A prospective randomized study: day 2 versus day 5 embryo transfer

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BACKGROUND: This randomized controlled study was performed in an unselected IVF/ICSI population to test the hypothesis that blastocyst transfers result in higher clinical pregnancy rates (CPR) per oocyte retrieval when compared with day 2 transfers. METHODS: Blind randomization for transfer on day 2 (group 1) or day 5/6 (group 2) was performed before stimulation. Oocytes and embryos were cultured in sequential media in 5.5% CO₂, 5% O₂, 89.5% N₂ and 90% humidity. A maximum of two embryos was transferred. RESULTS: The two groups were similar for age, IVF indication, number of treatment cycles, rate of ICSI/IVF, number of fertilized oocytes and number of embryos transferred. The CPR/oocyte retrieval was comparable in group 1 (32%) and in group 2 (44%), while the CPR/embryo transfer was significantly higher (P < 0.01) in group 2 (60%) than in group 1 (35%). Similarly, the implantation rate per embryo transferred was significantly higher (P < 0.03) in group 2 (46%) than in group 1 (29%). The cryo-augmented delivery rate/oocyte retrieval was comparable in group 2 (36.3%) and in group 1 (28.6%). CONCLUSION: This randomized study in an unselected population showed a significantly higher CPR/embryo transfer and a tendency toward a higher CPR/oocyte retrieval in patients receiving blastocysts when compared with day 2 transfers.

Keys words: blastocyst/embryo transfer/human/sequential media

Introduction

Before the introduction of sequential media, human embryos were routinely cultured for only 2 or 3 days in simple Earle’s balanced salt solutions or equivalents, often supplemented with serum. These simple culture media, however, did not sufficiently support the metabolic needs of the embryo (Bavister, 1995; Gardner, 1996; Gardner and Lane, 1997) and therefore only a small number of embryos reached the blastocyst stage, mostly with a delay in development. Co-culture of human embryos with a variety of somatic cells has resulted in higher blastocyst development rates (30–50%) and fairly good implantation rates (20–30%) (Kaufmann et al., 1995; Guerin and Nicollet, 1997; Simon et al., 1999), but there is a lack of randomized studies to prove clinical benefit and there is anxiety about safety in these culture conditions.

Recently, new sequential culture media without serum supplementation have been developed, taking into account the physiological and metabolic needs of the pre- and post-compaction embryos and the changes in the environment of the female reproductive tract (Gardner and Lane, 1997). In these culture media, 25–50% of fertilized ova reach the blastocyst stage with an implantation rate per blastocyst of ≥50% (Gardner, 1998a,b; Jones, 1998; Trounson, 1998). However, other investigators (Mortimer et al., 1998) achieved an almost equally high implantation rate of 38% by culturing embryos in these new culture media but with transfers on day 2.

Previous randomized studies comparing day 2 or 3 versus day 5 or 6 transfers were performed in suboptimal culture conditions (Scholtes and Zeilmaker, 1996a,b; Gudmundsson, 1998). These authors claimed good pregnancy results where they were able to transfer a blastocyst, but often no blastocysts were available, or the embryos were in developmental delay. One study (Alves da Motta et al., 1998) cultured embryos in sequential media and found a 30% implantation rate per transferred blastocyst compared with 19% implantation rate for day 3 embryos. Another team (Gardner et al., 1998a), using further modified sequential media, obtained significantly higher implantation rates after embryo transfer on day 5 (57%) versus embryo transfer on day 3 (38%), but this randomized study was performed in a selected group of good prognosis patients with >10 follicles. In contrast, other investigators (Coskun et al., 2000; Plachot et al., 2000) could not detect significant advantages after blastocyst transfer when compared with day 3 transfers, but they used different culture media in both groups. Although blastocyst transfer seems to be a promising development in IVF, prospectively controlled randomized studies are needed to determine whether prolonged
culture to the blastocyst stage is really an advantage for an unselected population. Moreover, to the best of our knowledge, no studies have been published comparing day 2 versus day 5 transfers where both groups of embryos are cultured in the same sequential culture media. Since we do not know how often transfers will be cancelled because of a lack of blastocysts in an unselected population and how cryopreservation of blastocysts will influence the final result, it is not clear if a higher cryo-augmented delivery rate per oocyte aspiration will be reached after blastocyst transfer when compared with a day 2 transfer. Therefore, a randomized study was started in an unselected population to test the hypothesis that clinical pregnancy rate and delivery rate per oocyte retrieval and per embryo transfer is higher after embryo transfers on day 5/6 when compared with embryo transfer on day 2, using new sequential culture conditions in both groups.

