Optimal blastocyst transfer: the embryo and the endometrium

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Chapter 5

Quantitative grading of a human blastocyst: optimal inner cell mass size and shape

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Fertility and Sterility, 2001 76:1157-1167.
Abstract

Objective: To investigate the predictive value of quantitative measurements of blastocyst morphology on subsequent implantation rates following transfer.

Design: Prospective observational study.

Setting: Private ART center.

Patient(s): One hundred seventy four IVF patients receiving transfers of expanded blastocyst-stage embryos on day 5 (n = 112) or day 6 (n = 62) after oocyte retrieval.

Intervention(s): None.

Main Outcome Measure(s): Blastocyst diameter, number of trophectoderm cells, inner cell mass (ICM) size, ICM shape, implantation and pregnancy rates.

Result(s): Blastocyst diameter and trophectoderm cell numbers were unrelated to implantation rates. Day-5 expanded blastocysts with ICMs greater than 4,500 µm² implanted at a higher rate than those with smaller ICMs (55% vs. 31%). Day-5 expanded blastocysts with slightly oval ICMs implanted at a higher rate (58%) compared to those with either rounder ICMs (7%) or more elongated ICMs (33%). Implantation rates were highest (71%) for embryos with both optimal ICM size and shape. Pregnancy rates were higher for day 5 transfers of optimally shaped ICMs compared to day 5 transfers of optimally sized ICMs.

Conclusion(s): Quantitative measurements of the inner cell mass are highly indicative of blastocyst implantation potential. Blastocysts with relatively large and/or slightly oval inner cell masses are more likely to implant than other blastocysts.
INTRODUCTION

The practice of growing and transferring blastocyst-stage rather than younger embryos (day 3 post oocyte retrieval or earlier) has become more common in the field of IVF with the development of sequential culture media capable of maintaining healthy growth past the third day of development (1-4). The higher rates of implantation and pregnancy associated with the transfer of blastocyst-stage embryos compared to earlier cleavage stage embryos (2, 3, 5-7) have led many fertility centers to adopt this procedure for some or all of their IVF patients. Determination of characteristics of blastocyst stage embryos that are indicative of viability will further improve the ability to distinguish those embryos more likely to implant. Improvements in the ability to identify the most viable embryos will allow high pregnancy rates to be maintained with the transfer of fewer embryos (ideally only one), reducing the potential for multiple pregnancies.

A variety of characteristics are known to be indicative of the viability of human embryos that are no more than three days old. First polar body morphology (8), pronuclear morphology (9-11), cleavage rate (12-19), blastomere size and shape (15, 17), cellular fragmentation (13, 15, 17, 18, 20), blastomere multinucleation (21-23), and zona thickness variation (22) are among the features which have been shown to be related to implantation rates for IVF embryos transferred within three days. Implantation rates as high as 40-50% have been achieved with transfers of "top quality" day 3 embryos (24, 25), as defined by a combination of several different morphological criteria (rapid cleavage rate, low fragmentation, and lack of multinucleated blastomeres), each of which has individually been
shown to be predictive of implantation. In addition, metabolic measurements such as pyruvate uptake by embryos (26) and production of platelet-activating factor (27, 28) have been shown to be related to cleavage-stage embryo viability.

The higher degree of differentiation of blastocysts, and the fact that the newly formed embryonic genome has only just begun to function by day 3 (29), suggest that features not visible until the blastocyst stage may be more useful in evaluating viability than features of day 3 embryos. However, with the exception of the well-documented relationship between development rates and implantation rates (1, 30-36), relatively little is known about traits specific to blastocyst-stage embryos that are indicative of implantation potential. Implantation rates are higher for transfers of hatching compared to non-hatching blastocysts (37, 38). Necrotic areas within blastocysts (39), and in particular within the inner cell mass (37), have been reported to be associated with poor implantation potential. One study demonstrates that grading of zygote pronuclear morphology may improve the ability to select viable embryos at the blastocyst-stage (40).

