Objective: To assess the value of routine screening of nuclear status of day-2 four-cell preembryos for single embryo transfer (SET) in predicting implantation.

Design: Retrospective analysis.

Setting: Private IVF unit.

Patient(s): A total of 1,985 fresh embryo transfers on day 2 or day 3 were performed from January 2002 to November 2004. In 1,295 (65.2%) of these transfers, SET was performed. All day-2 four-cell preembryos transferred in SET cycles (n = 861) were analyzed retrospectively for outcome in terms of implantation rate and its relation to the number of visible mononucleate blastomeres (MNBs), the degree of fragmentation, and equality of blastomeres.

Intervention(s): Light microscopic evaluation of preembryos before transfer on day 2.

Main Outcome Measure(s): Implantation rate.

Result(s): The number of MNBs was found to be related to implantation, whereas blastomere equality and rate of embryo fragmentation were not. The implantation rate was statistically significantly higher in cycles where a four-cell preembryo with four MNBs was transferred than after transfer of a four-cell preembryo with zero to three MNBs (42% versus 22%). In a logistic regression analysis, nucleation of all blastomeres was the only morphologic parameter that was associated with the implantation rate.

Conclusion(s): Evaluation of nuclear status of four-cell preembryos is important in predicting implantation potential. Visualization of four MNBs in a four-cell preembryo predicted a statistically significant higher implantation rate than in cases where not all four blastomeres were mononucleate. (Fertil Steril 2005;84: 584–9. ©2005 by American Society for Reproductive Medicine.)

Key Words: In vitro fertilization, single embryo transfer, implantation potential, preembryos, mononucleation.
considered to be low. Before the above-mentioned legislation, regional regulation had been introduced in southern Sweden in the year 2002 stating that only one embryo should be transferred in all patients treated under the regional health services. As a consequence of these decrees, our rate of SET increased from 20% to 25% (mostly because only one embryo was available for ET) to approximately 70% during the year 2003 (18) and close to 80% during 2004.

As a result of the two decrees, we have now had the opportunity to analyze retrospectively the outcome of day-2 four-cell preembryos in relation to three different morphologic markers in a consecutive cohort of patients undergoing fresh ET.

MATERIALS AND METHODS

During the period January 2002 to November 2004, a total of 1,985 fresh IVF/ET cycles were carried out at our clinic. Of these, SET was performed in 1,295 cycles (65.2%). Among the 1,295 SETs, 981 (76%) were performed on day 2 and 314 (24%) on day 3 (on Monday when ovum pick-up was carried out on a Friday). Of the patients receiving a four-cell preembryo (n = 867), six were excluded from the study because data on the nuclear status had not been recorded. All patients receiving a four-cell preembryo on day 2 and in whom the nuclear status was recorded (n = 861) were included in the study. The results of SET on day-2 of embryos that were not in the four-cell stage were also analyzed.

In preparation for IVF treatment, all women with no contraindication to estrogen were given oral contraceptive pills (Follimin, containing 30 μg ethinylestradiol and 150 μg levonorgestrel) for 25 days from the first day of bleeding in the cycle preceding ovarian stimulation. In our IVF program, the long protocol with the GnRH agonist buserelin (Suprecur nasal spray; Hoechst AG, Frankfurt, Germany; 0.3 mg four times daily, started on day 21 in the contraceptive pill cycle) is standard, although a minority of the patients were treated with a GnRH antagonist (Cetrotide, 0.25 mg daily injected SC, when the leading follicle had reached 14 mm in mean diameter until the day of hCG administration). For ovarian stimulation, recombinant FSH (Gonal F; Serono Laboratories, Geneva, Switzerland; or in a few patients Puregon; Organon, The Netherlands) in daily doses ranging from 75–450 IU SC was used.

The ovarian response was monitored with serial ultrasound scanning of follicular growth and, in low and high responders, in combination with serum E2 measurements. Oocyte maturation was induced with recombinant or urinary hCG (250 μg Ovitrelle SC or 10,000 IU Profasi SC; Serono Laboratories) when the leading follicles were 18 to 20 mm in diameter; ovum pick-up was scheduled 36 hours later. Oocyte retrieval was performed vaginally under ultrasound guidance (Pie Medical Scanner 150, Maastricht, The Netherlands) and general anesthesia (fentanyl 1 μg/kg and propofol 2–3 mg/kg IV). Cumulus–oocyte complexes were identified in the follicular fluids and washed in conventional media (Medicult, Jyllinge, Denmark, or Vitrolife, Gothenburg, Sweden) and then incubated in approximately 6% CO2 in air at pH 7.40 until the time of insemination or microinjection. The semen sample was collected in a plastic jar with 2 mL of medium. The sample was allowed to liquefy for 30 minutes before preparation for conventional IVF or intracytoplasmic sperm injection (ICSI).