Materials and methods

**Fresh IVF cycles**

This randomized study was approved by the Ethical Committee of the Department of Medicine at the Catholic University Hospital Gasthuisberg, Leuven. IVF and ICSI patients (n = 136) who started their cycle between February 1999 and September 2000 were randomized for a transfer on either day 2 (group 1) or day 5 (group 2).

After the patients gave informed consent, blind randomization (with sealed envelopes) was performed at the beginning of the hormonal stimulation, before the hormonal response was known. Ovarian stimulation was performed as follows. In the previous cycle an oral contraceptive pill was given for 25 days (Cilest®; Janssen-Cilag, Beerse, Belgium), followed by pituitary down-regulation with buserelin acetate (Suprefact®, 600 µg daily; Hoechst, Frankfurt, Germany) from day 21 onwards. HMG (Humegon®, Organon, Oss, The Netherlands) were started on the second day after the subsequent menstruation while ovulation was induced with 10,000 IU HCG (Pregnyl®; Organon). Ultrasound-guided oocyte aspiration was performed 36 h later. Luteal supplementation was given either by 1500 IU HCG, every 3 days, starting on the third day after oocyte retrieval, or by vaginal progesterone (600 mg/day, Utrogestan®; Janssen-Cilag, Beerse, Belgium) when there was an increased risk for hyperstimulation syndrome (estradiol level >3500 pg/ml at the time of HCG injection, or patients with polycystic ovarian syndrome). Oocytes and embryos were cultured in either sequential media from Cook (Fertilization, Cleavage and Blastocyst medium; Cook IVF, Queensland, Australia) or sequential media from Vitrolife (IVF-500, G1.2 and G2.2, Scandinavian IVF Science AB, Göteborg, Sweden) as part of a simultaneously randomized study comparing these two sequential culture media (Van der Auwera et al., 2001). Culture was performed in a modified overflow Cellstar incubator at 37°C and 90% humidity in 5.5% CO2, 5% O2 and 89.5% N2.

In group 1, up to five pronucleate ova were cultured in vitro, while the others were frozen at the pronuclear stage. This was done to optimize the cryo-augmented pregnancy rate per oocyte retrieval. A maximum of two selected embryos was transferred on day 2, while the remaining embryos (maximum of three) were cultured for another 3–4 days and frozen at the blastocyst stage if available. In group 2, all fertilized ova were cultured in vitro to achieve blastocysts. A maximum of two blastocysts was transferred while those remaining were frozen on day 5 or 6.

**Frozen–thawed embryo replacement cycles**

All frozen-thawed embryos from the study period were included in the evaluation of the cryo-augmented pregnancies per oocyte retrieval. During the replacement cycles, mild ovarian stimulation was given using 75 or 150 IU HMG and ovulation was triggered by an injection of 10,000 IU HCG as described before (Van der Auwera et al., 1994). When a spontaneous LH surge occurred in the presence of a mature follicle, an additional injection of 5000 IU HCG was given the same day. Frozen pronucleate ova were thawed 40 h after HCG administration or 24 h after the onset of the LH surge (Mandelbaum, 1987). HCG (1500 IU) was given as luteal supplementation, every 3 days, from the fifth day after HCG injection.

