSI of human cryopreserved oocytes, a rate of PA similar to that observed in non-frozen oocytes was reported (Porcu et al., 2000), confirming a low rate of PA associated with the PROH cryopreservation procedure. The limited evidence available suggests that the incidence of PA is not significantly increased due to cryopreservation of human oocytes using PROH.

Abnormalities of the oocyte cytoskeleton and organelles
Microtubules and meiotic spindle
During normal fertilization resumption of meiosis occurs separating the chromatids attached to the meiotic spindle, expelling one set into the second polar body and the other into the female pronucleus. The meiotic spindle has been shown to depolymerize when exposed to reduced temperatures (Magistrini and Szollosi, 1980; Sathananthan et al., 1992). Subsequent impaired repolymerization on rewarming may lead to scattering of chromosomes or lesions in the reformed spindle resulting in misaggregation of chromatids following resumption of meiosis.

In mouse oocytes, reduction to room temperature has a dramatic effect on the spindle; resulting in the majority (>75%) of spindles acquiring an abnormal configuration (Pickering and Johnson, 1987; Van der Elst et al., 1988). Further reduction in temperature (to 4°C) resulted in an abnormal spindle in all oocytes. On returning to 37°C, repolymerization was observed, but numerous chromosomes were observed scattered throughout the cytoplasm (Pickering and Johnson, 1987; Sathananthan et al., 1992). Depolymerization of the spindle following exposure to reduced temperature has been observed in oocytes from other species although the rate and the extent differ (Richardson and Parks, 1992; Aman and Parks, 1994; Tharasantit et al., 2006; Wu et al., 2006). Repeating their mouse study in human, Pickering et al. (1990) observed a high proportion (50–75%) of oocytes with abnormal spindles following brief exposure to reduced temperature and a low frequency (29%) of normal repolymerization on return to 37°C. This has been confirmed by direct visualization of the human oocyte (Wang et al., 2001).

Unlike the pattern observed in the mouse, the chromosomes in the human remained in a discrete cluster whether an abnormal or normal spindle was present (Sathananthan et al., 1988a; Pickering...
et al., 1990; Gook et al., 1993). This difference relates to the formation of microtubule asters produced from the high concentration of free tubulin in the cytoplasm following depolymerization of the spindle in the mouse (Maro et al., 1985; Johnson and Pickering, 1987), which was not observed in human oocytes (Sathananthan et al., 1988a; Pickering et al., 1990; Gook et al., 1993). This is a significant difference between mouse and human oocytes. In human oocytes, the association between kinetochores and microtubule organizing centres, which anchor the chromosomes, is maintained and it is from these centres that repolymerization of the spindle is initiated.

Addition of cryoprotectants (DMSO or PROH) appeared to have a protective effect against the temperature-dependent depolymerization of the spindle, reducing the proportion of cooled mouse oocytes with an abnormal spindle (Van der Elst et al., 1988; Joly et al., 1992; George and Johnson, 1993). Cryoprotectants appeared to stabilize both the spindle and free tubulins and facilitate normal reformation of spindles within asters (Van der Elst et al., 1988; Joly et al., 1992; Pajot-Augy, 1993). Similar protection was observed following cryopreservation with PROH in human oocytes (Gook et al., 1993). Van der Elst et al. (1988) observed an interesting phenomenon associated with cooling in the presence of PROH; on rewarming both the spindle and asters were absent in all mouse oocytes but the fertilization rate was similar to that observed in non-cooled oocytes. Since a spindle must be present for normal fertilization to occur, this indicated that the spindle must have reformed during the 4 h insemination period. This delay in reformation of the spindle (Aigner et al., 1992; Eroglu et al., 1998b) has been confirmed in human oocytes by direct visualization after cryopreservation using PROH/sucrose (Rienzi et al., 2004; Bianchi et al., 2005b).

Although hyperosmotic conditions which occur during cryopreservation result in an increase in abnormal spindles in human oocytes (Mullen et al., 2004), a high proportion (76%) of normal spindles have been shown to reform following cryopreservation with PROH even in conjunction with elevated (0.5 M) sucrose concentrations (Rienzi et al., 2004; Bianchi et al., 2005b). Although the majority of human oocytes have a normal spindle appearance following cryopreservation with PROH, disruption of a single microtubule in the spindle may not be identified with the techniques used above.

