Day 3 versus day 5 embryo transfer: a prospective randomized study

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Abstract
Transfer of embryos at the blastocyst stage has been associated with exceptionally high implantation rates. There are, however, only a few prospective randomized studies comparing day 3 versus day 5 embryo transfer. Furthermore, the number of embryos replaced in the day 3 group transfer is often higher than the number of blastocysts replaced, thereby affecting implantation rates. A total of 118 patients undergoing standard IVF/intracytoplasmic sperm injection who had developed at least three 8-cell embryos showing <20% extracellular fragmentation on day 3 were randomized for day 3 or day 5 transfer. A maximum of two embryos were replaced. In this prospective, randomized study the implantation and pregnancy potential of embryos transferred on day 3 or day 5 were compared. Equal numbers of embryos were replaced in the two groups. There was no statistically significant difference between day 3 and day 5 transfer regarding positive human chorionic gonadotrophin rates (70 versus 67%), clinical pregnancy rates (61 versus 51%), implantation rates (44 versus 37%), twinning rates (42 versus 41%) and rates of early pregnancy loss (15 versus 29%). Transfer of embryos on day 3 or 5 showed similar implantation rates when equal numbers of embryos were transferred. Embryo transfer at the blastocyst stage seems to have no advantage over day 3 transfer in patients with more than two 8-cell embryos showing less than 20% fragmentation on day 3.

Keywords: blastocyst, culture, embryo, IVF, sequential media, transfer
Embryos with <20% extracellular fragments were randomly selected to have their embryos cultured for either 3 or 5 days in the sequential media system used in the standard IVF/ICSI programme (IVF-100™, G1.2™ and G2.2™; Vitrolife, Gothenburg, Sweden). Randomization was performed by drawing lots (sealed envelopes).

IVF-100™ was used in the first step of culture, from oocyte retrieval to fertilization; the oocytes were then rinsed twice in G1.2™ and cultured in G1.2™ until the morning of day 3. In cases of blastocyst culture, G2.2™ was used from the 8-cell stage on day 3 until embryo transfer. Embryo transfer was performed in G2.2™ for both groups.

A maximum of six fertilized oocytes were cultured in 20 µl media droplets under oil (OVOIL™; Vitrolife). In cases with fewer than six oocytes, 10 µl droplets were used. A gas phase of 6% CO2 and 5% O2 and 89% N2 was used in a humidified incubator.

**Sperm preparation**

Semen analysis was performed according to the World Health Organization (WHO) guidelines (WHO, 2000) and a standard density gradient centrifugation method, 45 and 90% PureSperm (Nidacon Ltd, Gothenburg, Sweden) diluted in SpermRinse™ (Vitrolife), was used for sperm preparation. The washing procedure and dilution was performed in IVF-100™.

**Insemination procedure**

In cases of conventional IVF, spermatozoa at a final concentration of 150,000 × 10⁶/ml were added to the oocytes. After incubation for 90 min, the oocytes were washed three times before further culture in IVF-100™ until time of assessment for fertilization.

**Intracytoplasmic sperm injection**

In cases of microinjection (ICSI) denudation of cumulus cells was performed by exposure of the oocytes to HYASE™ (Vitrolife) for a maximum of 30 s. Denudation of cumulus cells was performed by the use of glass denuding pipettes (SweMed Lab, Billdal, Sweden) immediately before injection. The oocytes were washed four times in Gamete™ (Vitrolife) after denudation. ICSI was performed in Gamete™ by commercially available ICSI pipettes (Cook, Brisbane, Australia). A 5 µl ultra-micro droplet of polyvinylpyrrolidone (PVP), ICSI-100™ (Vitrolife) was spread out in a thin layer on the Petri dish. A 1 µl aliquot of the sperm preparation was introduced to the centre of the PVP droplet. After injection, the oocytes were washed twice in G1.2™ medium before further culture in G1.2™.

