Human Reproduction Vol.18, No.7 pp. 1417–1421, 2003 DOI: 10.1093/humrep/deg297

The births of five Spanish babies from cryopreserved donated oocytes

N.Fosas, F.Marina, P.J.Torres, I.Jové, P.Martín, N.Pérez, N.Arnedo and S.Marina[1]

Instituto de Reproducción CEFER, Marquesa de Vilallonga 12, 08017 Barcelona, Spain

[1]To whom correspondence should be addressed. E-mail: info@institutocefer.com

BACKGROUND: The technique of freezing oocytes is still not widely used. Reasons cited for this include the technique's low efficacy and the risk of aneuploidy. However, the introduction of technical changes (the type and concentration of cryoprotective substances; slow freezing and rapid thawing; and fertilization by ICSI) has led to improved results. We present four pregnancies obtained using mature oocytes (in metaphase II) that had been frozen and thawed. The oocytes were donated by young women who were not patients. METHODS: The frozen oocytes (n = 88) came from seven donors aged 18–25 years. The metaphase II oocytes, morphologically normal in appearance, were denuded of their cumulus–corona complex. The cryoprotective freezing solution contained 1,2-propanediol (1.5 mol/l) and sucrose (0.3 mol/l). Freezing was slow and thawing rapid. The oocytes were fertilized by ICSI. RESULTS: Seventy-nine of the 88 thawed oocytes survived (89.8%); 58 were fertilized (73.4% of all those microinjected); and 26 were transferred (44.8% of all those fertilized). Four pregnancies were produced after seven transfers (57.1%). Five children were born from four pregnancies. CONCLUSIONS: With the freezing/thawing technique used, oocyte survival was high (~90%). The pregnancy rate with frozen oocytes was similar to that obtained using fresh oocytes from donors (~50%).

Key words: birth/human/oocyte cryopreservation/oocyte donation

Introduction

In 1986 Chen published results of the first pregnancy achieved using frozen and thawed oocytes (Chen, 1986). In the following decade, publications about new pregnancies were sporadic (Al-Hasani *et al*., 1987; Van Uem *et al*., 1987; Serafini *et al*., 1995; Tucker *et al*., 1996). In 1995 ICSI was used for the first time to fertilize thawed oocytes (Gook *et al*., 1995; Kazem *et al*., 1995). ICSI has since been used to achieve a continuous string of pregnancies by the fertilization of frozen/thawed oocytes (Porcu *et al*., 1997; 1998, 1999a,b,c; 2000; 2002; Antinori *et al*., 1998; Borini *et al*., 1998; Polak de Fried *et al*., 1998; Tucker *et al*., 1998a; Vidali *et al*., 1998; Yang *et al*., 1998; 1999, 2002; Young *et al*., 1998a; Fabbri *et al*., 2001). As far as we know, all these children (more than 100) born from frozen oocytes are healthy. Freezing oocytes is an essential technique for preserving female fertility compromised by medical treatment (radiotherapy, chemotherapy, surgical removal of the ovaries) or the physiological effects of age. It is a useful technique for couples resorting to IVF who do not want to have any of their excess embryos frozen (Vidali *et al*., 1998). This situation is not unusual in Spain, where the decision about what to do with any excess embryos is not taken by the people who created them, but by the government (Law 35/1988). Freezing some of the oocytes obtained reduces or avoids the need to freeze embryos. Frozen oocytes are also useful in those sporadic cases of IVF in which the man unexpectedly cannot provide semen on the day scheduled for follicular puncture. In the oocyte donation process, having an oocyte bank would make it easier for a specific couple to choose the most appropriate donor; it would simplify the donation process, which is legal in Spain, and would make it possible to repeat the HIV blood test on the donor after the window period of viral infection has transpired, something that is not possible with fresh oocytes. The lower reproductive efficacy of frozen oocytes compared with fresh ones and the risk of aneuploidies have discouraged embryologists from freezing oocytes for reproductive purposes. In this article we present the results of freezing 88 oocytes in metaphase II for subsequent use in assisted reproduction. The oocytes came from seven follicular punctures performed on seven non-patient oocyte donors under the age of 30 years. Because these donors were young, non-sterile women and all the mature oocytes obtained were frozen, certain factors were eliminated that are unrelated to the practice of oocyte freezing, but which can bias results. This clinical research project was begun in December 2001 following a protocol approved by an ethics committee legally authorized to do so.

