Human oocyte cryopreservation

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Despite early success with sperm and embryo cryopreservation, human oocyte cryopreservation has met with limited success both in terms of survival and subsequent fertilization. However, compared with embryo cryopreservation, oocyte freezing has great potential for eliminating the ethical and legal problems of embryo storage.

As long-term survival rates for young cancer patients continue to improve, protection against iatrogenic infertility caused by chemotherapy and/or radiotherapy becomes a higher priority (Apperley and Reddy, 1995). There is currently no way of predicting whether or not a patient will remain fertile after being exposed to chemotherapy or radiotherapy. Occasionally there is only a temporary lull in menstrual rhythmicity, but when the treatment is particularly aggressive or the patient is over 30 years of age the risk of permanent sterility increases.

Embryo cryopreservation may sometimes be the only option available for patients undergoing aggressive high-dose chemotherapy and bone-marrow transplantation. However, this is far from satisfactory since a harvest of viable oocytes cannot be guaranteed and there may not be enough time for a complete in-vitro fertilization (IVF) cycle before cancer treatment begins. What is more, the procedure is inappropriate in children and unacceptable to many women who do not have a partner and reject donor sperm as an alternative. Donor oocytes can be used as a last resort after iatrogenic menopause, but they are scarce in most countries and add to the costs of treatment when available. In view of all these practical and ethical problems, it is highly desirable to develop a technology to store the unfertilized female gamete.

The difficulties in cryopreserving human oocytes are related to their large volume and variable membrane permeability. This makes it difficult to achieve sufficient dehydration during cooling to prevent ice formation, without disrupting other cytoplasmic and nuclear components. It has been suggested that cooling an oocyte on its own (Hunter et al, 1991; Pickering et al, 1990), exposure to cryoprotectants (Hunter et al, 1991; Pickering et al, 1991) and cryopreservation may increase the incidence of chromosomal loss from the meiotic spindle as the temperature-sensitive spindle, which holds the chromosomes in place, disaggregates and reforms abnormally on rewarming (Pickering et al, 1990; Baka et al, 1995). In addition, oocyte cryopreservation may initiate parthogenetic activation (development without a paternal genome) (Gook et al, 1995a) and stimulate the release of cortical granules (which are usually released at fertilization, causing the zona to harden, reducing the likelihood of polyspermy) (Trounson and Kirby, 1989; Vincent et al, 1990).

It should be noted, however, that healthy live offspring have been born from cryopreserved metaphase II mouse (Whittingham, 1977), rat (Kasai et al, 1979), monkey (DeMayo et al, 1985), rabbit (Al-Hasani et al, 1989) and human oocytes (Chen, 1986; Van Uem et al, 1987; Porcu et al, 1997, 1998; Polak de Fried et al, 1998; Antinori et al, 1998; Borini et al, 1998; Tucker et al, 1998b). These results clearly indicate that not all embryos derived from cryopreserved oocytes are abnormal. In some thawed oocytes, there may be a process of repolymerization that allows the spindle to recover so that subsequent processes associated with fertilization, cleavage and development can occur normally (Gook et al, 1993, 1994, 1995a,b; Baka et al, 1995).

Immature oocytes, aspirated from growing follicles, in stimulated (Mandelbaum et al, 1988; Toth et al, 1994a; Tucker et al, 1998a,b) or spontaneous cycles (Toth et al, 1994b; Son et al, 1996; Park et al, 1997) are less differentiated than metaphase II oocytes and may be less prone

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to microtubular disruption, since they have a disassembled microtubule apparatus and decondensed chromatin housed within a nuclear membrane. The disadvantage of immature oocyte cryopreservation is that after thawing, the cells must be matured in vitro before fertilization. In-vitro maturation (IVM) has proved highly successful in animals and ~50% of cattle blastocyts generated by IVM/IVF form fetuses after transfer to recipients (Trounson et al, 1994a). Furthermore, frozen-thawed mouse (Candy et al, 1994), bovine (Suzuki et al, 1996) and human (Tucker et al, 1998a,b) oocytes have survived, matured and fertilized in vitro to form blastocyts, which when transferred to recipients produced live young.

