A prospective evaluation of cryopreservation strategies in a two-embryo transfer programme


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of development at cryopreservation provides a clear advantage with respect to successful pregnancy outcome (Gelety and Surrey, 1993). Work by Fugger (1989) has demonstrated a clear advantage for the cryopreservation of embryos at the PN stage, compared with EC and blastocyst stages, with pregnancy rates of 17.4, 12.5 and 4.5% respectively. These findings have been supported by subsequent investigations demonstrating increased pregnancy and implantation rates with embryos cryopreserved at the PN stage (Gordts et al., 1990; Testart et al., 1990; Demoulin et al., 1991; Veeck et al., 1993). In contrast, Van den Abbeel et al. (1988) found no difference in pregnancy rates between PN and EC stage embryos.

The objectives of this prospective randomized study were to describe and compare the relative merits of embryo cryopreservation at the PN and EC stages and to determine the effect on the pregnancy rate in both the initial IVF cycle and the subsequent transfer of thawed embryos.

Materials and methods

Patients

All patients requesting IVF treatment at the Regional IVF Unit, St Mary’s Hospital, Manchester, UK in 1994, following informed consent, were randomly assigned to either a PN or EC embryo cryopreservation group. Patients requesting gamete intra-Fallopian transfer (GIFT), single embryo replacement or requiring micromanipulation of their oocytes were excluded from the study. A maximum of two fresh or frozen embryos were replaced throughout the study for each patient. All the patients were asked at the time of recruitment to return within 6–8 months for a frozen embryo replacement cycle if their treatment was unsuccessful so as to minimize the interval between treatment cycles.

Ovarian stimulation

Treatment was commenced 7 days before the next expected menstrual period, and this was defined as day 1 of treatment. Pituitary desensitization using the gonadotrophin-releasing hormone agonist buserelin (Suprefact; Hoechst Roussel Ltd, Uxbridge, UK) began by the daily administration of 500 mg s.c. from day 1; this continued until the ovulatory trigger. On day 12 of treatment the patient was assessed for pituitary desensitization and ovarian suppression. The criteria used for adequate suppression were: (i) no ovarian follicles or cysts >10 mm in diameter and (ii) the onset of menses. If both criteria were not met on day 12, then the patient returned weekly until pituitary desensitization and ovarian suppression were achieved.

Upon fulfilling the above criteria, ovarian stimulation commenced with daily injections of human menopausal gonadotrophin (HMG; Pergonal; Serono Laboratories, Welwyn Garden City, UK; or Humegon; Organon Laboratories, Oss, The Netherlands), usually two ampoules (150 IU follicle stimulating hormone). A higher dose of HMG was given to women with a previous poor response to the two ampoules dose. From day 8 of stimulation onwards, the ovarian response was monitored daily by serum oestradiol measurements and vaginal ultrasonography. When three or more follicles measured >20 mm in diameter, oovulation was triggered with the administration of 10 000 IU human chorionic gonadotrophin (HCG). The oocytes were collected 36 h later using ultrasonad oocyte retrieval. If the patient was deemed to be at risk of developing OHSS (Wada et al., 1992, 1993), then all the embryos were cryopreserved at the PN stage and stored.

The oocytes were inseminated 4–6 h after retrieval using a sperm concentration of 100 000/ml. Normal fertilization was characterized by the visualization of two clear pronuclei. Any oocyte containing three or more pronuclei, which was assumed to indicate polyspermy, was discarded.

On day 2 (48–50 h post-oocyte retrieval) the embryos had usually cleaved to the EC stage. The embryos were scored for cell number and quality using the following criteria: grade A, the blastomeres had an equal size, but allowance was made for blastomeres during division, and no anucleate fragments were present; grade B, not all the blastomeres had an equal size and anucleate fragments were present in <20% of the embryo volume; and grade C, the embryos were dark and contained >20% anucleate fragments by volume.

A further injection of 2000 IU HCG was given on day 4 post-oocyte recovery for luteal phase support.