The subsequent development of an oocyte with a minor aberration of the spindle could result in an aneuploid embryo. As outlined above, a high rate of aneuploidy may be expected in mouse embryos derived from cooled or cryopreserved oocytes, but Glenister et al. (1987) observed a low aneuploidy rate (1.5%) similar to that seen in those derived from non-frozen mouse oocytes and the inclusion of DMSO (Glenister et al., 1987) or PROH (Van der Elst et al., 1988) did not impact on aneuploidy rates. In contrast, Kola et al. (1988), using the same mouse oocyte cryopreservation procedure as that used by Glenister et al. (1987), reported a 3-fold increase in the aneuploidy rate of resultant zygotes (32%) compared with non-frozen controls, although no abnormal fetuses were developed. However, in a report from a larger study (Van Blerkom and Davis, 1994), no increase in the frequency of aneuploidy was reported for cryopreserved mouse and human oocytes. Assessment of cryopreserved (PROH and sucrose) human oocytes following fertilization, using a sensitive ultraviolet stain for DNA/chromatin, did not reveal any chromatin which had not been incorporated into the pronucleus or the second polar body (Gook et al., 1994). Normal karyotypes were also observed following fertilization of cryopreserved human oocytes (Gook et al., 1994). Fluorescent in situ hybridization using probes for five chromosomes in human embryos (n = 21) derived from cryopreserved oocytes detected a similar proportion with an abnormal chromosomal number to controls (28.6 versus 26.0%, respectively) (Cobo et al., 2001). Although attempts to assess the risk of aneuploidy in human cryopreserved oocytes have been limited, the high rate reported by Kola et al. (1988) has not been replicated. In essence, there is no clear evidence of a significantly elevated level of aneuploidy in human cryopreserved oocytes.

**Microfilaments**

Microfilaments, consisting predominately of actin, are involved in fertilization and cleavage and have also been shown to be altered by cryoprotectants and cooling. In a similar way to the microtubules of the spindle, microfilaments are absent following cryopreservation in PROH (Le Gal et al., 1994) and reform following removal of the cryoprotectant (Vincent et al., 1990b). PROH has been shown to decrease the length of actin filaments (Vincent et al., 1989, 1990b), and there is a suggestion that this may confer a benefit during cryopreservation by removing cytoskeletal restrictions, thereby permitting a lower cell volume during dehydration (Vincent et al., 1990b).

**Mitochondria**

Another change observed in the cortical region, following cryopreservation using the PROH/sucrose procedure, is the inability of mitochondria to form the normal aggregates observed in non-frozen oocytes (Jones et al., 2004). This phenomenon appears to have no impact on ATP production in the oocyte and may relate to diminished ability to regulate intracellular free calcium levels which are also altered within cryopreserved oocytes (Jones et al., 2004). It has also been suggested that microvacuolization in human cryopreserved oocytes may be more evident in the presence of elevated sucrose concentrations (Nottola et al., 2007).

**Embryogenesis and genetic abnormalities**

Although many of these processes or organelles are affected during cryopreservation, the key question is whether normal function has been preserved. Although the ability to undergo normal fertilization appears to be preserved, at least with the PROH/sucrose cryopreservation regimen, this does not ensure subsequent normal development. In a study which assessed subsequent cleavage following normal fertilization of cryopreserved human oocytes (Gook et al., 1995b), all fertilized oocytes (n = 16) cleaved following 24 h in culture, although the majority (56%) had undergone only one cleavage division. Subsequent cleavage occurred in the majority following another 24 h of culture with 50% reaching the 8-cell stage without exhibiting significant cytoplasmic fragmentation. The proportion continuing to cleave reduced with further time but 25% had formed normal blastocysts at the same time as non-frozen oocyte controls and subsequent hatching was observed. Development to the blastocyst stage was confirmed later by Cobo et al. (2001) with oocytes cryopreserved using the same procedure but with a higher sucrose concentration (0.2 M). A similar rate of blastocyst formation (30%) to that...
described above and to embryos derived from non-frozen oocytes (35%) was observed.