For standard IVF, groups of a maximum of five oocytes were inseminated with 200,000 motile swim-up sperm in individual medium droplets with a volume of 50 μL under mineral oil and incubated overnight. Insemination was performed 4 to 5 hours after oocyte retrieval. A check was made 18 to 20 hours later for signs of fertilization, which was verified by the presence of two pronuclei. The zygotes were then further cultured in groups of a maximum of five in 20-μL droplets under mineral oil. Using an Eppendorf ICSI apparatus (Eppendorf AG, Hamburg, Germany), ICSI was performed, and a maximum of five oocytes were thereafter transferred to a 20-μL droplet under oil and further incubated as above. Inclusion criteria for ICSI were a low sperm count (less than 1 million sperm/mL after preparation), or low motility (on a subjective scale of 1 to 4), or previous failure of fertilization after standard IVF.

Grading and selection of the preembryos for transfer were based on a morphologic evaluation under an inverted light microscope (Olympus CK 2; Olympus Microscopy, Melville, NY) at ×200 magnification on a single occasion 48 to 51 hours after insemination/ICSI and 5 to 10 minutes before ET. The grading and selection were performed by either of the authors throughout the study period. Preembryos were graded according to the total number of blastomeres, degree of cytoplasmic fragmentation score (0 = 0 to 9%, 1 = 10% to 19%, 2 = 20% to 30%, 3 = >30%), number of visible mononucleate blastomeres (zero to four), equality of blastomere size (0 = equal size, 2 = not equal size), and presence or absence of multinucleated blastomeres. Preembryos with multinucleate blastomeres were excluded from transfer. A four-cell preembryo with zero to three MNBs was chosen only if no preembryo with four MNBs was available.

Embryo transfer was performed with a Sydney IVF catheter (K-JETS-7019-SIVF, Cook, Australia) or an Edwards-Wallace replacement catheter (Smiths Medical, Hythe, Kent, United Kingdom). The catheter was loaded by either of the authors with the embryo(s) placed in the distal third of 15–20 μL of medium. The catheter was inserted through the cervical canal and the embryo or embryos were expelled at a level 6.0 to 6.5 cm from the external cervical os. Ultrasound guidance was not used. After removal, the inner catheter was flushed and checked under the microscope to ascertain that the catheter was empty.

For luteal phase support, natural micronized P pessaries, 400 mg three times a day (Apoteksbolaget, Stockholm, Sweden), were used. The P treatment was initiated in the afternoon on the day of oocyte retrieval and continued until the
day of the pregnancy test. A urinary pregnancy test (hCG urine test; Abbot Laboratories, Abbot Park, IL) was performed 18 days after oocyte retrieval. Implantation was defined as vaginal ultrasound verification of a gestational sac/sacs at 7 weeks’ gestation. A positive β-hCG test alone was not considered as evidence of implantation.

Statistical analyses of nominal variables were performed with the chi-square test, and of continuous variables with the Mann-Whitney U test. Logistic regression was used to estimate the impact of the morphologic parameters (fragmentation, degree of equality of blastomere size, and number of MNBs) on the implantation rate. P<.05 was considered statistically significant, and was adjusted for ties. The statistical analyses were performed with the aid of StatView (SAS Institute, Cary, NC) and MedCalc (MedCalc Software, Mariakerke, Belgium) computer software.

### RESULTS

During the study period, SET constituted 65.2% (1,295 out of 1,985) of all ETs. The implantation rate in the SET group was 427 out of 1,295 (33.0%). The relationship between the number of blastomeres of the preembryo selected for transfer and the implantation rate after SET on day 2 is shown in Table 1.