**Assessment of fertilization**

Fertilization was determined 18–20 h after the insemination procedure. The oocytes were considered fertilized when two distinct pronuclei were visible. Cleavage and classification of morphology was first assessed 24 h later.
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Embryo morphology classification, embryo transfer and criteria for cryopreservation

A maximum of two embryos were transferred on day 3 or 5 after retrieval according to the randomization in the morning of day 3. On day 3, embryos were scored using criteria set up by Ziebe et al. (1997). Strict criteria for cryopreservation were used. Only embryos containing at least seven blastomeres and <20% intracellular fragments were cryopreserved on day 3. On day 5, embryos were assessed according to scoring criteria for blastocysts (Gardner and Schoolcraft, 1999). Only expanded blastocysts were cryopreserved.

All embryo transfers were performed with a Cook Soft 5000 catheter (Cook, Australia).

Luteal phase support and pregnancy test

Luteal phase support was given by daily vaginal administration of micronized progesterone, either 400 mg twice a day (Cyklogest; Hoechst, Copenhagen, Denmark) or 90 mg once a day (Crinone 8%; Serono Nordic, Denmark) starting on the day following oocyte retrieval and continuing until the day of the pregnancy test (i.e. day 12 after embryo transfer). A positive pregnancy test was defined by a plasma β-HCG concentration >10 IU/l. A clinical pregnancy was defined as an intrauterine gestational sac with a heart beat 3 weeks after a positive HCG test. An early pregnancy loss was defined as a preclinical or a clinical abortion before gestational week 12. The implantation rate was calculated as the ratio of gestational sacs determined by ultrasound after 7 weeks in relation to the total number of embryos transferred.

Statistical methods

Results are expressed as mean ± SD, and for statistical analysis STATGRAPHICSTM software (Manugistics, Inc., Rockville, MD, USA) was applied using either Student’s t-test or chi-squared test where appropriate.

### Table 1. Demographic data.

<table>
<thead>
<tr>
<th></th>
<th>Group I (day 3)</th>
<th>Group II (day 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients included (n)</td>
<td>57</td>
<td>61</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>31.3 (22.0–39.0)</td>
<td>31.2 (22.5–39.3)</td>
</tr>
<tr>
<td>Mean body mass index (kg/m²) (range)</td>
<td>23.6 (17.9–29.5)</td>
<td>22.9 (16.9–30.0)</td>
</tr>
<tr>
<td>Patients undergoing first or second IVF/ICSI treatment (%)</td>
<td>84.0</td>
<td>88.0</td>
</tr>
<tr>
<td>Mean (±SD) pre-treatment levels of FSH (IU/l)</td>
<td>6.5 ± 1.7</td>
<td>6.5 ± 1.8</td>
</tr>
</tbody>
</table>

### Table 2. Oocytes, fertilization and embryo quality.

<table>
<thead>
<tr>
<th></th>
<th>Group I (day 3)</th>
<th>Group II (day 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients included (n)</td>
<td>57</td>
<td>61</td>
</tr>
<tr>
<td>Mean (±SD) FSH-consumption (IU/l)</td>
<td>2074.1 ± 860.1</td>
<td>1968.5 ± 620.5</td>
</tr>
<tr>
<td>Mean no. (±SD) oocytes retrieved</td>
<td>12.8 ± 4.4</td>
<td>13.5 ± 5.3</td>
</tr>
<tr>
<td>Cycles with ICSI (%)</td>
<td>51</td>
<td>64</td>
</tr>
<tr>
<td>Mean no. (±SD) of oocytes fertilized (2PN)</td>
<td>7.7 ± 3.2</td>
<td>8.2 ± 3.2</td>
</tr>
<tr>
<td>Blastocysts of 2 PN (day 5) (%)</td>
<td>–</td>
<td>55.2</td>
</tr>
<tr>
<td>Mean no. (±SD) ≥8 cells 68 h post-insemination</td>
<td>4.6 ± 2.1</td>
<td>5.8 ± 2.3</td>
</tr>
<tr>
<td>Mean no. embryos transferred</td>
<td>2.0</td>
<td>1.96</td>
</tr>
<tr>
<td>Patients with embryo cryopreservation (%)</td>
<td>95</td>
<td>59</td>
</tr>
<tr>
<td>Mean no. (±SD) embryos cryopreserved</td>
<td>3.4 ± 2.4</td>
<td>1.4 ± 1.6</td>
</tr>
</tbody>
</table>

*P < 0.001.