MATURE OOCYTE CRYOPRESERVATION

Soon after the first reports that human embryos could be successfully frozen and thawed (Trounson and Mohr, 1983; Zeilmaker et al, 1984) interest rapidly turned to the mature oocyte. The first successful pregnancy after human oocyte cryopreservation was reported in 1986 by Chen, who obtained high rates of survival (80%) and fertilization (83%) after oocytes were exposed to 1.5 M dimethylsulphoxide (DMSO) at 0°C for 20 minutes, seeded at -7°C, before slow cooling at 0.3°C/min to -40°C and transferred to liquid nitrogen. Thawing was achieved rapidly by warming in a 45°C water-bath, followed by dilution of DMSO as a single step.

After the transfer of embryos obtained from oocytes frozen using this protocol, one twin and one singleton pregnancy went to term. In the following year, two additional pregnancies were reported (Chen, 1987; Van Uem et al, 1987). Subsequent studies, however, did not report similar levels of success with low post-thaw survival (25–35%) and poor fertilization rates after classical insemination (32–54%) (Al-Hasani et al, 1987; Mandelbaum et al, 1988) together with poor embryo developmental ability (Trounson and Kirby, 1989).

This experience has raised several questions about the ability of the mature oocyte to be cryopreserved successfully. Much of the apprehension surrounding the cryopreservation of the mature oocyte concerns the vulnerability of particular organelles in the oocyte cytoplasm, such as the meiotic spindle and cortical granules. Microtubules of the second meiotic metaphase spindle have been shown to be highly sensitive to thermal change. Exposure of mouse oocytes to a reduced temperature of 20°C or 25°C resulted in 89% or 75% abnormal spindles, respectively (Pickering and Johnson, 1987; Van der Elst et al, 1988). Pickering and Johnson also observed chromosomes scattered within the cytoplasm of the oocytes, while Van der Elst et al (1988) reported that 27% of the embryos exposed to this treatment had abnormal chromosome numbers. In the human oocyte, Pickering et al (1990) observed that cooling to room temperature for 10 minutes followed by return to 37°C caused disruption of the spindle in 75% of cases; in this study, scattered chromosomes were observed in a discrete cluster. The sensitivity of the spindle to minor reductions in temperature suggests a more acute response when exposed to subzero temperatures attained in cryopreservation.

Al-Hasani et al (1987) postulated that the high rate of polyploid embryos, which resulted after IVF of human frozen-thawed oocytes, might be the result of cryopreservation-induced damage to the cortical granules, which are responsible for preventing the entry of more than one sperm into the cytoplasm. The migration of cortical granules from the cortex back to the centre of the oocyte, thereby permitting multiple sperm entry, is another explanation for polyploidy in frozen-thawed oocytes (Sathananthan and Trounson, 1982). Both a reduced temperature of 4°C or exposure to DMSO decrease the relative number of cortical granules in the cortex of mouse oocytes (Vincent et al, 1990), indicating that a possible consequence of cryopreservation may be the spontaneous migration of the cortical granules.

TABLE 1.
Advantages of oocyte cryopreservation

Avoid the ethical dilemmas associated with embryo cryopreservation
Allow donated oocytes to be used in a more cost-effective manner
Provide a means to reconstitute fertility after successful cancer therapy
Avoid the unnecessary wastage of surplus oocytes in routine in-vitro fertilization treatment
Immature oocyte storage in combination with in-vitro maturation could reduce the cost of infertility treatment and avoid the risk of ovarian hyperstimulation syndrome

TABLE 2.
Potential problems for freeze-storing mature oocytes

The spindle in metaphase II oocytes depolymerizes on cooling and, although reversible on rewarming, it is possible that not all chromosomes will reassemble on the same spindle and aneuploidy might occur after the second maturation division
Cryopreservation hardens the zona pellucida and, unless this can be avoided by adding serum or substitutes to the medium, intracytoplasmic sperm injection is required for fertilization
The exceptionally large volume of the mature oocyte prolongs the process of thermal and chemical equilibration