Materials and methods

Egg donors

Articles on the process of attracting non-patient oocyte donors and the medical tests they undergo before being accepted have been published previously (Marina *et al*., 1999). In summary, potential oocyte donors were selected from among university students age range 18–25 years who were not adopted and not virgins. The donors knew what the oocytes were to be used for and gave their written consent. They were given a complete medical check-up consisting of a detailed personal and family medical history, gynaecological exploration and ultrasound scan, a bacteriological study of cervicovaginal secretion and a blood test to check for HIV, hepatitis B and C and syphilis; a karyotype analysis and a test for coagulation factor VIII were carried out and a fetal haemoglobin assay was performed to test for thalassaemia. Donation was anonymous as dictated by Spanish law. The seven donors were single women who had never been pregnant.

Ovarian stimulation

Stimulation with rFSH was started on day 3 of the natural cycle, and was performed thereafter with dose adjustments based on the individual's ovarian response. Leuprolide acetate (0.15 mg daily, s.c.; Procrin, Abbott, Madrid, Spain) was administered from day 2 onward, up to and including the day of HCG. The donors came to the CEFER Reproduction Institute every day to receive ovarian stimulation treatment to ensure treatment was applied correctly. Ovarian response was monitored following conventional procedures by means of transvaginal ovarian ultrasound scans and serial 17-β-estradiol readings. Follicular puncture was performed 36 h after transvaginal, ultrasound-guided administration of 10 000 IU of HCG (Profasi HP; Serono, Madrid, Spain) under anaesthesia with propofol on an outpatient basis. Each donor was paid €900. An article containing the opinions of 100 donors compiled from a questionnaire they answered after follicular puncture has been published previously (Expósito *et al*., 2001).

Oocyte selection

Once the oocytes were identified in the follicular fluids, they were denuded by exposing them briefly to hyaluronidase (10 IU/ml) with the help of polycarbonate pipettes (Flexipet; Cook Spain, Madrid, Spain). The oocytes were checked for the presence of the first polar body in the perivitelline space (indicating that the oocytes were in metaphase II) and the morphological appearance of the gamete was evaluated (size of the perivitelline space, cell membrane and ooplasm). All the oocytes in metaphase II that were morphologically normal were frozen.

Cryoprotective media

The solutions of cryoprotectant agents used were as described by Fabbri *et al*. (2001). All the solutions were prepared using a home-made human tubal fluid medium buffered with HEPES (HTF/HEPES) and supplemented with human serum albumin (HSA). The permeating cryoprotectant used was 1,2-propanediol (PROH) and sucrose was used as the non-permeating cryoprotectant. Three solutions were used in the freezing phase: O1, O2 and O3. The composition of these solutions was: O1, HTF/HEPES + 20% HSA; O2, HTF/HEPES + 20% HSA + 1.5 mol/l PROH; and O3, HTF/HEPES + 20% HSA + 1.5 mol/l PROH + 0.3 mol/l sucrose.

Another three solutions were used in the thawing phase: O4, O5 and O6. The composition of these solutions was as follows: O4, HTF/HEPES + 20% HSA + 1.0 mol/l PROH + 0.3 mol/l sucrose; O5, HTF/HEPES + 20% HSA + 0.5 mol/l PROH + 0.3 mol/l sucrose; and O6, HTF/HEPES + 20% HSA+ 0.3 mol/l sucrose.

Freezing the oocytes

The mature, denuded oocytes were washed in O1 solution at room temperature (RT) and then immediately submerged in O2 equilibration medium for 10 min to begin dehydration. After 10 min, the oocytes were transferred to O3 solution, immediately loaded into plastic straws and introduced into a Kryo 10 series II biological freezer (Planner Kryo 10/1.7 GB). O3 solution was therefore the loading and freezing solution. The initial temperature was 20°C. The temperature was lowered at a rate of 2°C/min until –7°C was reached, at which point manual seeding was performed. At that moment ice crystals began forming outside the oocytes. The temperature continued to be lowered at a rate of 0.3°C/min until –30°C was reached, and then at 50°C/min until –80°C was reached. The straws were then submerged in liquid nitrogen (–196°C).