Embryo cryopreservation and thawing protocols

The cryopreservation and thawing of embryos at the PN and EC stages were performed using PROH (Sigma, Poole, UK)/sucrose in EFM1 (Medi-Cult, Redhill, UK) freezing medium and a fast freeze–fast thaw method (Lassalle et al., 1985; Troup et al., 1990). Only grade A and B EC embryos were frozen; grade C embryos were deemed to be unsuitable and discarded.

The cryopreservation procedure involved exposure of the embryos to the cryoprotectants through a series of solutions. The process was performed at room temperature. The freezing solutions (F) were first made up as follows: F1, EFM1; F2, EFM1 + 1.5 M PROH; and F3, EFM1 + 1.5 M PROH + 0.1 M sucrose. The embryos were washed initially in F1, and then transferred to F2 for 10–15 min. Finally they were transferred to F3, loaded immediately into freezing straws and plunged. The straws were then transferred to a programmable controlled-rate freezer and frozen at the following rates: ramp 1, –2°C/min to –7°C; ramp 2, hold for 10 min; ramp 3, –0.3°C/min to –30°C; ramp 4, hold for 5 min; ramp 5, –50°C/min to –190°C; ramp 6, hold for 20 min.

An embryo was thawed by removing the straw from liquid nitrogen and leaving it at room temperature for 40 s. It was then immersed in a 30°C water bath for 1 min. The embryo, now at room temperature, was passed through a series of solutions (T): T1, EFM1 + 0.2 M sucrose + 1 M PROH; T2, EFM1 + 0.2 M sucrose + 0.5 M PROH; T3, EFM1 + 0.2 M sucrose; and T4, EFM1.

A PN stage embryo was considered to have survived the freeze–thaw process if there was no obvious damage to the zona pellucida and if it divided after overnight culture. EC embryos were cultured for 2–4 h after thawing before replacement, and were considered to have survived the freeze–thaw process if at least 50% of the blastomeres remained intact.

Replacement cycles

Two protocols were followed for the replacement of frozen–thawed embryos: (i) The first protocol was a natural cycle which involved the daily measurement of serum luteinizing hormone (LH; LH Miaclone; Biodata Diagnostics, Rome, Italy) to detect the LH surge (i.e. a LH concentration >12 mIU/ml). In this way the embryos and the stage of endometrial development were synchronized (Mandelbaum et al., 1988). Once the LH surge was observed, PN embryos were thawed 2 days later and, if suitable, replaced on the third day. EC embryos were thawed and replaced on the third day after the LH surge; (ii) Following desensitization with buserelin, endometrial development was stimulated by the administration of exogenous steroids, namely oral oestradiol valerate (Climaval; Sandoz Pharmaceutica, Camberley, UK) and vaginal progesterone ( Cyclogest; Hoechst Roussel Ltd) (Table 1). A retrospective study had previously concluded that there was no
on the first day of the menstrual cycle, buserelin injections were started. On day 12 (following menses), hormone replacement therapy was started (day 1 in the table).

A total of 364 patients entering the study were randomly divided into the two cryopreservation strategies. From these patients, 134 (36.8%) treatment cycles met both study criteria of having an embryo replacement and also embryo cryopreservation. There was no difference in the demographic details of the two groups or the response to ovarian stimulation, as shown in Table II.

Results

Patient details

A total of 364 patients entering the study were randomly divided into the two cryopreservation strategies. From these patients, 134 (36.8%) treatment cycles met both study criteria of having an embryo replacement and also embryo cryopreservation. There was no difference in the demographic details of the two groups or the response to ovarian stimulation, as shown in Table II.

Source cycle results

The two study groups (PN and EC strategies) contained 72 and 62 patients respectively (Table III). The overall fertilization rate in the EC strategy compared with the PN strategy was significantly different (P < 0.01). There was no significant difference between the two groups with respect to the number of oocytes retrieved, the number of embryos replaced, the number of oocytes fertilized and the number of embryos frozen (Table III).