### Table 3. Pregnancy and implantation rate.

<table>
<thead>
<tr>
<th></th>
<th>Group I (day 3)</th>
<th>Group II (day 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo transfer (ET)</td>
<td>57</td>
<td>61</td>
</tr>
<tr>
<td>Positive HCG (% per ET)</td>
<td>40 (70.2)</td>
<td>41 (67.2)</td>
</tr>
<tr>
<td>Clinical pregnancies (% per ET)</td>
<td>36 (63.2)</td>
<td>32 (52.5)</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>50/114 (43.9)</td>
<td>44/120 (36.7)</td>
</tr>
<tr>
<td>Double implantations (%)</td>
<td>15/36 (41.6)</td>
<td>13/32 (40.6)</td>
</tr>
<tr>
<td>Early pregnancies lost (%)</td>
<td>6 (15.0)</td>
<td>12 (29.2)</td>
</tr>
</tbody>
</table>

Values are numbers unless otherwise stated.
Results

A total of 118 patients were included in the study. Embryo transfers was performed in 57 patients in the day 3 group (group I) and in 61 in the day 5 group (group II). Demographic data are given in Table 1. No statistical differences were seen between the two groups concerning age; body mass index (BMI), number of previous IVF/ICSI attempts or pre-treatment levels of FSH.

Table 2 shows that the two groups were comparable regarding rec-FSH consumption, the number of oocytes retrieved, the frequency of IVF/ICSI fertilization rates, the number of 8-cell embryos present on day 3 and as the number of embryos transferred. Significantly more patients had embryos cryopreserved on day 3 compared with day 5 (95 versus 59%) \((P < 0.001)\). A tendency for a higher number of embryos cryopreserved was seen in the day 3 group, but the difference was not statistically significant \((P > 0.10)\) (Table 2).

All randomized patients in the day 3 group had two embryos transferred, according to the protocol, whereas in the day 5 group two patients had only one embryo transferred, due to lack of other viable embryos for transfer (Table 2). Moreover, four of the patients in the day 5 group did not have two blastocysts available for transfer; instead, two morulae or combined blastocyst/morulae were transferred. There were no statistical differences regarding rates of positive HCG (70.2 versus 67.2%), clinical pregnancy (63.2 versus 52.5%), implantation (43.9 versus 36.7%), twinning (41.6 versus 40.6%) and early pregnancy loss (15.0 versus 29.2%) (Table 3). The power of the statistical tests comparing the clinical pregnancies is 0.32 and the total number of observations should be 726 to obtain a power of 0.90. For the implantation rate, the power is 0.30 and a total of 1592 observations are required to avoid making a type II error at a level of 0.90.

The rate of blastocyst formation on day 5 was 55.2%.