TABLE 3.
Summary of published data on the cryopreservation of mature (metaphase II) human oocytes

Author	Year	Cryoprotectant	Cumulus	Oocytes	Survival (%)	Fertilization (%)	Cleavage (%)	Pregnancy/birth
Chen	1986	DMSO (1.5 M)	Yes	40	32 (80)	25 (83) IVF	18 (60)	Yes/twins
Van Uem et al	1987	DMSO (1.5 M)	No	4	ND	2 IVF	2 (100)	Yes/female
Al-Hasani et al	1987	DMSO (1.5 M) and PROH (1.5 M)	Yes Yes	144 38	40 (28) 12 (31)	20 (50) IVF 9 (75) IVF	ND ND	No transfer
Mandelbaum et al	1988	PROH (1.5 M)	Yes	56	20 (36)	ND	ND	No transfer
Siebzehnruebl et al	1989	DMSO (1.5 M)	Yes	38	14 (37)	7 (50) IVF	ND	Yes (2)/ND
Todorow et al	1989	DMSO (1.5 M)	Yes	48	20 (42)	3 (15) IVF	ND	No transfer
Hunter et al	1991	DMSO (1.5M)	Yes	15	11 (73)	5 (45) IVF	1 (20)	No transfer
Imoedemhe and Sigue	1992	PROH (2 M) and sucrose (0.25 M)	Yes No	33 30	18 (55) 8 (27)	4 (22) IVF 1 (12) IVF	1 (25) 0	No transfer
Gook et al	1993	PROH (1.5 M) and sucrose (0.15 M)	No Yes	131 48	91 (69) 23 (48)	ND ND	ND ND	No transfer
Gook et al	1994	PROH (1.5 M) and sucrose (0.15 M)	No	134	55 (41)	25 (46) IVF	ND	No transfer
Gook et al	1995b	PROH (1.5 M) and sucrose (0.15 M)	No	26 IVF 20 ICSI	18 (69) 19 (95)	9 (50) IVF 7 (50) ICSI	9 (100 7 (100)	No transfer
Kazem et al	1995	PROH (1.5 M)	Yes	220	74 (34)	1/37 (2.7) IVF 16/37 (46) ICSI	0 (0) 7 (43)	No transfer
Porcu et al	1997	PROH (1.5 M)	Yes	12	4 (33)	2 (50) ICSI	1 (50)	Yes/female
Polak de Fried et al	1998	PROH (1.5 M) and sucrose (0.1 M)	Yes	10	3 (30)	2 (66) ICSI	2 (100)	Yes/male
Young et al	1998	PROH (1.5 M)	No	9	8 (89)	8 (100) ICSI	5 (62)	Triplets/miscarriage
Porcu et al	1998	PROH (1.5 M)	No	709	396 (56)	248 (63) ICSI	224 (90.3)	Yes/six children
Antinori et al	1998	PROH (1.5 M) and sucrose (0.1 M)	No	335	188 (56)	105 (56) ICSI	79 (75)	Yes (2)/male
Borini et al	1998	PROH (1.5 M) and sucrose (0.15 M)	No	129	63 (49)	34 (51) ICSI	32 (94)	Yes/three children
Tucker et al	1998b	PROH (1.5 M) and sucrose (0.1 M)	No	241	75 (31)	38 (51) ICSI	ND	Yes (5)/twins
Nawroth and Kissing	1998	PROH (1.5 M) and sucrose (0.1 M)	Yes	7	3 (43)	1 (33) ICSI	1 (100)	Yes/miscarriage

DMSO = dimethyl sulphoxide; ICSI = intracytoplasmic sperm injection; IVF = in-vitro fertilization; ND = not defined; PROH = propandiol

Meanwhile, Carroll et al (1989) suggested that the elevated rate of polyploidy recorded in fertilized, frozen-thawed metaphase II mouse oocytes was mainly the result of increased frequency of digyny, the retention of the second polar body and/or its chromosomes.