In 981 out of 1,295 women, SET was performed on day 2; of these, a four-cell preembryo was transferred in 867 (88.2%) of the patients. All three morphologic markers were recorded in 861 of these cycles. The nuclear status was found to be related to implantation, whereas blastomere size, equality, and embryo fragmentation were not (Table 2). The number of MNBs was found to be related to both equality (chi-square test, P<.0001) and fragmentation (P=.001) and all three morphologic markers were therefore included in a logistic regression analysis. In this analysis, nucleation of all four blastomeres was the only morphologic sign that was associated with the implantation rate (P=.005). In 665 (77.2%) of the four-cell preembryos, four visible MNBs were recorded, while in 196 (22.8%) zero to three MNBs were visualized.

The outcome of transfer of a four-cell/four-nuclei preembryo or a four-cell/0–3 nuclei preembryo in relation to age is given in Table 3.

There was no difference in mean age, body mass index, or infertility duration between the group of women in whom a four-cell preembryo with four MNBs was transferred and the group with zero to three MNBs (Table 4). The mean number of oocytes at retrieval and the number of normally fertilized oocytes were statistically significantly higher in the group with four MNBs (see Table 4).

### Table 1

<table>
<thead>
<tr>
<th>No. of blastomeres</th>
<th>Implantation rate</th>
<th>Implantation rates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0/41</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2/23</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>321/867</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>9/38</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>1/10</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>0/2</td>
<td>0</td>
</tr>
</tbody>
</table>


### Table 2

The influence of morphologic markers on implantation rates in 861 single embryo transfer cycles with day 2 four-cell preembryos.

<table>
<thead>
<tr>
<th>Score</th>
<th>Significance of difference* (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Equalityb</td>
<td>316/840</td>
</tr>
<tr>
<td>(37.3)</td>
<td>(23.8)</td>
</tr>
<tr>
<td>Fragmentationc</td>
<td>85/218</td>
</tr>
<tr>
<td>(38.0)</td>
<td>(37.3)</td>
</tr>
<tr>
<td>Mononucleate blastomeresd</td>
<td>9/45</td>
</tr>
<tr>
<td>(20.0)</td>
<td>(20.8)</td>
</tr>
</tbody>
</table>

Note: Values within parentheses are percentages.

*a Statistical analysis with the chi-square test.

b Score 0 = equal size blastomeres, 1 = not equal size blastomeres.

c Score 0 = 0–9%, 1 = 10%–19%, 2 = 20%–30%, 3 = >30%.

d Number of visible mononucleate blastomeres (0–4).

TABLE 3

<table>
<thead>
<tr>
<th>Nuclear Status</th>
<th>Implantation Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 cells/4 nuclei</td>
<td>260/608 (42.8)</td>
</tr>
<tr>
<td>4 cells/0–3 nuclei</td>
<td>40/172 (23.3)</td>
</tr>
</tbody>
</table>

Note: Values within parentheses are percentages.


DISCUSSION

This study shows that the number of MNBs in a day-2 four-cell preembryo is a strong predictor of the implantation potential, and the presence of four MNBs predicts a significantly higher implantation rate compared with other markers, such as degree of equality of blastomeres and rate of fragmentation. Further, visualization of four MNBs predicted almost twice as high an implantation rate as a finding that not all blastomeres showed a nucleus. We therefore no longer consider a day-2 four-cell preembryo as a top quality embryo unless all four blastomeres are mononucleate. However, caution should also be observed when considering DET with two four-cell/0–3 nucleipreembryos, because such preembryos still have a fairly high potential to implant (22%).

We have recently found that, after the new legislation on SET in our country, it has been possible to increase the SET rate from 20% to 25% to approximately 70% in our clinic and still maintain an overall delivery rate of approximately 30%. As a consequence of the decrees, the twinning rate was reduced from 23% to 6% (18). The legislation restricting ET to a single embryo put pressure on our ability to select the most viable embryo for transfer; as mentioned, MNBs have since long been our prime criterion for selection of a day-2 preembryo in the four-cell stage. True data on the fate of an embryo with particular characteristics can only be obtained after transfer of a single embryo.

Blastocyst transfers have resulted in high success rates, with pregnancy rates of 50% to 60% in selected patients even after transfer of only one blastocyst (19–21). However, culture of preembryos to the blastocyst stage before ET also has disadvantages. Obviously it is more time-consuming and makes more demands on staff, and therefore is more costly. Blastocyst transfer has also been associated with an increased risk of monozygotic twinning (22). Further, a slight increase in the number of patients who do not have an embryo available for transfer has been noted (23). Moreover, only selected groups of patients may benefit from blastocyst transfer. Transfers at an early cleavage stage have therefore not completely been abandoned, even in units with a high rate of blastocyst transfers. Meta-analyses have so far failed to establish a significant difference in outcome between early cleavage stage and blastocyst transfers (24). Apart from these aspects, many units have hesitated to shift to blastocyst transfer in spite of reports of good results; for various reasons, such as convenience or storage problems, they have adhered to the more established day-2 or day-3 transfers.