Oocyte thawing

The straws were thawed for 40 s at RT and for 1 min at 30°C. The oocytes were then placed in O4 solution for 5 min at RT; in O5 solution for 5 min at RT; and in O6 solution for 2.5 min at RT and for 2.5 min at 37°C. The last wash was done in O1 solution at 37°C and they were then cultured in HTF at 37°C in a 5% CO_2 atmosphere.

IVF by ICSI

After thawing, the oocyte morphology, cytoplasm, cell membrane and perivitelline space were evaluated. The oocytes were considered to have survived if their zona pellucida and cell membrane were intact, the perivitelline space was of a normal size and there was no evidence of ooplasm alterations. ICSI with frozen sperm from the husband of the recipient woman was performed on the oocytes considered survivors 1 or 2 h after thawing, and 17–20 h after sperm microinjection the presence of pronuclei was observed. Embryonic division was evaluated on days +2 and +3 after oocyte thawing. On day +3, assisted hatching was performed with acid Tyrodes on all the embryos to be transferred. Ultrasound-guided transfer into the vagina was carried out on day +3 using abdominal ultrasound. The woman then rested in the same bed for 2 h.

Recipients

The recipients in the study needed donor oocytes because they presented with a low response to ovarian stimulation, physiological menopause or repeated failures with IVF. All the couples gave their informed written consent to the use of donated and frozen oocytes.

Endometrial preparation

The endometrium was prepared to receive the embryos through the oral administration of estrogens at increasing doses until 6 mg/day was reached (Progynova; Schering Spain, Madrid, Spain). Endometrial response was monitored by ultrasound to measure endometrial thickness. The woman was considered to be ready to receive the embryos when the endometrium was at least 7 mm thick and ultrasonography showed a triple-line pattern; the serum estradiol level was $\geqslant$200 pg/ml. All the women were administered a 1 g dose of antibiotic the day before the transfer (Zytromax; Pfizer, Madrid, Spain) and 16 mg of corticoids (Urbason; Hoechst-Marion-Roussel, Barcelona, Spain) for 4 days starting on the day before transfer.

Luteal phase and pregnancy monitoring

Every 8 h, all the patients were administered 300 mg of progesterone vaginally (Utrogestan; Seid, Barcelona, Spain). A blood test was performed to check β-HCG levels 13 days after transfer. In the cases in which pregnancy was confirmed, amniocentesis was performed so the chromosomes of the fetal cells could be analysed.

Results

Oocyte donors received stimulation for an average of 11.9 days (range 7–15), and the mean total dose of rFSH was 1696.4 IU (range 900–3000). Table I shows the results of the freezing/thawing cycles and subsequent ICSI with oocytes in metaphase II. The most relevant clinical data on the receiving women are shown in Table II. Fetal chromosome studies were normal in the four pregnancies achieved, one of which involved twins. Each of the follicular punctures performed produced oocytes that were frozen, and subsequent embryo transfer was successful. Therefore, the rates of pregnancy per follicular puncture and per transfer were the same: 57.1% (four of seven). Five healthy children have been born from four deliveries: three boys and two girls.

Discussion

Frozen semen

In our oocyte donation programme we use frozen semen to avoid a meeting between the oocyte donor and recipient woman's husband. The oocyte donor must be anonymous, and in the clinical practice it is easier for patient and for biologist to work with frozen semen.

Alterations to the oocyte in the freezing–thawing process

Alterations that may arise during the freezing–thawing process include those affecting the following.

(i) The zona pellucida, which becomes harder, thus reducing the fertilization rate (Vincent and Johnson, 1992; Dumoulin *et al*., 1994; Kazem *et al*., 1995).