**Freezing and thawing procedures**

Pronucleate ova had been frozen by a slow freezing method (Lassalle et al., 1985) in 1.5 mol/l 1,2-propanediol (PROH; Sigma, St Louis, MO, USA) and 0.125 mol/l sucrose. Blastocysts were exposed for 10 min to 5 and 9% glycerol in HEPES-buffered human tubal fluid (HTF) medium supplemented with 15% human donor serum. Both were frozen with the slow freezing protocol. Frozen pronucleate ova were thawed in a 37°C waterbath and the cryoprotectant was diluted stepwise by decreasing the PROH concentrations (1.5, 1, 0.5 and 0 mol/l respectively) in phosphate-buffered saline with 0.25 mol/l sucrose at 5 min intervals. Frozen blastocysts were thawed at room temperature and the cryoprotectant was diluted stepwise by decreasing the glycerol concentrations (6, 4, 2 and 0% respectively) in a HEPES-buffered HTF solution with 0.25 mol/l sucrose at 10 min intervals. Up to two morphologically normal embryos were cultured for 24 h in sequential medium from Cook or from Vitrolife.

**Outcome variables**

Implantation rates, pregnancy rates and pregnancy outcome were compared between the two experimental groups for fresh IVF cycles and after freezing and thawing. Finally, the cryo-augmented baby take home rates per oocyte aspiration were compared between the two groups.

Statistical significance of differences was performed using Students t-test, Fisher’s exact χ2 and Biggers’ χ2 where appropriate.

**Results**

A total of 136 patients was randomized for the study. Three patients were excluded for analysis from group 1 because they wanted an elective blastocyst culture while four patients were excluded from group 2 because they wanted an elective day 2 transfer. After randomization, no differences were found for age, duration of infertility, type of infertility or IVF indication, nor for number of treatment cycles, ratio of ICSI:IVF cycles or mean oestradiol at HCG injection (Table I). Both groups had a comparable mean number of oocytes at oocyte retrieval, the same proportion of mature oocytes and fertilized oocytes, and a comparable mean number of embryos per transfer (Table II).

A significantly lower number of embryo transfers was performed in group 2 (73%) compared with group 1 (90%, P < 0.01; Table III). More clinical pregnancies (P < 0.01) and more deliveries (P < 0.05) per embryo transfer were observed in group 2 than in group 1 due to a higher implantation rate per embryo transferred in group 2 (46%) than in group 1 (29%, P < 0.03; Table III). Although 27% of the patients in group 2 did not receive a transfer, a trend towards a higher clinical pregnancy rate per oocyte retrieval and a higher
had already been transferred. The percentage total blastocyst probably because the morphologically best-looking embryos in group 2: fewer embryos reached the blastocyst stage on delayed in development in group 1 when compared with those cultured until day 5/6. These supernumerary embryos were variables shown.

Furthermore, embryo quality in both groups was comparable:

Embryos transferred 106 90

No. of seven-cell embryos 23.7 41.5

No. of embryos transferred 106 ND

No. of embryos per transfer (mean ± SD) 1.86 ± 0.28 1.87 ± 0.22

The differences between the two groups were not significant for any of the variables shown.

**Table II. Results after oocyte aspiration with respect to number of oocytes and embryos**

<table>
<thead>
<tr>
<th></th>
<th>Day 2</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with oocyte retrieval (n)</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>Oocytes at oocyte retrieval (n)</td>
<td>761</td>
<td>761</td>
</tr>
<tr>
<td>Mature oocytes (n)</td>
<td>652</td>
<td>652</td>
</tr>
<tr>
<td>Fertilized oocytes (n)</td>
<td>423</td>
<td>423</td>
</tr>
<tr>
<td>Embryos transferred</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>No. of oocytes at oocyte retrieval (mean ± SD)</td>
<td>10.7 ± 5.4 11.5 ± 5.1</td>
<td></td>
</tr>
<tr>
<td>No. of mature oocytes (mean ± SD)</td>
<td>9.0 ± 5.0 9.9 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>No. of two-pronucleate embryos (mean ± SD)</td>
<td>5.5 ± 3.6 6.4 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>No. of embryos per transfer (mean ± SD)</td>
<td>1.86 ± 0.28 1.87 ± 0.22</td>
<td></td>
</tr>
</tbody>
</table>

The differences between the two groups were not significant for any of the variables shown.

number of deliveries per oocyte retrieval was observed (44 and 36% in group 2 versus 32 and 27% in group 1 respectively), but this trend was not significantly different.

