Introduction

The first clinical pregnancy ever established during assisted conception involved the transfer of a blastocyst (Edwards and Brody, 1995). However, due to difficulties in culturing human embryos for a period of 5 days, it became common practice to transfer embryos at cleavage stages, i.e. day 2 or 3, despite relatively low implantation rates of 10–20% (Edwards and Brody, 1995). In contrast, recent studies have reported exceptionally high implantation rates after blastocyst culture and embryo transfer (Gardner, 1998; Gardner et al., 1998a). Prolonged culture of human embryos has been advocated to increase the efficiency of IVF treatments by improving the selection of embryos with the best chances of implantation and by reducing the number of embryos replaced, thereby reducing the risk of high-order pregnancy (Gardner et al., 1998a). Due to recent advances in the culture media used for IVF/intracytoplasmic sperm injection (ICSI) (Bertheussen et al., 1997; Jones et al., 1998; Gardner et al., 1998a), many clinics have been encouraged to postpone embryo transfer to day 5 (Scholtes and Zeilmaker, 1996; Marek et al., 1999; Huismann et al., 2000). Conflicting results have been reached, however, concerning the superiority of day 5 embryo transfer as compared with transfer on day 2 or day 3 (van Os et al., 1989; Scholtes and Zeilmaker, 1996; Gardner et al., 1998b; Cruz et al., 1999; Coskun et al., 2000; Huismann et al., 2000; Milki et al., 2000; Laverge et al., 2001; Lundquist et al., 2002; Van der Auwera et al., 2002). So far, eight prospective randomized studies comparing day 3 to day 5 embryo transfers have been published (Scholtes and Zeilmaker, 1996; Gardner et al., 1998b; Coskun et al., 2000; Huismann et al., 2000; Karaki et al., 2002; Levron et al., 2002; Rienzi et al., 2002; Utsunomiya et al., 2002). However, the design of some of these studies has been questioned in the ongoing debate. The studies of Scholtes and Zeilmaker (1996) and Huismann et al. (2000) consisted of an unselected study population and the
studies by Gardner et al. (1998b), Coskun et al. (2000), Karaki et al. (2002), Levron et al. (2002), Rienzi et al. (2002) and Utsunomiya et al. (2002) analysed a selected group of patients. Only two of these prospective randomized studies were able to demonstrate an improved outcome in favour of blastocyst transfer (Gardner et al., 1998b; Karaki et al., 2002). The number of transferred embryos in both of these studies, however, was lower in the blastocyst group, contributing to improved implantation rates of day 5 embryo transfer. The policy of the clinic is to transfer a maximum of two embryos. Patients showing at least three high quality embryos on day 3, i.e. good prognosis patients, were included in this study. This set-up would potentially result in the transfer of equal numbers of embryos in the day 3 and the day 5 groups, allowing a direct comparison of implantation rates. In addition, unlike previous studies, the outcome of treatment was compared in these two groups of women. The primary aim of this study was to investigate whether embryos from good prognosis patients have a different implantation potential comparing day 3 to day 5 embryo transfer when equal numbers of embryos are transferred.

Materials and methods
Patients, hormonal treatment and study design

During the period December 2001 to May 2002, a total of 118 patients undergoing standard IVF or ICSI were included in the study. Patients fulfilling the following inclusion criteria were prospectively enrolled in a consecutive manner: (i) three or more 8-cell embryos with <20% extracellular fragments on day 3; (ii) female age <40 years; (iii) body mass index (BMI) <30; (iv) baseline FSH <12 IU/l; (v) standard hormonal treatment as follows: pituitary down-regulation with gonadotrophin-releasing hormone agonist (GnRHa) (Suprefact; Hoechst, Hørsholm, Denmark), 0.8 mg s.c. daily from the mid-luteal phase for 14 days. After pituitary down-regulation, the dose of GnRHa was reduced to 0.4 mg s.c. daily and ovarian stimulation was initiated with recombinant FSH (rec-FSH) (Gonal F; Serono Nordic, Copenhagen, Denmark or Puregon; Organon, Skovlunde, Denmark) using an individualized dose of between 100 IU and 375 IU s.c. per day according to age, ovarian volume, baseline FSH and body mass index (BMI). The ovarian response was monitored by ultrasound examination starting on day 8 of stimulation, and the dose of FSH administered was adjusted if necessary. The Ethics committee of Viborg County approved this study.

Oocyte retrieval

When at least three follicles had reached a diameter of 17 mm or more, 10,000 IU of human chorionic gonadotrophin (HCG) (Profasi; Serono Nordic, Denmark) was administered to induce final follicular maturation. Oocyte retrieval was performed 35 h later by vaginal ultrasound-guided follicle aspiration. Gamete™ (Vitrolife, Gothenburg, Sweden), a HEPES-buffered medium, was used to rinse the oocytes.

Culture media and procedure for culturing

On the morning of day 3, patients with three or more 8-cell 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.
### 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) (range)</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>
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</table>

*P < 0.001.

### 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 cryopreservationa (%)</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>
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<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 a 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

<table>
<thead>
<tr>
<th>Day of transfer</th>
<th>3</th>
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<th>5</th>
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</tr>
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<tbody>
<tr>
<td><strong>Fertilization type</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Embryo transfer (ET) (n)</td>
<td>IVF</td>
<td>ICSI</td>
<td>IVF</td>
<td>ICSI</td>
</tr>
<tr>
<td>Mean no. (±SD) of oocytes fertilized (2PN)</td>
<td>27</td>
<td>30</td>
<td>25</td>
<td>36</td>
</tr>
<tr>
<td>Blastocysts of 2 PN (%)</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>
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<tr>
<td><strong>Positive HCG (% per ET)</strong></td>
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<tr>
<td>—</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><strong>Clinical pregnancy (% per ET)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>—</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>
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<tr>
<td><strong>Implantation rate (%)</strong></td>
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<td></td>
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<tr>
<td>—</td>
<td>24/54 (44.4)</td>
<td>26/60 (43.3)</td>
<td>23/49 (46.9)</td>
<td>20/71 (28.2)</td>
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<tr>
<td><strong>Early pregnancies lost (%)</strong></td>
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<tr>
<td>—</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 ($P < 0.05$).
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 (45.3 versus 18.5% and 26 versus 13% respectively). Higher implantation rates with transfer on day 5 compared with 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, however, implantation rates were compared to increase the blastocyst rates and the number of cells in the inner cell mass and the trophectoderm (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.

The blastocyst formation rate varies considerably between different studies (Schoelte 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 Ménézé, 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%).
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.

Acknowledgements

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