**Clinical application of oocyte cryopreservation**

**Slow cooling in 1.5 M PROH with 0.1 M sucrose**

Based on the above biological validation studies and following the report of the first live birth from oocytes cryopreserved using PROH/sucrose (Porcu et al., 1997), a number of reports detailing clinical experience with the protocol were published. Prior to the first birth, Tucker et al. (1996) reported the application of this procedure to a small series of donated oocyte cycles. Survival was low (25%) but fertilization following ICSI (65%) was similar to that reported previously (Gook et al., 1995b), and all normally fertilized oocytes developed into good quality embryos with 4–12 cells on Day 3. Transfer of all embryos (13) resulted in two implantations (fetal hearts), both of which miscarried during the first trimester although normal karyotype was confirmed in both. The series had achieved an implantation rate of 15%, but attrition at each step reduced the success to only 2.5 implantations per 100 oocytes thawed. Although this is not the standard way to express ART outcomes, it is a useful way to compare success using different methods for oocyte cryopreservation. Following on from the first birth (Porcu et al., 1997), a number of other groups reported case studies which resulted in either pregnancies (Nawroth and Kissing, 1998; Young et al., 1998; Chia et al., 2000; Allan, 2004) or births (Polak de Fried et al., 1998; Wurfel et al., 1999; Huttelova et al., 2003; Notrica et al., 2003; Miller et al., 2004). Variation in results reported in these case studies make it difficult to gauge the success of the procedure. However, larger studies reported survival rates that were either similar to that reported by Tucker et al. (1996), i.e. ~25% (Kazem et al., 1995; De Santis et al., 2007) or similar to that reported by Gook et al. (1993), i.e. ~50% (Antinori et al., 1998; Borini et al., 1998, 2004, 2006a; Porcu et al., 1998, 2000, 2001, 2002). ICSI has been used in all clinical experience with cryopreserved oocytes. The majority of zygotes (range 75–100%) cleaved by Day 2 and, in the one study in which cleavage was assessed on Day 3, a majority (7/9) had continued to cleave with six having reached the 8-cell stage (Miller et al., 2004). Numerous pregnancies and births were reported and, in the largest study (Borini et al., 2006a) of over 700 thawed oocytes, an implantation rate of 12% was reported although a quarter of the pregnancies subsequently aborted. In this clinic, the implantation rate was much lower than observed with fresh oocytes (23%), but the abortion rate was similar. To date, in the above studies, 61 pregnancies (64 implantations) and 38 births have been reported (Table 1).

However, oocyte cryopreservation using this procedure was relatively inefficient, mainly as a consequence of the low survival rate. To provide an overall assessment of the efficiency, the information reported in the above studies has been combined and attrition rates calculated (see Table 5 for final summary). Based on the published data on 3537 oocytes cryopreserved, the cumulative impact of the reported survival, fertilization and cleavage rates results in 23 embryos being generated per 100 thawed oocytes and the proportion of transferred embryos which resulted in implantation was 10%. In studies where the number of embryos transferred has not been reported, an assumption has been made that all embryos were transferred. The overall yield from cryopreserved oocytes is, therefore, 2.3 implantations per 100 oocytes thawed. This disappointing overall attrition rate, which appeared to be associated with the use of this protocol in a clinical context (Gook and Edgar, 1999), led to a search for modifications to the methodology in the hope of improving clinical efficiency.

**Slow cooling in 1.5 M PROH in the presence of elevated sucrose concentrations**

The possibility of increasing the extent of dehydration of human oocytes prior to cooling was considered as a possible approach to improving outcome. This was aimed at overcoming the variation in hydraulic permeability coefficients observed in individual human oocytes (Hunter et al., 1992a) and increasing the stabilization of the phospholipid bilayer during cryopreservation (Crowe et al., 1990). Survival was enhanced by increasing the sucrose concentration from 0.1 M (39%) to 0.2 M (58%) or 0.3 M (83%) in the first report of this approach (Fabbri et al., 2001). Higher survival rates could also be achieved by extending the dehydration time in 0.2 M sucrose from 5 min (55%) to >10 min (70%). Additionally, higher fertilization and cleavage rates have been reported with 0.2 M when compared with 0.1 M sucrose (Chen et al., 2004). Therefore, significantly more embryos may be generated when oocytes are cryopreserved in 0.2 M relative to 0.1 M sucrose. Similarly, survival (71 versus 24%), fertilization (80 versus 53%) and cleavage (91 versus 80%) rates were all significantly increased when oocytes were cryopreserved in 0.3 M relative to 0.1 M sucrose (De Santis et al., 2007) resulting in an overall 5-fold increase in available embryos. The fertilization (76%) and cleavage (94%) rates of oocytes cryopreserved in 0.2 M sucrose have been reported to be similar to the rates for fresh oocytes (80 and 97%, respectively) from the same clinic (Bianchi et al., 2007). Similar fertilization (67 versus 67%) and cleavage (89 versus 98%) rates have also been reported for oocytes frozen in 0.3 M sucrose relative to fresh controls (Levi Setti et al., 2006).
Although more comparisons may be required to fully substantiate these results, they suggest that oocyte cryopreservation does not impact on the fertilization rate or the proportion of embryos, which cleave if a sufficient level of dehydration is achieved prior to cooling. However, this does not preclude an impact on embryo quality.