<table>
<thead>
<tr>
<th>Day</th>
<th>Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–5</td>
<td>Oestradiol valerate 1 mg/day</td>
</tr>
<tr>
<td>6–9</td>
<td>Oestradiol valerate 1 mg twice a day</td>
</tr>
<tr>
<td>10 onwards</td>
<td>Oestradiol valerate 1 mg three times a day</td>
</tr>
<tr>
<td>15</td>
<td>Attend the in-vitro fertilization unit for serum oestradiol, scan and last buserelin injection; 200 mg progesterone pessary</td>
</tr>
<tr>
<td>16</td>
<td>400 mg progesterone pessary</td>
</tr>
<tr>
<td>17</td>
<td>400 mg progesterone pessary twice a day</td>
</tr>
<tr>
<td>18</td>
<td>Possible day of embryo replacement</td>
</tr>
</tbody>
</table>

On the first day of the menstrual cycle, buserelin injections were started. On day 12 (following menses), hormone replacement therapy was started (day 1 in the table).

A total of 55 women returned for 68 cycles in which embryos were thawed (Table IV). Of these thaws, 59 (86.8%) resulted in a live birth. The livebirth rate with the EC strategy (27.4%) was significantly higher than that with the PN strategy (11.1%; P < 0.05) (Table III). This may have been because of the significantly higher pregnancy wastage with the PN strategy (42.8%) than with the EC strategy (10.5%; P < 0.05). The implantation rate also appeared to be higher with the EC strategy (15.3%; 19/124) than with the PN strategy (10.1%; 14/138), although this did not reach statistical significance.

Survival and transfer of thawed embryos

Pronucleate strategy

A total of 55 women returned for 68 cycles in which embryos were thawed (Table IV). Of these thaws, 59 (86.8%) resulted in a live birth. The livebirth rate with the EC strategy (27.4%) was significantly higher than that with the PN strategy (11.1%; P < 0.05) (Table III). This may have been because of the significantly higher pregnancy wastage with the PN strategy (42.8%) than with the EC strategy (10.5%; P < 0.05). The implantation rate also appeared to be higher with the EC strategy (15.3%; 19/124) than with the PN strategy (10.1%; 14/138), although this did not reach statistical significance.

Statistical analysis

The two-tailed Mann–Whitney U-test was used to compare details of the initial treatment cycles. The data were also compared using chi-square tests, with any differences regarded as significant if P < 0.05.
in at least one embryo surviving and being replaced. A total of 129 embryos were thawed in the 68 cycles, with 96 (74.4%) surviving and being replaced.

**Early cleavage strategy**
A total of 40 patients had embryos thawed on 50 occasions. This resulted in at least one embryo being replaced in 46 (92.0%) of the cycles. These patients had a total of 102 embryos thawed, of which 79 (77.4%) survived and were replaced (Table IV).

### Implantation and outcome after transfer of thawed embryos

#### Pronucleate strategy
Overall, 22.0% of embryo transfers resulted in a positive pregnancy test, with 84.6% of these resulting in a live birth (Table V). The performance of the individual embryos transferred was that 14.6% of embryos transferred implanted and developed into a live infant. This includes the only multiple implantation recorded in the study.

#### Early cleavage strategy
Overall, 13.0% of cycles resulted in a positive pregnancy test, with 50.0% of these going on to a live birth. Of the embryos implanted, 7.6% went on to develop into a live baby (Table V).

### Cumulative livebirth rates

It can be seen from Table VI that the actual cumulative livebirth rate for IVF cycles after one fresh and two frozen–thawed embryo replacements was the same irrespective of the stage at which the embryos were cryopreserved. There was also no significant difference between the anticipated cumulative livebirth rate for the two embryo stages.

**Discussion**

There have been many retrospective studies describing the benefits that embryo cryopreservation has provided to IVF/GIFT units (Veeck et al., 1993; Wang et al., 1994). Usually the data have described groups of patients participating in the routine laboratory protocol and who have had embryos frozen at the PN (Veeck et al., 1993) or EC (Wang et al., 1994) stage. However, direct comparison between the data has been difficult, because of differing individual clinic stimulation regimens, embryo culture conditions, freezing protocols, cryoprotectants and interpretation and documentation of the results.