Table I. Results of oocyte freezing cycles (n = 7)

	N	Mean ± SD	Range	%
Metaphase II frozen	88	12.6 ± 3.0	9–20	100
Survived	79	11.3 ± 3.0	8–17	89.8
2 PN	58	8.3 ± 2.0	6–11	73.4[a]
1 PN	6	0.9 ± 1.4	0–4	7.6[a]
No PN	4	0.6 ± 1.1	0–3	5[a]
3 PN	3	0.4 ± 0.8	0–2	3.8[a]
>3 PN	2	0.3 ± 0.5	0–1	2.5[a]
Degenerated	6	0.9 ± 1.1	0–3	7.6[a]
No. embryos transferred	26	3.7 ± 0.5	3–4	44.8[b]

[a]Percentage of the total number of oocytes that survived cryopreservation.
[b]Percentage of embryos transferred out of all those fertilized.
PN = pronucleate.

(ii) The cortical granules are released prematurely (Van Blerkom and Davis, 1994), thus facilitating polyspermy.

(iii) The integrity of the fibres in the meiotic spindle (Pickering *et al*., 1990; Wang *et al*., 2001a). Moreover, freezing and thawing human oocytes in metaphase II showed no increase in aneuploidies (Gook *et al*., 1994; Cobo *et al*., 2001).

(iv) Ooplasm organelles can be altered when ice crystals form during the freezing process (Mazur *et al*., 1984).

(v) Changes in oocyte volume are produced during freezing due to the different osmotic pressures between the intracellular and extracellular solutions (Bernard *et al*., 1988).

Factors that result in improved results when frozen oocytes are used in assisted reproduction

(i) The oocyte's state of maturity. Freezing oocytes in metaphase II has been shown to be more effective than freezing them in prophase I (Hwu *et al*., 1998), in which case, in-vitro maturation is required after thawing. Experience with the process of maturing human oocytes *in vitro* is progressing, but only a limited number of pregnancies has been achieved with the association of freezing human oocytes and maturing them *in vitro* (Tucker *et al*., 1998b; Cha *et al*., 2000). Freezing denuded oocytes in metaphase II without granulosa cells and the corona has, according to Young *et al*. (1998b), produced better results than freezing oocytes with the cumulus and corona. However, Fabbri *et al*. (2001) did not observe a statistically significant difference.

(ii) Using the ICSI technique rather than insemination to fertilize thawed oocytes. The first birth from frozen oocytes fertilized by the ICSI technique was achieved in Bologna, Italy (Porcu *et al*., 1997). The ICSI technique solves problems related to the hardness of the zona pellucida and avoids polyspermy.

(iii) The cryprotectant agents. The movement of water through the cell membrane and the effects of freezing are controlled by the permeating cryoprotective substances (PROH), together with the action of the non-permeating cryoprotectant agent, sucrose.

In the protocols for freezing oocytes in the literature, the main permeating cryoprotectant agent used is PROH and the most common non-permeating cryoprotectant agent used is sucrose (Fabbri *et al*., 2001). The permeating cryoprotectant agent (PROH) is present in the O2 solution at a concentration

Table II. Clinical data on receiving women

	Patient						
	1	2	3	4	5	6	7
Age (years)	38	40	42	43	44	50	53
Previous children	No	Yes	No	No	No	No	Yes
Previous IVF attempts	–	3	1	1	–	1	–
Previous oocyte donation	–	–	–	–	–	1	2
Indication	LR	IF	LR	LR	LR	PM	PM
No. embryos transferred	4	3	4	3	4	4	4
Pregnancy	No	Yes	Yes	No	Yes	No	Yes
Children born	–	1 boy	1 girl	–	1 girl, 1 boy	–	1 boy

LR = low responder; PM = physiological menopause; IF = IVF failures.