Although protocols using both 0.2 and 0.3 M sucrose have been applied clinically (Table 2), more extensive experience has been reported for 0.3 M sucrose which has been used for oocytes from over 800 patients in 11 clinics (Chen et al., 2002, 2005; Fosas et al., 2003; Li et al., 2005; Tjer et al., 2005; Chamayou et al., 2006; La Sala et al., 2006; Levi Setti et al., 2006; Borini et al., 2006b; Barritt et al., 2007; De Santis et al., 2007; Konc et al., 2007). Outcomes from oocyte cryopreservation using 0.2 M sucrose have been reported for 200 patients in 6 clinics (Yang et al., 1998, 2002; Porcu et al., 1999; Kyono et al., 2001; Winslow et al., 2001; Montag et al., 2006; Bianchi et al., 2007; Gook et al., 2007). Clinical experience of higher sucrose concentration procedures has substantiated the initial reports of higher survival rates with reported rates using 0.2 M sucrose ranging from 68–76% and using 0.3 M sucrose ranging from 69–90%.

The first birth following cryopreservation using an elevated (0.2 M) sucrose concentration was reported by Porcu et al. (1999). Minor modifications to the 0.2 M sucrose procedure initially described by Porcu et al. (1999) have also been reported including freezing the oocyte within the intact cumulus complex (Kyono et al., 2001; Montag et al., 2006), dehydration and rehydration at 37°C (Yang et al., 1998, 2002; Winslow et al., 2001; Gook et al., 2007) and the use of 0.3 M sucrose in the initial rehydration step after thawing (Bianchi et al., 2007). The pooled results from reports using 0.2 M sucrose are presented in the final summary (Table 5). An overall survival rate of 72%, a fertilization rate of 80% and a cleavage rate of 93% are reflected in a yield of 53 embryos per 100 thawed oocytes. A subsequent implantation rate of 17% results in an overall yield of 9.1 implantations per 100 thawed oocytes, which surpasses the overall outcome reported from the original 0.1 M sucrose protocol. There have been 46 pregnancies and 37 births reported (with one further unpublished birth and an ongoing pregnancy from the authors’ clinic). In a comparison of outcomes from oocytes cryopreserved in 0.2 M sucrose with those from fresh oocytes, Bianchi et al. (2007) reported similar fertilization (76 versus 80%), cleavage (94 versus 97%), implantation (13 versus 14%) and abortion (12 versus 10%) rates. Although the report did not include an assessment of the quality of embryos derived from the fresh oocytes, >40% of embryos resulting from the thawed oocytes were at the 4-cell stage, 36% at the 2-cell stage and 86% had <25% cytoplasmic fragmentation on Day 2 of development, suggesting that embryo quality was within normal limits. The robust nature of the zygotes/embryos derived from this oocyte cryopreservation procedure is illustrated by two reported births, one following subsequent refreezing at the pronuclear stage (Montag et al., 2006) and the other following subsequent refreezing at an early cleavage stage (Gook et al., 2007).

Three studies have reported clinical results for a large number of patients (>100) using 0.3 M sucrose during oocyte cryopreservation (Borini et al., 2006b; La Sala et al., 2006; Levi Setti et al., 2006). Although survival, fertilization and cleavage rates appear normal in these and two other studies (Chamayou et al., 2006; De Santis et al., 2007), the implantation rate is poor (~5%). The pooled data for all reports to date using 0.3 M sucrose (Chen et al., 2002, 2005; Fosas et al., 2003; Li et al., 2005; Tjer et al., 2005; Chamayou et al., 2006; La Sala et al., 2006; Levi Setti et al., 2006; Borini et al., 2006b; Barritt et al., 2007; De Santis et al., 2007; Konc et al., 2007) are summarized in Table 5 and show clearly that, although the number of embryos generated (49 from 100 thawed oocytes) is consistent with outcomes from the use of 0.2 M sucrose, relatively few of the resultant embryos (5%) implant. The overall yield (2.4 implantations per 100 thawed oocytes) is lower than that achieved with the use of 0.2 M sucrose and similar to that achieved with 0.1 M sucrose. A number of reports (La Sala et al., 2006; Levi Setti et al., 2006; De Santis et al., 2007) also indicate that approximately a third of these implantations may abort. A total of 47 babies have been born using this procedure from over 4900 oocytes thawed.