Recent reappraisals of the effects of freezing on the mature oocyte's infrastructure gave a more optimistic outlook (Gook et al, 1993, 1994). Using 1,2-propandiol (PROH) as cryoprotectant, Gook et al (1993) found that at least 60% of surviving MII oocytes had normal spindles and chromosome configurations, and there was no evidence of an increased frequency of freezing-associated aneuploidy as assessed by fluorescence or cytogenetics. In addition, cortical granule distribution was similar in cryopreserved and non-cryopreserved human oocytes, excluding spontaneous cortical reactions resulting from cryo-injury (Gook et al, 1993).

Furthermore, Gook and colleagues (1994) obtained normal and abnormal fertilization rates roughly approaching those of conventional IVF, indicating that in the surviving oocytes there appears to be minimal damage to the zona pellucida, plasma membrane, cytoskeleton or cortical granules as a result of the procedure. The investigators concluded that the spindle of the human oocyte appears to be less sensitive to cryopreservation than that of the mouse, since cryodamage previously reported in animal studies did not occur to the same extent in human oocytes.

In a subsequent study, Gook et al (1995a) showed that human oocytes were more stable and resistant to parthogenetic activation when exposed to agents such as PROH, which are known to induce activation in mouse oocytes. This indicates that digynic fertilization of human oocytes is unlikely and polyploidy following fertilization is more likely to result from polyspermic fertilization. Using PROH as the cryoprotectant to preserve MII oocytes, Gook and her colleagues (Gook et al, 1995b) achieved embryo development (following intracytoplasmic sperm injection (ICSI) fertilization) to the blastocyst and early hatching stages, indicating that cryopreservation of the mature human oocyte has not affected subsequent embryo development.

These encouraging results, together with subsequent reports of normal pregnancies (Porcu et al, 1997, 1998; Polak de Fried et al, 1998; Young et al, 1998; Antinori et al, 1998; Borini et al, 1998; Tucker et al, 1998b; Nawroth and Kissing, 1998) and deliveries (Porcu et al, 1997, 1998;

TABLE 4.
Summary of published data on the cryopreservation of immature (GV-stage) human oocytes

Author	Year	Ovarian stimulation	Cryoprotectant	Cumulus	Oocytes	Survival (%)	Oocytes reached MII in vitro (%)	Fertilization (%)	Cleavage (%)	Pregnancy/birth
Mandelbaum et al	1988	No	DMSO (1.5 M)	Yes	27	10 (37)	2 (20)	ND	ND	No
Toth et al	1994a	No	PROH (1.5 M) and sucrose (0.2M)	Yes	67	22 (43)	6 (27)	ND	ND	No
Toth et al	1994b	Yes	PROH (1.5 M) and sucrose (0.2M)	Yes	123	72 (58)	60 (83)	30/52 (57)	16 (53)	No
Baka et al	1995	Yes	PROH (1.5 M) and sucrose (0.2M)	Yes	98	62 (63)	42 (68)	ND	ND	No
Son et al	1996	No	PROH (1.5 M) One-step freezing	Yes	98	54 (55)	32 (59)	6/14 (43)	1 (17)	No
Park et al	1997	No	PROH (1.5 M) and sucrose (0.1M)	Yes	128	77 (60)	47 (61)	ND	ND	No
Tucker et al	1998a	Yes	PROH (1.5 M) and sucrose (0.1M)	No	13	3 (23)	2 (66)	2 (66) ICSI	2 (100)	Yes/female
Tucker et al	1998b	Yes	PROH (1.5 M) and sucrose (0.1M)	No	16	7 (44)	3 (43)	3 (100) ICSI	3 (100)	Yes/female

DMSO = dimethyl sulphoxide; ICSI = intracytoplasmic sperm injection; ND = not defined; PROH = propandiol

Polak de Fried et al, 1998; Antinori et al, 1998; Borini et al, 1998; Tucker et al, 1998b) have rekindled interest in mature oocyte cryopreservation. Nevertheless, in the two largest studies done on frozen-thawed mature human oocytes, the live birth rate was a disappointing 1–2%. Using a slow freeze rapid thaw protocol with PROH as the cryoprotectant, Porcu et al (1998) reported that only six births were obtained from more than 709 cryopreserved mature oocytes, a success rate of less than 1% of all thawed oocytes.