Furthermore, embryo quality in both groups was comparable: 60% (group 1) and 62% (group 2) of cultured two-pronucleate embryos reached the 4-cell stage on the morning of day 2 (Table IV). In group 1, the two morphologically best embryos out of a maximum of five cultured embryos were transferred on day 2. The remaining embryos (n = 93) were further cultured until day 5/6. These supernumerary embryos were delayed in development in group 1 when compared with those in group 2: fewer embryos reached the blastocyst stage on day 5 in group 1 (11%) than in group 2 (21%, P < 0.05), probably because the morphologically best-looking embryos had already been transferred. The percentage total blastocyst formation per cultured embryo was slightly higher (P = 0.08) in group 2 (45%) than in group 1 (34%).

Pregnancy outcome in relation to the number of fertilized ova is shown in Table V. Patients with four or fewer fertilized ova had comparable delivery rates per oocyte retrieval in both groups (15 and 16% respectively, not significant). For patients with more than four fertilized ova, the delivery rate/oocyte retrieval was 10% higher in group 2 (45%) than in group 1 (35%) but this difference was not significantly different. In group 2, significantly more clinical pregnancies (P = 0.02) and implantations (P = 0.03) were obtained when blastocysts were already available on day 5 in comparison with day 6 (Table VI).

In group 2, a significantly lower total number of embryos could be frozen (P < 0.0001) compared with group 1 (Table VIIa). One year after the end of the study (evaluation in July
Table V. Pregnancy outcome in relation to the number of fertilized ova for day 2 versus day 5 transfer

<table>
<thead>
<tr>
<th>No. of fertilized ova</th>
<th>Deliveries/clinical pregnancies/no. of oocyte retrievals (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 5</td>
</tr>
<tr>
<td>0</td>
<td>0/0/4 (0)</td>
<td>0/0/4 (0)</td>
</tr>
<tr>
<td>1</td>
<td>0/1/5 (0)</td>
<td>0/0/1 (0)</td>
</tr>
<tr>
<td>2</td>
<td>1/3/6 (17)</td>
<td>0/0/3 (0)</td>
</tr>
<tr>
<td>3</td>
<td>1/1/3 (33)</td>
<td>1/2/5 (20)</td>
</tr>
<tr>
<td>4</td>
<td>2/2/8 (25)</td>
<td>2/2/6 (33)</td>
</tr>
<tr>
<td>≤4 (total)</td>
<td>4/7/26 (15)</td>
<td>3/4/19 (16)</td>
</tr>
<tr>
<td>&gt;4</td>
<td>13/17/37 (35)</td>
<td>21/25/47 (45)</td>
</tr>
</tbody>
</table>

Values in parentheses (%) are the delivery rates per oocyte retrieval. NS = not significant.

Table VI. Pregnancy outcome in relation to the availability of a blastocyst on day 5 or on day 6

<table>
<thead>
<tr>
<th>Day 5</th>
<th>Day 6</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of embryo transfers</td>
<td>37</td>
<td>11</td>
</tr>
<tr>
<td>No. of clinical pregnancies (%)</td>
<td>26 (70)</td>
<td>3 (27)</td>
</tr>
<tr>
<td>No. of deliveries (%)</td>
<td>21 (57)</td>
<td>3 (27)</td>
</tr>
<tr>
<td>No. of blastocysts transferred</td>
<td>69</td>
<td>21</td>
</tr>
<tr>
<td>No. of gestational sacs (% per embryo)</td>
<td>36 (52)</td>
<td>5 (24)</td>
</tr>
<tr>
<td>No. of children born (% per embryo)</td>
<td>27 (42)</td>
<td>4 (19)</td>
</tr>
</tbody>
</table>

NS = not significant.