The expression of the results of embryo cryopreservation varies enormously. Indeed Jones et al. (1995) described nine separate formulae and concluded that five of these were essential to give a comprehensive evaluation of the efficacy of cryopreservation. However, in our study, formulae were selected to allow the following parameters to be clearly represented and compared: (i) the survival and implantation of individual embryos; (ii) the pregnancy rate achieved at each transfer of frozen–thawed embryos; (iii) the actual cumulative pregnancy rate achieved from the original collection of oocytes, to include all transfers of resulting fresh and frozen embryos; and (iv) the projected cumulative pregnancy rate, to include embryos still remaining frozen, given that not all patients will have used all their embryos at the time of analysis.

It is important to note that embryo cryopreservation affected 40.9% (134/328) of treatment cycles commenced and 47.9% (134/280) of cycles reaching oocyte collection; 62.0% (134/216) of patients reaching embryo replacement generated sufficient embryos for cryopreservation. Therefore, the effect of embryo cryopreservation upon an IVF unit is substantial, and the benefits to those patients with supernumerary embryos must not be taken lightly.

There was no significant difference between the embryo survival/replacement rates for the two embryo development stages. However, this figure does not have the same definition for both groups, because it is essentially a true embryo survival rate for the PN stage, whereas it represents an embryo survival rate for the EC stage.

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### Table IV. The progress of patients having embryos thawed

<table>
<thead>
<tr>
<th>Replacement cycle</th>
<th>PN strategy</th>
<th>EC strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive pregnancy test per embryo transfer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural</td>
<td>12/55 (21.8)</td>
<td>EC</td>
</tr>
<tr>
<td>HRT</td>
<td>1/4 (25.0)</td>
<td>EC</td>
</tr>
<tr>
<td>Total</td>
<td>13/59 (22.0)</td>
<td>EC</td>
</tr>
<tr>
<td>Viable pregnancy per positive pregnancy test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural</td>
<td>11/12 (91.7)</td>
<td>EC</td>
</tr>
<tr>
<td>HRT</td>
<td>0/1 (0.0)</td>
<td>EC</td>
</tr>
<tr>
<td>Total</td>
<td>11/13 (84.6)</td>
<td>EC</td>
</tr>
<tr>
<td>Implantation rates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural</td>
<td>13/90 (14.4)</td>
<td>EC</td>
</tr>
<tr>
<td>HRT</td>
<td>1/6 (16.7)</td>
<td>EC</td>
</tr>
<tr>
<td>Total</td>
<td>14/96 (14.6)</td>
<td>EC</td>
</tr>
</tbody>
</table>

### Table V. The outcome of frozen embryo replacement cycles

<table>
<thead>
<tr>
<th>Replacement cycle</th>
<th>PN strategy</th>
<th>EC strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>55</td>
<td>40</td>
</tr>
<tr>
<td>No. of thawed</td>
<td>68</td>
<td>50</td>
</tr>
<tr>
<td>No. of replacements</td>
<td>59 (86.8)</td>
<td>46 (92.0)</td>
</tr>
<tr>
<td>No. of embryos thawed</td>
<td>129</td>
<td>102</td>
</tr>
<tr>
<td>No. of embryos survived/replaced</td>
<td>96 (74.4)</td>
<td>79 (77.4)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages. PN strategy = embryos frozen at the pronuclear stage; EC strategy = embryos frozen at the early cleavage stage.

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### Table VI. Cumulative viable pregnancy rate for patients having fresh and frozen embryo replacements