Using a similar technique, Tucker et al (1998b) achieved a live birth rate of 2% following freeze-thaw of more than 400 oocytes. Indeed, the overall combined data in the literature indicate that of 2477 mature oocytes that were frozen, only 48% (1195/2477) survived the freeze-thaw process. Using conventional IVF, the fertilization rate was 42% (111/266) compared with 58% (460/796) when ICSI was used. From an untold number of transfers, only 23 pregnancies were reported, leading to the birth of 17 healthy children (*Table 3*). Thus, despite considerable effort, mature human oocyte cryopreservation poses several technical problems and, unlike embryo cryopreservation, is far from becoming incorporated into routine clinical practice.

IMMATURE OOCYTE CRYOPRESERVATION

Germinal vesicle oocytes can be obtained from Graafian follicles via the transvaginal route, but a modified needle and lower aspiration pressure than used in IVF are needed to improve oocyte recovery rates (Trounson et al, 1994b). Immature oocyte cryopreservation carries many advantages. They are smaller and less differentiated, and there is time for repair of sub-lethal damage before they are ready for fertilization. Most importantly, since they are suspended at the prophase stage of meiosis I, they do not possess a spindle and the risks of cytogenetic errors are less. There is still a risk of zona hardening and damage to the cytoskeleton during exposure to cryoprotectants, freezing and thawing.

Mandelbaum et al (1988) reported the first successful cryopreservation of immature human oocytes using DMSO, although it is unclear as to the technique of freezing and whether the oocytes were collected from stimulated or unstimulated follicles. In that study, 37% of oocytes survived the freezing and thawing process and 20% matured in vitro to metaphase II. Subsequently, Toth et al (1994a) reported the survival and maturation of prophase I human oocytes obtained from unstimulated ovarian tissue using PROH. Using a one-step addition of PROH followed by a slow-freeze and thaw protocol, the authors obtained a low post-thaw survival of 15%, but a high maturation in surviving oocytes of 58%. By contrast, when PROH was added in a stepwise fashion, with the cryopreservation performed in the presence of 0.2M sucrose, they obtained a higher cryosurvival rate of 43% but a lower maturation rate of 27%.

Toth et al (1994b), using the stepwise addition of PROH, also reported the maturation, fertilization, and cleavage rates of immature human oocytes from stimulated IVF cycles after cryopreservation. In that study, cryopreserved oocytes achieved a 58% survival rate, an 83% maturation rate, a 57% fertilization rate and a 3% blastocyst rate. Other investigators (Cha et al, 1991; Gomez et al, 1993) have also demonstrated the benefit of using immature oocytes collected from stimulated ovaries.

Cha and co-workers (1991) reported higher fertilization rates for immature oocytes obtained from stimulated compared with non-stimulated ovaries. Similarly, Gomez et al (1993) obtained higher maturation rates of germinal vesicle-stage oocytes retrieved from gonadotrophin-stimulated cycles. Indeed, pooled data from the literature on 570 frozen immature human oocytes showed that 43% (107/250) of them collected in stimulated cycles were able to reach metaphase II in vitro compared with only 27% (87/320) of oocytes recovered from natural cycles (*Table 4*).

The decreased potential seen in the oocytes collected from unstimulated ovaries may be the result of the collection of oocytes outside the normal cohort recruited by the ovaries during the menstrual cycle. Oocytes may have been collected in stages of follicle growth or atresia that made them either more susceptible to cryodamage or with less potential for IVM (Wynn et al, 1998). Therefore, the purposeful collection of immature GV-stage oocytes after ovarian stimulation may afford a larger, clinically useful, biologically intact pool of oocytes for cryopreservation.