With fresh oocytes, a correlation has been demonstrated between the developmental rate of embryos and their implantation potential (Ziebe et al., 1998; Edgar et al., 2000). Early cleavage stage embryos with 4 cells on Day 2 have a significantly higher implantation rate than those that exhibit a slower growth rate. Unfortunately, this information is rarely reported for embryos derived from cryopreserved oocytes, with most authors merely reporting that cleavage has occurred. Borini et al. (2006b) reported that, in oocytes cryopreserved in 0.3 M sucrose, a high proportion (90%) cleaved but the majority (68%) of resultant embryos were only at the 2-cell stage on Day 2, and only 14% of embryos were at the 4-cell stage. Although retarded in their development, these embryos did not exhibit high levels of fragmentation with only 20% of embryos classified as grade III or IV (Borini et al., 2006b). In the absence of parallel control data for fresh oocytes from the same clinic, however, it is difficult to attribute this retarded development to oocyte cryopreservation alone. In

Table 2: Reports of clinical outcomes from human oocytes cryopreserved using 1.5 M PROH with elevated sucrose

<table>
<thead>
<tr>
<th>Study (no. of patients)</th>
<th>No. of oocytes thawed</th>
<th>Survival rate, %</th>
<th>Implantations</th>
<th>Births</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porcu et al. (1999) (1)</td>
<td>10</td>
<td>70</td>
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</tr>
<tr>
<td>Kyono et al. (2001) (1)</td>
<td>5</td>
<td>60</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Winslow et al. (2001) (33)</td>
<td>324</td>
<td>68</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>Yang et al. (2002) (24)</td>
<td>158</td>
<td>71</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>Montag et al. (2006) (1)</td>
<td>12</td>
<td>42</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bianchi et al. (2007) (141)</td>
<td>403</td>
<td>76</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td>Gook et al. (2007) (1)</td>
<td>6</td>
<td>67</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.3 M sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fosas et al. (2003) (7)</td>
<td>88</td>
<td>90</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Chen et al. (2005) (22)</td>
<td>159</td>
<td>75</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Li et al. (2005) (28)</td>
<td>81</td>
<td>90</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Tjer et al. (2005) (1)</td>
<td>14</td>
<td>71</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Borini et al. (2006b) (146)</td>
<td>927</td>
<td>74</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Chamayou et al. (2006) (40)</td>
<td>337</td>
<td>78</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>La Sala et al. (2006) (414)</td>
<td>1647</td>
<td>73</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>Levi Setti et al. (2006) (120)</td>
<td>1087</td>
<td>69</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>Barritt et al. (2007) (4)</td>
<td>79</td>
<td>86</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>De Santis et al. (2007) (66)</td>
<td>396</td>
<td>71</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Konc et al. (2007) (25)</td>
<td>87</td>
<td>80</td>
<td>7</td>
<td>3</td>
</tr>
</tbody>
</table>
another report (Levi Setti et al., 2006), the proportion of embryos generated from oocytes cryopreserved in 0.3 M sucrose (89%) is similar to that from fresh oocytes, but no information is given with respect to comparative developmental rates although the implantation rate is four times higher for embryos from fresh oocytes. There is only one clinical report of a small number of embryos from cryopreserved oocytes, which were cultured for an extended period and transferred at the blastocyst stage (Tjer et al., 2005), making further analysis of the impact on developmental rate difficult. Another report which suggests that cryopreservation in 0.3 M sucrose may retard subsequent development is the observation that very few resultant embryos (7%) had undergone early cleavage at 25 h post-insemination when compared with the majority of embryos (59%) from fresh oocytes (Bianchi et al., 2005a). Early cleavage, when assessed at 25–27 h post-insemination, is an independent indicator of developmental potential (Lundin et al., 2001; Sakkas et al., 2001; van Montfoort et al., 2005).

**Slow cooling in 1.5 M PROH with sucrose in sodium-depleted medium**

Based on the evidence that the removal of sodium from the medium used to cryopreserve mouse oocytes resulted in higher survival (>90%) and pregnancy (>40%) rates (Stachecki et al., 1998, 2002), a number of clinical studies have been reported using cryopreservation media in which sodium is replaced by choline (Table 3). However, no equivalent comparative data are available on human oocytes. Cryopreservation in sodium-depleted medium containing PROH and 0.1 M sucrose resulted in two births (Quintans et al., 2002), a further nine births have been reported in similar medium containing 0.2 M sucrose (Azambuja et al., 2005; Boldt et al., 2006; Petracco et al., 2006), and five births have been reported from the application of this approach in the presence of 0.3 M sucrose (Boldt et al., 2006). These clinical studies are on small series of patients (<25). Cryopreservation in the choline containing media did not appear to impact adversely on the fertilization rate, proportion of embryos cleaving or the level of fragmentation in the resulting embryos when compared with fresh oocytes. However, retarded development on Day 2 was again observed with 55% of the resultant embryos containing only two blastomeres on Day 2 when compared with 28% of embryos from fresh oocytes (Petracco et al., 2006). Although there are no parallel sodium-based media controls in the above studies, the survival, fertilization and proportion of embryos cleaving appear similar to those reported with equivalent sucrose concentrations in sodium-containing media (final summary Table 5). The high implantation rate reported with choline in conjunction with 0.1 M sucrose is based on a small number of embryos (33), and a high abortion rate (33%) is also reported (Quintans et al., 2002). A recent paper by Stachecki et al. (2006) suggested that subsequent modification of the original choline-containing media in conjunction with the inclusion of 0.2 M sucrose and PROH may improve survival to 90%.