of 1.5 m/l (Fabbri *et al*., 2001). This high concentration of PROH in the O2 solution induces water to be released from the oocyte and allows the PROH to enter, so the concentration of PROH on either side of the cell membrane is balanced. The O2 solution is therefore known as the equilibration solution. Ten minutes at RT is considered the ideal length of time to expose the oocyte to the equilibration solution (Al-Hasani and Diedrich, 1995). Dehydration of the oocyte begins during this phase. The non-permeating cryoprotectant agent, sucrose, a component of the loading and freezing solution (O3), remains outside the oocyte and increases extracellular osmotic pressure in relation to intracellular pressure. This osmotic imbalance induces even more intracellular water to leave the cell. The concentration of the non-permeating cryoprotectant agent, sucrose, in the loading solution has been shown to be more effective at 0.3 mol/l than at 0.2 mol/l (Fabbri *et al*., 2001)

(iv) Freezing speed. The slow freezing described (at the rate of 0.2°C/min until –7°C is reached; and then at 0.3°C/min until –30°C is reached) allows the oocyte to dehydrate and reduces the formation of intracellular ice crystals, which are harmful to the oocyte.

(v) Thawing. The thawing speed is critical in the oocyte cryopreservation process. If any intracellular water remains when the oocyte is frozen, small ice crystals form when it is submerged in liquid nitrogen (–196°C). These ice crystals act as crystallization nuclei when the oocyte is thawed. If thawing is slow, crystals grow and the oocyte is damaged. Therefore, the thawing process must be very rapid (almost 275°C/min) to allow for rapid dispersion of the intracellular ice crystals. The extracellular ice melts and the resulting liquid water enters the oocyte and rehydrates it (Friedler *et al*., 1988). We introduced a change in the rehydration times published by Fabbri *et al*. (2001). In our work, the oocytes remained in the rehydration solutions at room temperature for only 12.5 min, compared with the 30 min described by Fabbri *et al*. (2001). This reduction in the rehydration time at RT does not seem to affect the oocyte survival rate or the number of pregnancies. If this short period of time does not prevent rehydration from occurring correctly, it would seem logical to avoid exposing oocytes to RT for long periods, because an increase in the number of aneuploidies has been reported to be related to the length of time oocytes are exposed to RT (Pickering *et al*., 1990; Wang *et al*., 2001a).

(vi) The age of the woman whose oocytes are frozen. The rate of spontaneous aneuploidy in fresh oocytes increases with age (Battaglia *et al*., 1996; Wang *et al*., 2001b). The oocyte aneuploidy rate is 17% in women aged ⩽25 years and 79% in women aged >40 years (Battaglia *et al*., 1996).

The same results are not expected when a 25-year-old woman's oocytes are frozen as when a 35-year-old woman's are frozen.

Comparison of results

The results of using fresh or frozen oocytes have revealed similar findings (Porcu *et al*., 2002; Yang *et al*., 2002). The average number of fresh oocytes required to obtain one pregnancy at IVF centres in the UK was 50 (Ahuja *et al*., 1998). Porcu *et al*. (1999a) needed almost 100 frozen oocytes (16 pregnancies from 1502 thawed oocytes) to achieve one pregnancy. Yang *et al*. (1999) needed 17 frozen oocytes for every pregnancy achieved (seven pregnancies from 120 thawed oocytes). Tucker *et al*. (1998a), using donated oocytes, need 62 frozen oocytes per pregnancy (five pregnancies from 311 thawed oocytes). In that work the oocyte donors were patients, and the average age was 32.6 years. In the present study the oocyte donors were not patients, and were 10 years younger; we needed 22 frozen oocytes to achieve one pregnancy, i.e. we achieved four pregnancies using 88 thawed oocytes using young oocyte donors.

The pregnancy rates in terms of the number of embryo transfers using fresh oocytes and oocytes frozen in our oocyte-donation programme, are similar: they reveal an ~50% chance of pregnancy per transfer. It can be concluded from the data presented and the arguments made that freezing human oocytes for reproductive purposes is safe and effective enough to be applied in clinical situations where it is indicated.

Legal aspects

There are no legal restrictions to freezing oocytes at the international level (although the situation is not the same when it comes to donating oocytes) except in Spain, Norway and Singapore (Jones and Cohen, 2001). In Spain, the Assisted Reproduction Act was passed 15 years ago, in 1988. It authorized the donation of oocytes and, according to some legal experts, established a moratorium on freezing oocytes, while others interpret the law to mean that freezing oocytes is prohibited. The legal aspects of freezing oocytes in Spain and the administrative vicissitudes experienced by the CEFER Reproduction Institute when it started freezing oocytes for reproductive purposes have been described in other articles (Marina and Marina, 2002; 2003).