Discussion

To the best of our knowledge, this is the first randomized study comparing day 2 versus day 5 embryo transfers in an unselected population, independent of their ovarian response, and with embryos cultured in the same new sequential complex culture media in both groups. This study clearly demonstrates that blastocyst transfers resulted in a higher clinical pregnancy rate per transfer, a higher delivery rate per transfer and a higher implantation rate per transferred embryo. This study also showed a trend towards a higher clinical pregnancy rate per oocyte retrieval, but significant differences were not obtained for several reasons.

First, fewer patients were recruited than originally planned. Power calculation before initiation of this study had shown that 175 patients were needed in each group to demonstrate a significant difference of 15% in pregnancy rate/oocyte retrieval between both groups. However, recruitment of patients was stopped earlier because of high multiple pregnancy rates in both groups and problems in patient recruitment. Indeed, some patients wanted an elective day 5 transfer whereas others wanted an elective day 2 transfer out of fear that they might have no embryos left on day 5/6.

Second, this study was designed for unselected IVF patients, irrespective their ovarian response. As a result, 27% of the patients in group 2 did not receive an embryo transfer due to a lack of blastocysts on day 6. By comparison, another study (Gardner et al., 1998a) showed only 4.5% of transfer failures but selected only patients with ≥10 mature follicles. This resulted in a mean number of 11.6 fertilized oocytes in his...
study compared with only 6.0 in our study. If we had included only patients with more than four fertilized ova, only 15% of patients would have had an oocyte retrieval without transfer and probably fewer patients would be needed to detect significant differences in pregnancy rate per oocyte retrieval between both groups.

Third, the implantation rate per embryo transferred on day 2 was very high (29%) in our study and therefore it was more difficult to obtain significant differences between day 2 and day 5/6 transfers. Our results on day 2 in an unslected population were comparable with those of other investigators on day 2 or 3 [38% (Mortimer et al., 1998); 37% in good prognosis patients (Gardner et al., 1998a)] who also cultured the embryos in media containing amino acids. In contrast, in other randomized studies (Demylle et al., 2000; Plachot et al., 2000) embryos from the two groups of patients were cultured in different culture media (day 2/3 in simple media without amino acids and day 5/6 in complex sequential media). In these studies (Demylle et al., 2000; Plachot et al., 2000), the day 5/6 implantation rate per embryo was 35–45%, but the day 2/3 implantation rate per embryo was only 15–20%, considerably lower than the day 2 implantation rate per embryo of 29% in our study. Therefore, the higher pregnancy rate per oocyte retrieval after day 5/6 transfers that has been reported in these studies (Demylle et al., 2000; Plachot et al., 2000) can be largely explained by the relatively low implantation rate per transferred embryo on day 2/3.

The results of our study are in contradiction with the results of another study (Coskun et al., 2000) that did not report any difference in clinical pregnancy rates between a day 2–3 and a day 5 transfer. In this study (Coskun et al., 2000), the overall blastocyst formation was only 28% and the implantation rate per blastocyst was only 24% in good prognosis patients. This low blastocyst formation and implantation rate was probably due to culture conditions, since embryos were cultured under high oxygen tension and culture media were sequentially used from two different companies (Medicult and Vitrolife), which could affect blastocyst rate and viability. Good results with prolonged blastocyst culture can probably only be obtained when embryos are cultured in optimal laboratory conditions.