**Alternative approaches**

**Cryopreservation of immature oocytes**

Given the temperature-sensitive nature of the spindle in MII oocytes, cryopreservation of immature stages may offer a theoretical advantage. In early studies (Mandelbaum et al., 1988a,b) it was shown that survival of germinal vesicle (GV) stage oocytes was equivalent to that of mature oocytes (30%) following cryopreservation. However, only 20% of the surviving GV oocytes underwent subsequent maturation. Although higher survival rates (50–60%) have been reported, cryopreservation appears to significantly impair the ability to mature (Toth et al., 1994b; Son et al., 1996; Park et al., 1997), fertilize and cleave (Son et al., 1996) relative to controls. When maturation occurred after cryopreservation, a normal spindle was detected in less than a third of the oocytes, and high rates of aneuploidy and polyploidy were observed (Park et al., 1997). Some animal studies have shown that subsequent development is impaired in oocytes cryopreserved at the GV stage (Schroeder et al., 1990; Van der Elst et al., 1993; Martino et al., 1996a) and the frequency of spindle abnormalities does not appear to be different to controls (Frydman et al., 1997; Eroglu et al., 1998a), whereas others have shown increased frequencies of spindle abnormalities (Saunders and Parks, 1999; Tharasanit et al., 2006). In the human studies described above, immature oocytes were collected from unstimulated cycles. Similar survival was observed when GV oocytes were derived from stimulated cycles, but maturation, fertilization (Toth et al., 1994a) and the proportion of oocytes with normal spindles (Baka et al., 1995) appeared to be unaffected. In contrast, others have found maturation to be impaired (Goud et al., 2000) and the spindle to be affected (Boiso et al., 2002; Stachecki et al., 2004). Although similar survival rates to mature oocytes can be achieved, surviving oocytes may be compromised and the developmental competence of in vitro matured GV oocytes appears to be compromised irrespective of cryopreservation (Gook et al., 1996). One birth has been reported from a cryopreserved GV oocyte from an IVF patient (Tucker et al., 1998), and a pregnancy has been reported from an oocyte cryopreserved at the MI stage (Kan et al., 2004).

**Cryopreservation of follicular oocytes within ovarian cortex**

The limited time available and the limited number of mature oocytes which may be collected from women facing the prospect of fertility loss as a consequence of cytotoxic therapy, has led to investigations into the possibility of cryopreserving relatively large numbers of primordial follicles within slices of ovarian cortex.
cortex. It has been established in histological studies that ovarian tissue can be cryopreserved successfully (Gook et al., 1999; Eyden et al., 2004; Martinez-Madrid et al., 2004; Rimon et al., 2005). Functional survival as assessed by developmental competence has also been demonstrated by xenografting of thawed tissue (Oktay et al., 1998, 2000; Kim et al., 2000, 2002; Nisolle et al., 2000; Gook et al., 2001, 2003, 2005; Van den Broecke et al., 2001a, b). Mature oocytes have been identified following xenografting of cryopreserved ovarian tissue (Gook et al., 2003, 2005) and following heterotopic autografting (Oktay et al., 2004; Schmidt et al., 2004; Poirot et al., 2006; Rosendahl et al., 2006). Fertilization and embryo development occurred in oocytes recovered from heterotopic autografts and, following transfer, one resulted in a biochemical pregnancy (Rosendahl et al., 2006). Three pregnancies have been reported from orthotopic grafting of cryopreserved ovarian tissue (Donnez et al., 2004; Meirow et al., 2005; Demeestere et al., 2006), two of which continued to term (Donnez et al., 2004; Meirow et al., 2005).