In Table 4, the data are split into day and method of fertilization. The group of patients in whom ICSI was performed had significantly lower blastocyst formation rate than the IVF group (51 versus 60.3%) \((P < 0.001)\). The ICSI group consisted mainly of male factor infertility (89%). There was no effect of ICSI, however, on positive HCG rates (74.1 versus 66.7%), clinical pregnancy rates (55.5 versus 66.7%) or implantation rates (44.4 versus 43.3%) day 3. Five out of 20 patients (25%) in the IVF group had an early pregnancy loss, compared with one out of 20 patients (5%) in the ICSI patients. The difference, however, was not statistically significant \((P > 0.1)\). For patients having embryo transfer on day 5, the rates of positive HCG (74.1 versus 66.7%), and the rates of clinical pregnancy rates (55.5 versus 66.7%) were equal. Implantation rates for IVF was significantly higher than after ICSI (46.9 versus 28.2%) \((P < 0.05)\). In all, 17.4% of the day 5 IVF group had an early pregnancy loss compared with 34.6% of the day 5 ICSI group \((P > 0.1)\) (Table 4). Power calculations of the rate of blastocyst formation, the rate of positive HCG, the rate of clinical pregnancies, the implantation rate and the rate of early pregnancy loss comparing day 5 IVF versus ICSI are 0.64, 0.26, 0.12, 0.67 and 0.4 respectively. The numbers of observations needed to avoid a type II error (i.e. achieve a power of 0.9) are 972, 512, 2362, 222, 218.

Discussion

So far as is known, this is the first prospective, randomized study to compare the implantation and pregnancy potential of embryos transferred on day 3 or day 5 with equal numbers of embryos to each patient in the two groups. Replacement of equal numbers of embryos in the two groups was accomplished by selecting a group of patients who had more than two 8-cell embryos with <20% extracellular fragments on day 3. For this group of patients, the present study demonstrates that embryos have similar implantation potential whether transferred on day 3 or day 5. Although the power of the present study is limited, prolonging the in-vitro period to 5 days does not seem to provide additional information in selecting embryos with the highest likelihood of implantation. The results of the present study therefore do not support the use of blastocyst transfer in order to improve implantation and pregnancy rates for good prognosis patients. On the other hand, the results obtained do not justify a general conclusion that embryos from all patient categories show an equal implantation potential on days 3 and 5.

Despite differences in design, patient selection and culture conditions, the results of the present study are in overall agreement with the majority of previous published studies (Scholles and Zeilmaker, 1996; Coskun et al., 2000; Huismann et al., 2000; Bungum et al., 2002). This is the first randomized study comparing the implantation and pregnancy potential of embryos transferred on day 3 or day 5, with equal numbers of embryos to each patient in the two groups. Replacement of equal numbers of embryos in the two groups was accomplished by selecting a group of patients who had more than two 8-cell embryos with <20% extracellular fragments on day 3. For this group of patients, the present study demonstrates that embryos have similar implantation potential whether transferred on day 3 or day 5. Although the power of the present study is limited, prolonging the in-vitro period to 5 days does not seem to provide additional information in selecting embryos with the highest likelihood of implantation. The results of the present study therefore do not support the use of blastocyst transfer in order to improve implantation and pregnancy rates for good prognosis patients. On the other hand, the results obtained do not justify a general conclusion that embryos from all patient categories show an equal implantation potential on days 3 and 5.

Table 4. IVF/ICSI results.

<table>
<thead>
<tr>
<th>Day of transfer</th>
<th>3</th>
<th>3</th>
<th>5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertilization type</td>
<td>IVF</td>
<td>ICSI</td>
<td>IVF</td>
<td>ICSI</td>
</tr>
<tr>
<td>Embryo transfer (ET) (n)</td>
<td>27</td>
<td>30</td>
<td>25</td>
<td>36</td>
</tr>
<tr>
<td>Mean no. (±SD) of oocytes fertilized (2PN)</td>
<td>9.0 ± 3.5</td>
<td>7.0 ± 2.5</td>
<td>9.0 ± 5.7</td>
<td>7.5 ± 2.4</td>
</tr>
<tr>
<td>Blastocyst of 2 PN (%)</td>
<td>–</td>
<td>–</td>
<td>126/209 (60.3)a</td>
<td>127/249 (51.0)b</td>
</tr>
<tr>
<td>Positive HCG (% per ET)</td>
<td>20/27 (74.1)</td>
<td>20/30 (66.7)</td>
<td>15/25 (60.0)</td>
<td>26/36 (72.2)</td>
</tr>
<tr>
<td>Clinical pregnancy (% per ET)</td>
<td>15/27 (55.5)</td>
<td>20/30 (66.7)</td>
<td>14/25 (56.0)</td>
<td>18/36 (50.0)</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>24/54 (44.4)</td>
<td>26/60 (43.3)</td>
<td>23/49 (46.9)c</td>
<td>20/71 (28.2)d</td>
</tr>
<tr>
<td>Early pregnancies lost (%)</td>
<td>5/20 (25.0)</td>
<td>1/20 (5.0)</td>
<td>4/23 (17.4)</td>
<td>9/26 (34.6)</td>
</tr>
</tbody>
</table>