References

Ahuja, K.K., Simons, E.G., Mostyn, B.J. and Bowen-Simpkins, P. (1998) An assessment of the motives and morals of egg share donors: policy of 'payments' to egg donors requires a fair review. *Hum. Reprod.*, **13**, 2671–2678.

Al-Hasani, S. and Diedrich, K. (1995) Oocyte storage. In Grudzinskas, J.G. and Yovich, J.L. (eds) *Gametes: The Oocyte*. Cambridge University Press. Cambridge, UK, pp. 376–394.

Al-Hasani, S., Diedrich, K., van der Ven, H., Reinecke, A., Hartje, M. and Krebs, D. (1987) Cryopreservation of human oocytes. *Hum. Reprod.*, **2**, 695–700.

Antinori, S., Dani, G., Selman, H.A., Vidali, A., Antinori, M., Cerusico, C. and Versaci, C. (1998) Pregnancy after sperm injection into cryopreserved human oocytes. *Hum. Reprod.*, **13** (Abstract book 1), 157–158.

Battaglia, D.E., Goodwin, P., Klein, N.A. and Soules, M.R. (1996) Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women. *Hum. Reprod.*, **11**, 2217–2222.

Bernard, A., McGrath, J.J., Fuller, B.J., Imoedemhe, D. and Shaw, R.W. (1988) Osmotic response of oocytes using a microscope diffusion chamber: a preliminary study comparing murine and human ova. *Cryobiology*, **25**, 495–501.

Borini, A., Baffaro, M.G., Bonu, M.A., Di Stratis, V., Sereni, E., Sciajno, R. and Serrao, L. (1998) Pregnancies after freezing and thawing human oocytes. Preliminary data. *Hum. Reprod.*, **13** (Abstract book 1), 124–125.

Cha, K.Y., Chung, H.M., Lim, J.M., Ko, J.J., Han, S.Y., Choi, D.H. and Yoon, T.K. (2000) Freezing immature oocytes. *Mol. Cell. Endocrinol.*, **169**, 43–47.

Chen, C. (1986) Pregnancy after human oocyte cryopreservation. *Lancet*, **i**, 884–886.

Cobo, A., Rubio, C., Gerli, S., Ruiz, A., Pellicer, A. and Remohi, J. (2001) Use

of fluorescence in situ hybridization to assess the chromosomal status of embryos obtained from cryopreserved oocytes. *Fertil. Steril.*, **75**, 354–360.

Dumoulin, J.C., Bergers-Janssen, J.M., Pieters, M.H., Enginsu, M.E., Geraedts, J.P. and Evers, J.L. (1994) The protective effects of polymers in the cryopreservation of human and mouse zonae pellucidae and embryos. *Fertil. Steril.*, **62**, 793–798.

Expósito, R., Marina, S., Marina, F., Torres, P.J. and Jové I. (2001) What do egg donors think of egg donation after follicular puncture? *Reprod. Technol.*, **10**, 335–337.

Fabbri, R., Porcu, E., Marsella, T., Rocchetta, G., Venturoli, S. and Flamigni, C. (2001) Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Hum. Reprod.*, **16**, 411–416.

Friedler, S., Giudice, L.C. and Lamb, E.J. (1988) Cryopreservation of embryos and ova. *Fertil. Steril.*, **49**, 743–764.

Gook, D.A., Osborn, S.M., Bourne, H. and Johnston, W.I.H. (1994) Fertilization of human oocytes following cryopreservation; normal karyotypes and absence of stray chromosomes. *Hum. Reprod.*, **9**, 684–691.

Gook, D.A., Schiewe, M.C., Osborn, S.M., Asch, R.H., Jansen, R.P.S. and Johnston, W.I.H. (1995) Intracytoplasmic sperm injection and embryo development of human oocyte cryopreserved using 1,2-propanediol. *Hum. Reprod.*, **10**, 2637–2641.