**Vitrification**

More recently, interest in vitrification of oocytes as an alternative to slow freezing has been stimulated by a number of reports. Vitrification is the process by which water is prevented from forming ice due to the viscosity of a highly concentrated cryoprotectant cooled at an extremely rapid rate (Taylor, 1987). To reduce exposure to the toxic cocktail of cryoprotectants and prevent extreme dehydration, cells are exposed to the cryoprotectants for a very short period. In three early studies of human oocyte cryopreservation, vitrification was also assessed (Trounson, 1986; Al-Hasani et al., 1987; Pensis et al., 1989). Large variation in survival (4 to 75%) suggested that technical variations may have a major impact. Hunter et al. (1995) showed that brief exposure of human oocytes to a cryoprotectant cocktail (Rall and Fahy, 1985) had no impact on fertilization and embryo development to the morula stage. However, following vitrification using this cocktail, although 65% of oocytes survived and 45% of those fertilized, all arrested at the pronuclear stage. Effects observed following vitrification of human oocytes included damage to the zona pellucida and oolemma, disorganization of organelles, spindle disorganization from the pronuclei (Sathananthan et al., 1987).

However, a small study of seven human oocytes vitrified at the GV stage suggested that the resulting spindle was normal (Neri et al., 2003). In animal oocytes, spindle disruption has been observed following vitrification (Chen et al., 2000; Rojas et al., 2004; Cai et al., 2005; Shi et al., 2006; Tharasanit et al., 2006; Wu et al., 2006) as have high rates of aneuploidy and polyploidy in resultant zygotes and reduced embryo development (Kola et al., 1988; Martino et al., 1996b; Chian et al., 2004; Rho et al., 2004; Cai et al., 2005). Conversely, it has been suggested (Gardner et al., 2007) that vitrification may be less traumatic to the meiotic spindle than slow freezing and may also have less effect on cell physiology. The most concerning result following vitrification of mouse oocytes was a reduced proportion of live fetuses and a reciprocal increase in the proportion of malformed fetuses (Kola et al., 1988). Although it is unclear whether this outcome was related to the specific cocktail of cryoprotectants or the overall vitrification procedure, it suggests that caution should be exercised when applying the technology clinically.

A number of innovations have been introduced to increase the rate of cooling achieved. A minimal volume of the cryoprotectant solution containing the oocyte is exposed directly to liquid nitrogen in either a thin open straw (Vajta et al., 1998), which has since been modified to the Cryotop (Kuwayama et al., 2005), or on an electron microscopy grid (Martino et al., 1996b), which has subsequently been modified to the Cryoloop (Lane et al., 1999). In addition, an extensive evaluation of cryoprotectant combinations for vitrification indicated that a very high concentration of ethylene glycol (EG; 5.5 M) and sucrose (1.0 M) could be applied with minimal toxicity (Ali and Shelton, 1993). Using this cryoprotectant solution in combination with thin straws, the first birth from mature human oocyte vitrification was reported (Kuleshova et al., 1999). This and subsequent reports of clinical outcomes are listed in Table 4. The same procedure, but using grids, resulted in a pregnancy from immature human oocytes (Wu et al., 2001) and seven births from mature oocytes (Yoon et al., 2003). In this study, vitrification of oocyte-cumulus complexes resulted in 69% survival and a fertilization rate of 72% following ICSI. Although 95% of fertilized oocytes cleaved, a low implantation rate of 6% suggested impaired developmental potential. The same group had previously reported similar results using human immature oocytes (Hong et al., 1999; Cha et al., 2000; Chung et al., 2000; Yoon et al., 2000). Resultant blastocysts that could be assessed in these studies (n = 7) all had normal karyotypes.

Using human mature oocytes and a slightly lower concentration of EG (5.0 M) with the Cryotop, high rates of survival (90%), fertilization (90%) and development to the blastocyst stage (~50%) have been reported in association with an implantation rate of 18.7% and seven births (Kuwayama et al., 2005). A modification of the above procedure in which the concentrations of EG and sucrose have been halved, and DMSO (2.1 M) added to the cryoprotectant mix has resulted in 2 births both with normal karyotypes (Katayama et al., 2003), a further 11 births (Kyono et al., 2005; Okimura et al., 2005; Antinori et al., 2007) and a number of other pregnancies (Lucena et al., 2006; Selman et al., 2006). No difference was observed in the fertilization rate (93 versus 97%), the proportion of cleaving embryos (97 versus 98%), the implantation rate (13 versus 10%) or the abortion rate (20 versus 18%)