The impact of cryopreservation of immature human oocytes on the configuration of the meiotic spindle following IVM was examined by Baka et al (1995). They obtained survival rates of roughly 60%, irrespective of whether the oocytes were matured in vitro before or after cryopreservation. However, only 43% of the frozen-thawed mature oocytes had a normal spindle configuration and chromosomes compared with 81% of frozen-thawed in-vitro

matured prophase I oocytes (P = 0.0004). Baka et al concluded that cryopreservation of the prophase I human oocyte does not significantly increase abnormalities in the meiotic spindle.

On the other hand, Park et al (1997) reported a significant increase in the chromosomal abnormalities, especially aneuploidy, in frozen-thawed in vitro matured prophase I oocytes from unstimulated ovaries. In 78% of these oocytes, the chromosomes appeared fuzzy, less condensed or dispersed compared with 32% in control oocytes. In a previous study, the same group of researchers (Son et al, 1996) found that frozen-thawed prophase I oocytes had significantly lower fertilization (43%) and cleavage (17%) rates compared with control (91% and 95%, respectively). These two studies lead the investigators (Son et al, 1996; Park et al, 1997) to speculate that cytoplasmic and nuclear perturbations may be induced in the germinal vesicle oocytes by freezing-thawing, and these changes may not be reversible during culture after thawing.

Conflicting results have been reported on the role of the cumulus oophorus on the outcome of oocyte cryopreservation (Chen, 1986; Van Uem et al, 1987; Gook et al, 1993). When human oocytes that were cryopreserved within their cumulus cells were compared with those that were denuded of cumulus before cryopreservation, a higher survival rate of 69% was obtained in the denuded-oocytes group compared with 48% in those that were intact (Gook et al, 1993). The reduced survival in the cumulus-intact group may indicate a differing rate and extent of dehydration during cryopreservation because of the presence of cumulus cells and the cumulus matrix, rendering the oocyte more fragile to the cryopreservation regimen. The cumulus-corona complex may also form a rigid structure that limits the distortion of oocyte shape, which occurs during ice formation in the cryoprotectant (Ashwood-Smith et al, 1988). On the other hand, it has been speculated that cumulus cells seem to have a protective effect on the oocyte in the freeze-thaw cycle and are beneficial to the subsequent IVM of the GV-stage oocytes before IVF (Candy et al, 1994). Indeed, the outcome of GV (Porcu et al, 1997, 1998) and M II (Polak de Fried et al, 1998) oocyte cryopreservation was not found to be any different with or without the presence of the cumulus complex.

It is possible that aneuploidy screening of embryos generated from frozen-thawed oocytes would permit accurate study and selective assessment of such embryos for transfer (Kuleshova et al, 1999) to lessen any negative impact that oocyte cryopreservation might have on the clinical application of such technology. The normal fertilization and development of frozen-thawed germinal vesicle human oocytes, culminating in the delivery of healthy infants (Tucker et al, 1998a,b), must be taken as grounds for cautious optimism about the clinical application of immature oocyte storage.

CONCLUSIONS

Despite considerable effort, human oocyte cryopreservation still poses several technical problems and, unlike embryo cryopreservation, has not reached the stage of routine clinical use. Insemination of frozen-thawed oocytes by ICSI has solved one of the major problems of oocyte cryopreservation, i.e. low fertilization rates. The other limiting step is post-thaw survival which, with continuing technical refinement, will be further improved. Cautious optimism of the clinical application of this technology is encouraged by the birth of healthy children following successful freeze-storage of mature and immature human oocytes. **HM**

Conflict of interest: none.

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KEY POINTS

- Compared with embryo cryopreservation, oocyte freezing has great potential for eliminating the ethical and legal problems of embryo storage.
- Oocyte storage could be offered to patients at risk of losing their reproductive capacity through premature ovarian failure or anti-neoplastic treatments.
- The establishment of oocyte banks would not only reduce the costs and labour involved in synchronising donor-recipient cycles, but also provide a better match between oocyte donor and recipient.
- The disposal of excess mature oocytes, inevitably created through in-vitro fertilisation treatment, could be avoided where the surplus cryopreserved oocytes will remain viable until their future use.
- Freezing of immature oocytes in combination with in-vitro maturation could support the further development of assisted reproductive technologies and lead to a reduction in the need for ovarian stimulation.