This study was designed to reach the highest cryo-augmented pregnancy rate per oocyte retrieval possible in both groups. Therefore, in group 1, supernumerary pronucleate ova were frozen if patients had more than five fertilized ova. This procedure limited the number of embryos available for transfer on day 2, which could have a negative impact on the pregnancy results in group 1 and is therefore an important weakness of this study. However, in group 1, 46% of the good prognosis patients (n = 26) with more than five pronucleate ova were clinically pregnant with an implantation rate of 39% per transferred embryo. This implantation rate is comparable with the day 3 implantation rate (37%) reported (Gardner et al., 1998a) in good prognosis patients and the overall implantation rate on day 2 reached almost 30%, which is higher than that reported in the Belgian Register for Assisted Procreation (14% implantation rate per embryo in 1998 and 1999) (BELRAP 1998, 1999) or in other randomized day 2/3 versus day 5/6 studies (Demylle et al., 2000; Plachot et al., 2000; Gardner et al., 1998b). So far, only 17% of all frozen stored embryos have been thawed, and therefore the total cryo-augmented pregnancy rate cannot yet be analysed. This can be explained by two reasons. First, the policy in our centre is to start with a frozen–thawed embryo replacement cycle only if there are at least five frozen stored embryos available, and many patients have too few stored embryos. Second, 37% (group 1) and 62% (group 2) of the patients with frozen stored embryos have already delivered from their fresh IVF cycle and have not yet come back for a second pregnancy. If we extrapolate our current cryopreservation results to the remaining frozen stored embryos in group 1 (Table VIIb), we expect that 20% of all frozen embryos will never be used (patients stop treatment and have their embryos destroyed), and that 60% of the remaining pronucleate ova will survive with a 14% live birth rate per transferred cryo-embryo. This extrapolation would result in eight extra children over probably six deliveries, resulting in a cryo-augmented delivery rate/oocyte retrieval of 38% (24/63) in group 1. A similar result (36% delivery rate/oocyte retrieval) has already been obtained in group 2 with fresh blastocyst transfers, without cryo-augmentation. At this moment, cryopreservation of blastocysts needs further improvement to increase the survival rate after freezing–thawing.

This study suggests that prolonged culture to the blastocyst stage may be an advantage for those patients who have more than four fertilized ova, since in this subgroup the delivery rate per oocyte retrieval is 10% higher than for patients who receive a transfer on day 2. For patients with a lower number of fertilized ova, prolonged culture to blastocysts does not compromise the pregnancy rate, but it does not seem to be better than a day 2 transfer. Selection of the most viable embryo for transfer is probably the key for a successful IVF programme. Since human embryonic gene expression only starts from day 3 onwards (Braude et al., 1988) it is not feasible to predict which embryo will be viable only according to morphological criteria on day 2 or 3. In one study (Rijnders and Jansen, 1998) it has been reported that only 47% of the morphologically best embryos reach the blastocyst stage on day 5 and that only half of the preselected embryos on day 3 were actually selected for transfer on day 5. However, other investigators (Racowsky et al., 2000) stated that the availability of three 8-cell stages is the key factor for choosing a day 5 transfer.

The most important advantage of blastocyst transfers is probably the opportunity to avoid multiple pregnancies by transferring only one blastocyst. In our study, blastocysts that were available on day 5 had an implantation rate of 52%, which makes the transfer of only one embryo feasible. The high implantation rates per embryo in both groups of our study resulted in a high proportion of twin pregnancies in both groups. Further analysis of the occurrence of twin pregnancies in our study resulted in the conclusion that patients at risk for multiple pregnancy were younger than 39 years old, were in their first or second treatment cycle and had good quality embryos. Based on these results, a new strategy was designed and started on January 1, 2001 in our centre to prevent multiple pregnancies. In this strategy, patients with fewer then five
fertilized ova receive an embryo transfer on day 2, while patients with five or more fertilized ova are scheduled for embryo transfer on day 5/6. Patients at risk for a twin pregnancy and scheduled for embryo transfer day 2, who have two morphologically perfect 4-cell stages on the morning of day 2, receive a single embryo transfer (SET) whereas the others receive a maximum of two embryos. Patients at risk for a twin pregnancy and scheduled for embryo transfer day 5, who have blastocysts available on day 5, have a SET on day 5, whereas the others receive two blastocysts on day 6. Preliminary results show that a SET on day 2 (n = 24) resulted in a 32% clinical pregnancy rate while a SET on day 5 (n = 28) resulted in 79% clinical pregnancies per transfer which encourages us to continue this strategy.

In conclusion, in an unselected IVF/ICSI population, prolonged blastocyst culture is an improvement for patients with more than four fertilized ova, whereas patients with a small number of embryos obtain no advantage from a prolonged culture. Blastocyst cultures may be useful for those centres wanting to avoid twin pregnancies.

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