a,b Groups with a different letter differ significantly \((P < 0.001)\).

c,d Groups with a different letter differ significantly \((P < 0.05)\).

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References

Scholles and Zeilmaker, 1996; Coskun et al., 2000; Huismann et al., 2002.
et al., 2000; Levron et al., 2002; Rienzi et al., 2002; Utsunomiya et al., 2002). A study by Gardner et al. (1998b) found exceptionally high implantation rates (50.5 versus 30.1%) in favour of blastocyst culture. In cycles with more than three 8-cell embryos on day 3, Racowsky et al. (2000) found that day 5 transfers resulted in an increased implantation rate (35.9 versus 24.2%). In addition, the studies of Abdelmassih et al. (2001) and Karaki et al. (2002) reported higher implantation rates with transfer on day 5 compared with day 3 (45.3 versus 18.5% and 26 versus 13% respectively). However, in these four studies more embryos were transferred on day 3 than on day 5, which influences the implantation rate for day 3 transfer in a negative manner. Other differences between the material of Gardner et al. (1998b) and the present study include the inclusion criteria; only high responders, i.e. patients who had at least 10 follicles ≥12 mm in diameter on the day of HCG injection, were included in the study by Gardner et al. Additionally, a majority of the embryos replaced on day 3 had undergone assisted hatching, whereas those replaced on day 5 had not (Gardner et al., 1998b). Moreover, two different culture media were used, one system for the day 3 transfers, another for the day 5 transfers. The use of different media in the group of patients transferred on day 3 and 5, now considered to be suboptimal for the metabolic requirements of the embryo after the 8-cell stage (Bertheussen et al., 1997; Gardner et al., 1998a; Jones et al., 1998) is a drawback for a number of the previous studies. In the present study, both groups were cultured in the same sequential media system (IVF-100, G1.2, G2.2; Vitrolife ab) and all other variables in the laboratory were constant.

It has been argued that blastocyst culture could be used to reduce the high multiple pregnancy rates, by selecting only one or two embryos with the best chance of implantation (Gardner et al., 1998a; Bergh et al., 1999; Abdelmassih et al., 2001; Racowsky et al., 2003). When replacing two embryos, the implantation rates in the present study resulted in twinning rates of 40.6% after day 5 transfer and 41.6% after day 3 transfer. Therefore, using the present methods, the strategy of replacing only two embryos is not a valid argument for reducing multiple gestation rates. On the contrary, the group of patients included in the present study should be considered for single embryo transfer as a measure to reduce the high rate of multiple pregnancies.

It has also been argued that blastocysts exhibit higher viability as compared with cleavage stage embryos, since transfer of blastocysts on day 5 coincides with entry of the embryos to the uterine cavity in the natural cycle, resulting in better synchrony between the endometrium and the blastocyst (Olivierennes et al., 1994; Kaufmann et al., 1995). However, some studies indicate that the human embryo in vivo actually enters the uterus as early as the 7- to the 12-cell stage in the natural cycle (Hertig et al., 1956; Croxatto, 1974; Trounson et al., 1984). Moreover, recent published studies (Ubaldi et al., 1997; Nikas et al., 1999; Kolibianakis et al., 2002) indicate a correlation between ovarian stimulation and earlier endometrial development as compared with the natural cycle.

