The births of five Spanish babies from cryopreserved donated oocytes


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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 transvaginal, ultrasound-guided 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-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% CO2 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 >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 ß-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).

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

<table>
<thead>
<tr>
<th>Metaphase II frozen</th>
<th>N</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survived</td>
<td>79</td>
<td>11.3 ± 3.0</td>
<td>8–17</td>
<td>89.8</td>
</tr>
<tr>
<td>2 PN</td>
<td>58</td>
<td>8.3 ± 2.0</td>
<td>6–11</td>
<td>73.4a</td>
</tr>
<tr>
<td>1 PN</td>
<td>6</td>
<td>0.9 ± 1.4</td>
<td>0–4</td>
<td>7.6a</td>
</tr>
<tr>
<td>No PN</td>
<td>4</td>
<td>0.6 ± 1.1</td>
<td>0–3</td>
<td>5a</td>
</tr>
<tr>
<td>3 PN</td>
<td>3</td>
<td>0.4 ± 0.8</td>
<td>0–2</td>
<td>3.8a</td>
</tr>
<tr>
<td>&gt;3 PN</td>
<td>2</td>
<td>0.3 ± 0.5</td>
<td>0–1</td>
<td>2.5a</td>
</tr>
<tr>
<td>Degenerated</td>
<td>6</td>
<td>0.9 ± 1.1</td>
<td>0–3</td>
<td>7.6a</td>
</tr>
<tr>
<td>No. embryos transferred</td>
<td>26</td>
<td>3.7 ± 0.5</td>
<td>3–4</td>
<td>44.8b</td>
</tr>
</tbody>
</table>

aPercentage of the total number of oocytes that survived cryopreservation.
bPercentage of embryos transferred out of all those fertilized.

Table II. Clinical data on receiving women

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

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**


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