Hwu, Y.M., Lee, R.K.K., Su, J.T., Lin, M.W. and Lin, S.P. (1998) Fertilization and embryonic development of cryopreserved human immature oocytes collected at time of Caesarean section following intracytoplasmic injection. *Fertil. Steril.*, **70** (Suppl. 1), S110–S111.

Jones, H.W. and Cohen, J. (2001) IFFS Surveillance 01. *Fertil. Steril.*, **76** (Suppl. 2), S14.

Kazem, R., Thompson, L.A., Srikantharajah, A., Laing, M.A., Hamilton, M.P.R. and Templeton, A. (1995) Cryopreservation of human oocytes and fertilization by two techniques in-vitro fertilization and intracytoplasmic sperm injection. *Hum. Reprod.*, **10**, 2650–2654.

Marina, S. and Marina, F. (2002) Congelación de ovocitos: una polémica estéril (I). *ASEBIR*, **7**, 39–41.

Marina, S. and Marina, F. (2003) Comments on oocyte cryopreservation. *Reprod. Biomed. Online*, **6**, Webpaper 845.

Marina, S., Expósito, R., Marina, F., Nadal, J., Masramon, M. and Vergés, A. (1999) Oocyte donor selection from 554 candidates. *Hum. Reprod.*, **14**, 2770–2776.

Mazur, P., Rall, W.F. and Leibo, S.P. (1984) Kinetics of water loss and the likelihood of intracellular freezing in mouse ova: Influence of the method of calculating the temperature dependence of water permeability. *Cell Biophys.*, **6**, 197–213.

Pickering, S.J., Brande, P.R. and Johnson, M.H. (1990) Transient cooling to room temperature can cause irreversible disruption to the meiotic spindle in human oocytes. *Fertil. Steril.*, **54**, 102–108.

PolakdeFried, E., Notrica, J., Rubinstein, M., Marazzi, A. and Gómez Gonzalez, M. (1998) Pregnancy after human donor oocyte cryopreservation and thawing in association with intracytoplasmic sperm injection in a patient with ovarian failure. *Fertil. Steril.*, **69**, 555–557.

Porcu, E., Fabbri, R., Seracchioli, R., Ciotti, P.M., Magrini, O. and Flamigni, C. (1997) Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Fertil. Steril.*, **68**, 724–726.

Porcu, E., Fabbri, R., Seracchioli, R., Ciotti, P.M., Petracchi, S., Savelli, L., Ghi, T. and Flamigni, C. (1998) Birth of six healthy children after intracytoplasmic sperm injection of cryopreserved human oocytes. *Hum. Reprod.*, **13** (Abstract book 1), 124.

Porcu, E., Fabbri, R., Ciotti, P.M., Marsella, T., Balicchia, B., Damiano, G., Caracciolo, D., Giunchi, S., De Cesare, R. and Flamigni, C. (1999a) Cycles of human oocyte cryopreservation and intracytoplasmic sperm injection: results of 112 cycles. *Fertil. Steril.*, **72** (Suppl. 1), S2.

Porcu, E., Fabbri, R., Petracchi, S., Ciotti, P.M. and Flamigni, C. (1999b) Ongoing pregnancy after intracytoplasmic sperm injection of testicular spermatozoa into cryopreserved human oocytes. *Am. J. Obstet. Gynecol.*, **180**, 1044–1045.

Porcu, E., Fabbri, R., Ciotti, P.M., Petracchi, S., Seracchioli, R. and Flamigni, C. (1999c) Ongoing pregnancy after intracytoplasmic sperm injection of epididymal spermatozoa into cryopreserved human oocytes. *J. Assist. Reprod. Genet.*, **16**, 283–285.

Porcu, E., Fabbri, R., Damiano, G., Giunchi, S., Fratto, R., Ciotti, P.M., Venturoli, S. and Flamigni, C. (2000) Clinical experience and applications of oocyte cryopreservation. *Mol. Cell. Endocrinol.*, **169**, 33–37.