The blastocyst formation rate varies considerably between different studies (Scholtes and Zeilmaker, 1996; Gardner et al., 1998b; Coskun et al., 2000; Huismann et al., 2000; Karaki et al., 2002; Rienzi et al., 2002; Utsunomiya et al., 2002), which could be due to differences in culture media and culture conditions. Several other factors, including impaired semen quality, have been suggested to influence blastocyst development in a negative manner. While some authors do not find differences in blastocyst formation rate between ICSI and IVF (Gardner et al., 1998b; Karaki et al., 2002), others have reported decreased blastocyst development in ICSI embryos compared with IVF embryos (Janny and Ménézo, 1994; Jones et al., 1998; Miller and Smith, 2001). The blastocyst formation rate in this study (55.2%) is comparable to earlier published reports (Gardner et al., 1998b; Schoolcraft et al., 1999; Rienzi et al., 2002). The group of patients in whom ICSI was performed, however, had a significantly lower blastocyst formation rate than the IVF group (51 versus 60.3%).

The present study indicates a possible negative influence of ICSI on the outcome of treatment for the day 5 group. No significant differences were seen in pregnancy rates. The implantation rates on day 5, however, were significantly better in the IVF group as compared with the ICSI group (46.9 versus 28.2%) (P < 0.05). Although the groups are small, day 5 transfers after ICSI may enhance early pregnancy loss. In the day 5 group, 34.6% of the ICSI transfers resulted in an early pregnancy loss as compared with 17.4% in the IVF-group. In the day 3 group, no difference was observed between the IVF and the ICSI groups. However, especially comparing day 5 IVF and ICSI results, the numbers are relatively low and additional information is required to exclude the risk of making a type II error when performing statistical tests.

Implantation rates are most correctly presented in single embryo transfer, because of the confounding factors present in embryo transfers with two or more embryos transferred. In the present study, however, implantation rates were compared between two groups who received the same numbers of embryos. The implantation rates, reported after embryo transfer on day 3 in other studies as well as the present, are high (Coskun et al., 2000; Huismann et al., 2000; Gerris and Van Royen, 2000; Scott et al., 2000; Gerris et al., 2001; Rienzi et al., 2002). The implantation rates in the present study, 43.9% for day 3 and 36.7% for day 5 transfer, were higher as compared with most of the other prospective randomized studies (Scholtes and Zeilmaker, 1996; Huismann et al., 2000; Coskun et al., 2000; Karaki et al., 2002; Levron et al., 2002; Rienzi et al., 2002; Utsunomiya et al., 2002). One exception is the earlier mentioned study by Gardner et al. (1998b). One explanation is of course the selection of a good patient population: patients having more than two 8-cell embryos with < 20% fragments transfer on day 3. Another explanation could be the culture system used in this study. A reduced O2 level compared with conventional protocols for IVF was used. The model for this choice has been the O2 concentration within the lumen of the female reproductive tract, which is significantly lower (3–8%) than that present in air (20%) (Mastroanni and Jones, 1965; Fisher and Bavister, 1993). Furthermore, the effect of the incubation volume:embryo ratio has been shown to increase the blastocyst rates and the number of cells in the inner cell mass and the trophoderm (Paria and Dey, 1990; Lane and Gardner, 1992). In this study, up to six embryos were cultured together in small droplets of culture medium (20 μl).

To summarize, high pregnancy and implantation rates can be achieved by embryo transfer on day 3 as well as day 5.
Implantation rates for day 3 and day 5 transfers were equal when equal numbers of embryos were transferred. The twinning rate was high in both groups, and it is suggested that only one embryo should be replaced in this group of patients. A tendency towards higher rates of early pregnancy loss was seen when ICSI embryos were transferred day 5 as compared with blastocysts following regular IVF treatment. For patients having more than two 8-cell embryos with <20% fragments, transfer on day 3 has no advantage as compared with day 5 transfer. However, further studies are needed to clarify whether selected groups of patients could benefit from transfer at the blastocyst stage.

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