<table>
<thead>
<tr>
<th>Study (no. of patients)</th>
<th>No. of oocytes thawed</th>
<th>Survival rate, %</th>
<th>Implantations</th>
<th>Births</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kuleshova et al. (1999) (4)</td>
<td>17</td>
<td>65</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Wu et al. (2001) (36)</td>
<td>79</td>
<td>59</td>
<td>1</td>
<td>0</td>
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<td>Yoon et al. (2003) (34)</td>
<td>474</td>
<td>69</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Kim et al. (2003) (16)</td>
<td>51</td>
<td>80</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chian et al. (2005) (15)</td>
<td>180</td>
<td>94</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Kuwayama et al. (2005) (67)</td>
<td>107</td>
<td>80</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>2.7 M EG + 2.1 M DMSO + 0.5 M sucrose</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Katayama et al. (2003) (6)</td>
<td>46</td>
<td>94</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Kyono et al. (2005) (1)</td>
<td>5</td>
<td>100</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Okimura et al. (2005)</td>
<td>64</td>
<td>91</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Lucena et al. (2006) (23)</td>
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<td>75</td>
<td>13</td>
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</tr>
<tr>
<td>Selman et al. (2006) (6)</td>
<td>24</td>
<td>75</td>
<td>3</td>
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<tr>
<td>Antinori et al. (2007) (120)</td>
<td>330</td>
<td>99</td>
<td>39</td>
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</tbody>
</table>
Table 5: Summary of clinical outcomes from oocyte cryopreservation using various protocols

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Survival, % (no. of thawed oocytes)</th>
<th>Fertilization (ICSI), %</th>
<th>Cleavage, %</th>
<th>Embryos per 100 thawed oocytes</th>
<th>Implantation rate, %</th>
<th>Implantations per 100 thawed oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M PROH + 0.1 M sucrose</td>
<td>50 (3537)</td>
<td>54</td>
<td>85</td>
<td>23</td>
<td>10</td>
<td>2.3</td>
</tr>
<tr>
<td>1.5 M PROH + 0.2 M sucrose</td>
<td>72 (926)</td>
<td>80</td>
<td>93</td>
<td>53</td>
<td>17</td>
<td>9.1</td>
</tr>
<tr>
<td>1.5 M PROH + 0.3 M sucrose</td>
<td>74 (4902)</td>
<td>73</td>
<td>90</td>
<td>49</td>
<td>5</td>
<td>2.4</td>
</tr>
<tr>
<td>1.5 M PROH + 0.1 M sucrose (Na depleted)</td>
<td>52 (127)</td>
<td>56</td>
<td>100</td>
<td>29</td>
<td>21</td>
<td>6.1</td>
</tr>
<tr>
<td>1.5 M PROH + 0.2 M sucrose (Na depleted)</td>
<td>62 (329)</td>
<td>58</td>
<td>86</td>
<td>31</td>
<td>11</td>
<td>3.4</td>
</tr>
<tr>
<td>1.5 M PROH + 0.3 M sucrose (Na depleted)</td>
<td>59 (190)</td>
<td>68</td>
<td>83</td>
<td>33</td>
<td>16</td>
<td>5.3</td>
</tr>
<tr>
<td>Vitrification 2.7 M EG + 2.1 M DMSO + 0.5 M sucrose</td>
<td>91 (628)</td>
<td>91</td>
<td>92</td>
<td>76</td>
<td>14</td>
<td>11</td>
</tr>
</tbody>
</table>

when compared with non-vitrified oocytes, respectively (Antinori et al., 2007).

It is evident from the above discussion that wide variation exists between oocyte vitrification methodology and outcomes and that this has changed significantly over time. An attempt to analyse the efficiency of vitrification by meta-analysis (Oktay et al., 2006) included studies using a range of different methodologies. We have restricted our summary analysis of human oocyte vitrification (Table 5) to reports using the most recent protocol (Katayama et al., 2003), which appears to be the most promising and is being used by a number of groups.

Conclusions

The ability to cryopreserve human oocytes confers significant advantages in clinical assisted reproduction. The validation of slow cooling/rapid thaw methods for oocyte cryopreservation in the 1990’s led to clinical application with limited success. Although attempts to improve the methodology by modification of the dehydration and cryopreservation media appeared to improve cryosurvival and fertilization rates, there is still insufficient evidence to determine whether this has increased clinical efficiency to a significant extent or to define optimal protocols for human oocyte cryopreservation (Oktay et al., 2006). Problems associated with assessment of clinical efficiency include a paucity of studies with appropriate control groups and, in circumstances where the legal framework dictates, restrictions on the ability to apply selection criteria to embryos derived from cryopreserved oocytes (Edgar and Gook, 2007). While vitrification may hold promise for future improvement, the limited experience reported to date requires more extensive confirmation. Given the relatively small number of births from this technology to date, there is a clear requirement for detailed follow up of children born, particularly with respect to cases involving vitrification where there is a lack of basic information on the biological implications.

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