Porcu, E., Fabbri, R., Ciotti, P.M., Frau, F., De Cesare, R. and Venturoli, S. (2002) Oocytes or embryo storage?. *Fertil. Steril.*, **169** (Suppl. 1), S15.

Serafini, P., Tran, C. and Tan, T. (1995) Cryopreservation of human oocytes. A clinical trial. *J. Assist. Reprod. Genet.*, **12** (Suppl. 1), 6S.

Tucker, M., Wright, G., Morton, P., Shanguo, L., Massey, J. and Kort, H. (1996) Preliminary experience with human oocyte cryopreservation using 1,2 propanediol and sucrose. *Hum. Reprod.*, **11**, 1513–1515.

Tucker, M.J., Morton, P.C., Wright, G., Sweitzer, C.L. and Massey, J.B. (1998a) Clinical application of human egg cryopreservation. *Hum. Reprod.*, **13**, 3156–3159.

Tucker, M.J., Wright, G., Morton, P.C. and Massey, J.B. (1998b) Birth after cryopreservation of immature oocytes with subsequent in vitro maturation. *Fertil. Steril.*, **70**, 578–579.

Van Blerkom. J., and Davis, P.W. (1994) Cytogenetic, cellular, and developmental consequences of cryopreservation of immature and human oocytes. *Microsc. Res. Tech.*, **27**, 165–193.

Van Uem, J.F., Siebzehnrubl, E.R., Schuh, B., Koch, R., Trotnow, S. and Lang, N. (1987) Birth after cryopreservation of unfertilized oocytes. *Lancet*, **i**, 752–753.

Vidali, A., Dani, G., Antinori, M., Cerusico, F., Versaci, C. and Antinori, S. (1998) Oocyte cryopreservation is a viable alternative option for patients who refuse embryo freezing. *Fertil. Steril.*, **70** (Suppl. 1), S138.

Vincent, C. and Johnson, M.H. (1992) Cooling, cryoprotectants, and the cytoskeleton of the mammalian oocyte. *Oxf. Rev. Reprod. Biol.*, **14**, 73–100.

Wang, W.H., Meng, L., Hackett, R.J., Odenbourg, R. and Keefe, D.L. (2001a) Limited recovery of meiotic spindle in living human oocytes after cooling–rewarming observed using polarized light microscopy. *Hum. Reprod.*, **16**, 2374–2378.

Wang, W.H., Cao, B., Meng, L., Hackett, R.J. and Keefe, D.L. (2001b) Imaging living, human MII oocytes with the polscope reveals a high proportion of abnormal meiotic spindles. *Fertil. Steril.*, **76** (Suppl. 1), S2.

Yang, D.S., Blohm, P.L., Winslow, L. and Cramer, L. (1998) A twin pregnancy after microinjection of human cryopreserved oocyte with a specially developed oocyte cryopreservation regime. *Fertil. Steril.*, **70** (Suppl. 1), S239.

Yang, D.S., Blohm, P.L., Cramer, L., Nguyen, K., Zhao, Y.L. and Winslow, K.L. (1999) A successful human oocyte cryopreservation regime: survival, implantation and pregnancy rates are comparable to that of cryopreserved embryos generated from sibling oocytes. *Fertil. Steril.*, **72** (Suppl. 1), S86.

Yang, D., Winslow, K.L., Blohm, P.L., Brown, S.E. and Nguyen, K. (2002) Oocyte donation using cryopreserved donor oocytes. *Fertil. Steril.*, **78** (Suppl. 1), S14.

Young, E., Kenny, A., Puigdomenech, E., Van Thillo, G., Tiverón, M. and Piazza, A. (1998a) Triplet pregnancy after intracytoplasmic sperm injection of cryopreserved oocytes: case report. *Fertil. Steril.*, **70**, 360–361.

Young, E., Kenny, A., Puigdomenech, E., Van Thillo, G., Tiverón, M. and Piazza, A. (1998b) Human oocyte cryopreservation and pregnancy. *Fertil. Steril.*, **70** (Suppl. 1), S16.

Submitted on February 7, 2003; accepted on April 4, 2003