Only a few studies have addressed the value of single nucleation of blastomeres in preembryos (6, 9). In a study by Palmstierna et al. (9), no consideration was paid to the question of how many blastomeres in a preembryo contained a single nucleus. In a study by Moriwaki et al. (6), a mixture of cleavage stage preembryos, in unknown proportions and with various characteristics, was included; furthermore, most of the transfers were DET, which makes it difficult to acer-

TABLE 4

<table>
<thead>
<tr>
<th>Details of 861 embryo transfer cycles in which a single four-cell preembryo was transferred.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A</strong></td>
</tr>
<tr>
<td>n = 665</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
</tr>
<tr>
<td>No. of oocytes</td>
</tr>
<tr>
<td>No. of fertilized oocytes</td>
</tr>
<tr>
<td>Women’s age</td>
</tr>
<tr>
<td>Women’s body mass index</td>
</tr>
<tr>
<td>Years of infertility</td>
</tr>
</tbody>
</table>

Note: Group A comprises patients in whom a single four-cell preembryo with four mononucleate blastomeres (MNBs) was transferred; Group B comprises patients in whom a single four-cell preembryo with 0–3 MNBs was transferred. Values are mean ± SD.

Statistical analysis with the Mann-Whitney U test.


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tain which preembryo implanted resulted in successful singleton pregnancies. In the latter study, a single nucleus in all blastomeres in day-2 preembryos was highlighted as an important criterion when an embryo or embryos were selected for transfer. Our study is exclusively a SET study, and data from such a study have a stronger impact than those from DET studies in which mixtures of preembryos in various cleavage stages have been transferred.

Evaluation of the nuclear status in a four-cell preembryo can be a swift, straightforward procedure, although there are some potential pitfalls. A nucleus can sometimes be difficult to view because of obscuring fragments. Rolling the embryo on the bottom of the Petri dish usually solves the problem of insufficient visualization of a cell. Further, a blastomere situated beneath another blastomere may be obscured from proper viewing. Alteration of the depth in focus is mostly sufficient to view a nucleus in a cell. Another obstacle in judging is the presence of vacuoles, which can resemble nuclei. However, a nucleus in the resting stage contains nucleoli, whereas a vacuole, which is more transparent than a nucleus, does not. Micronuclei are sometimes present together with a full-sized nucleus. Also, a micronucleus has one or a few nucleoli as a sign of a nucleus and can therefore be detected.

It cannot be ruled out that asynchrony in appearance of a nucleus in blastomeres in an early cleavage-stage embryo is a natural event. However, it is possible that asynchrony in nuclear appearance in a four-cell preembryo is followed by asynchrony in further cleavage (i.e., to five, six, or seven cells instead of to an eight-cell preembryo approximately 24 hours later).

Inevitably, a shift to a greater proportion of SETs must take place in the near future because it is not defensible to have a 50% complication rate (multiples) in successful treatments after IVF (25). Transfer of a single embryo is the ultimate way to obtain true data on the fate of a preembryo, or categories of preembryos, with particular characteristics. Data from SET studies on a larger scale, as in this study on day-2 four-cell preembryos, will thus add to the power of judging the potential of a preembryo with particular characteristics to implant. We will have important knowledge that will be of value when counseling couples on the number of preembryos to be transferred.

Early embryo cleavage has been shown to be a strong indicator of a preembryo’s potential to implant (8). Early cleavage in combination with single nucleation might be an even better predictor than either of the two markers separately when selecting the potentially most viable embryo for transfer on day 2. Further, even if single blastocyst transfers are becoming more common, it might still be of value to make an assessment regarding both early cleavage and single nucleation on day 2 to evaluate whether such preembryos are the ones that produce the most viable blastocysts.

Our study has shown that synchronous appearance of a single nucleus in all blastomeres in a day-2 four-cell preembryo yields a statistically significant higher implantation rate compared with a four-cell preembryo with zero to three MNBs (asynchronous appearance of nuclei) and is thus an important marker to consider when selecting a preembryo for transfer.

REFERENCES