<table>
<thead>
<tr>
<th>Replacement cycle</th>
<th>PN strategy</th>
<th>EC strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual cumulative viable pregnancy rate (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh embryos</td>
<td>11.1 (n = 72)</td>
<td>27.4 (n = 62)</td>
</tr>
<tr>
<td>First frozen embryo replacement</td>
<td>28.2 (n = 47)</td>
<td>31.3 (n = 37)</td>
</tr>
<tr>
<td>Second frozen embryo replacement</td>
<td>40.2 (n = 12)</td>
<td>41.1 (n = 7)</td>
</tr>
<tr>
<td>Anticipated cumulative viable pregnancy rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of patients</td>
<td>72</td>
<td>62</td>
</tr>
<tr>
<td>Fresh viable pregnancy rate</td>
<td>8/72 (11.1)</td>
<td>17/62 (27.4)</td>
</tr>
<tr>
<td>Frozen viable pregnancy rate</td>
<td>11/59 (18.6)</td>
<td>3/45 (6.7)</td>
</tr>
<tr>
<td>No. of embryos left in storage</td>
<td>98</td>
<td>95</td>
</tr>
<tr>
<td>Anticipated viable pregnancy rate (%)</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>Cumulative viable pregnancy rate</td>
<td>30/72 (41.7)</td>
<td>24/62 (38.7)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages. PN strategy = embryos frozen at the pronuclear stage; EC strategy = embryos frozen at the early cleavage stage.
replacement rate for the EC stage. For a PN embryo to be replaced it first has to divide following culture overnight, and therefore to survive totally the freeze–thaw process. In the case of EC embryos, all the embryos replaced were said to have survived. However, in some cases only 50% of the blastomeres of an individual embryo will have survived the freeze–thaw process.

The implantation rate of fresh embryos resulted in significantly more live births with the EC strategy compared with the PN strategy ($P < 0.05$), with almost a 3-fold increase. Similar results were shown by Sunde (1995), who demonstrated that selection of the best embryos (<10% volume anuclear fragments) for replacement resulted in an ~3-fold higher average implantation rate than when replacing inferior quality embryos. The EC strategy allows the embryologist to choose and replace the best embryos, whereas the PN strategy allows no choice because all the zygotes appear to be similar on the day after insemination.

The frozen embryo survival rates of the two strategies were not significantly different. However, the outcome per cycle of the frozen embryo replacement suggested that freeze–thawed PN embryos result in almost twice the number of viable pregnancies compared with the EC embryos; this was also reflected in a doubling of the implantation rate.

The cumulative viable pregnancy rates for the two cryopreservation strategies were not significantly different. The overall effect of embryo cryopreservation was that the cumulative livebirth rate after IVF and two or three frozen embryo replacement cycles was ~40%. This was a superb result when it has been demonstrated that the cumulative livebirth rate following four consecutive IVF cycles is 33.9% (Tan et al., 1992), although other studies have suggested a much higher value (Hull et al., 1992). These studies, however, did not take into account any live births generated from frozen embryos obtained from the IVF cycles.

The anticipated cumulative viable pregnancy rate was also not significantly different. The increased fresh livebirth rate for the EC strategy was offset by the increased freeze–thawed viable pregnancy rate of the PN strategy. The equation described by Veeck et al. (1993), to calculate the anticipated cumulative pregnancy rate, does not describe individual patient results, i.e. it describes the average number of live births from a total number of embryos based on the average embryo survival and implantation rates. It does not take into account possible improvements in post-thaw embryo culture and replacement techniques or possible embryo storage degradation. Patient age is not taken into account either, which has long been known to affect the livebirth outcome of IVF treatment (Human Fertilisation and Embryology Authority, 1993).

Despite isolated doubts about its long-term effects (Winston and Handyside, 1993; Dulouist et al., 1995), embryo cryopreservation has been accepted as an invaluable tool for IVF units, and similar results are now being achieved with the cryopreservation of embryos generated from intracytoplasmic sperm injection (Al-Hasani et al., 1996). It has also been suggested that embryo implantation and pregnancy rates may be improved using assisted hatching (Check et al., 1996).

In summary, our study has shown that the PN strategy compromises the fresh embryo replacement cycle but maximizes the potential of the frozen embryos. This would suggest that couples with low numbers of embryos (four or fewer) would be best served by leaving all embryos until the day of embryo transfer and cryopreserving only suitable supernumerary embryos (i.e. to maximize the potential of a fresh embryo replacement). However, couples with a large number of fertilized oocytes would probably improve their overall chance of achieving a viable pregnancy by having some embryos frozen at the PN stage (to maximize the performance of the subsequently thawed embryos) and leaving the remainder until the day of embryo transfer.

References

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strategies to select human embryos for cryopreservation. MSc Thesis, University of Manchester, Manchester, UK.


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