A study of mouse embryos suggests that cell numbers (particularly of the inner cell mass (ICM)) may be an important indicator of viability (41), but nondestructive assessment of cell numbers in living embryos is problematic. Other studies involving non-human embryos suggest a potential value of metabolic assessments of blastocyst viability, including glucose uptake (42, 43) and glycolytic activity (44). While promising, the value of metabolic measures such as these for assessing the quality of human blastocysts has yet to be demonstrated.
A recent attempt to grade human blastocysts combines qualitative grading of the
degree of blastocyst expansion and trichotomous qualitative assessments of both
the ICM and the trophectoderm (45). The ICM and trophectoderm scores used in
this system are vague and poorly defined, making this scoring system difficult to
replicate. And unfortunately, as we have noted previously (36), the contributions
of the ICM and trophectoderm grades in this study are impossible to evaluate.
Observed differences in implantation rates between their "top-quality" and "non-
top-quality" blastocyst transfers were consistent with differences in implantation
rates of embryos graded solely on the basis of developmental stage at the time of
transfer, suggesting that inclusion of ICM and trophectoderm grades may not have
improved the ability to identify viable embryos. Other studies suggest that the
morphology of both human (46) and non-human (47, 48) blastocyst-stage
embryos is related to viability, but also use grading systems that combine multiple
subjective criteria, preventing identification of the specific factors that reflect
developmental potential.

We suggest that the combination of multiple assessment factors into a single
grading system, without first demonstrating the importance of each factor
independently, is counterproductive and potentially misleading. Multi-factor
grading systems can obscure the relative importance of individual characteristics.
Without separate analysis of individual components of the grading system it is not
possible to determine which components contribute to the predictive ability of the
system, and to what extent. The inclusion of factors that do not significantly
increase the ability to identify viable embryos results in wasted time and effort
collecting unnecessary information, and can even introduce meaningless variation into the grading system, decreasing its accuracy.

In the current study we attempted to define some characteristics of viable blastocysts using quantitative measurements of various morphological features. A blastocyst is composed of a spherical layer of outer cells, the trophectoderm, surrounding an inner blastocoel cavity containing a tightly packed mass of cells, the inner cell mass. We analyzed quantitative measurements of blastocyst diameter, trophectoderm cell number, and ICM size and shape, collected prospectively for this purpose, in relation to implantation and pregnancy rates following embryo transfers. We first assessed the predictive value of each morphological measure independently, then combined features with significant predictive value into a unified grading system.

MATERIALS AND METHODS

Patients

Data from patients undergoing IVF-ET at a private assisted reproductive technology center between January 1999 and December 2000 were analyzed. During this time, all IVF embryos were cultured to the blastocyst stage before being transferred to patients. All patients being treated during this time period were included in the analysis, except for patients receiving thawed cryopreserved embryos.
For the main analysis of blastocyst morphology and implantation rates, data were limited to transfers of blastocysts that expanded on day 5 to eliminate variability in embryo viability associated with developmental rate. The more limited data from transfers of blastocysts that expanded on day 6 were examined separately in the context of patterns detected in the analysis of the blastocysts that expanded on day 5.

Patients receiving transfers of embryos derived from donated oocytes were included in the analysis. Oocyte donors were 33 years of age or younger, in good health, and had no prior evidence of infertility.

Data from only the first transfer were considered for patients undergoing more than one ET, and only one cycle of oocyte donation was included for egg donors undergoing multiple retrievals.

Institutional review board approval was not required due to the observational nature of this study.
After informed consent, patients or oocyte donors were stimulated with menotropins following pituitary down regulation with Lupron until at least 2 follicles had attained a mean diameter of 18 millimeters. Oocyte retrieval was performed 34-36 hours after hCG was administered at a dose of 5,000-10,000 U. Fertilization was performed by conventional insemination (or by ICSI for two embryos) 3-6 hours after oocyte retrieval in IVC-1 media (InVitroCare, San Diego, California) or in a few cases P-1 media (Irvine Scientific, Irvine, California). At 20-24 hours post retrieval normal fertilization was confirmed by the presence of two pronuclei.

Embryos were cultured in IVC-1 for the first three days and IVC-3 (InVitroCare) beginning on day 4, or in a few cases P-1 for the first three days and Blastocyst media (Irvine Scientific) beginning on day 4. All media was supplemented with 15% Human Serum Albumin (InVitroCare). Embryos were examined at 24-hour intervals until transferred. Characteristics including cell number, developmental stage, and amount of fragmentation were recorded for each embryo. Embryos were cultured in groups of two to four per 50 µl microdrop according to similarities in cell number and morphology, or occasionally individually when similar embryos were not present. Microdrops were overlaid with mineral oil (Sigma, Saint Louis, MO).
Embryo Transfer

Only blastocyst stage (at least cavitating) embryos were transferred to patients, and transfers were not conducted until at least one blastocyst expanded sufficiently to identify and measure a distinct inner cell mass within a well-developed blastocoel filling the embryo. One to three blastocysts (or four to a few poor-prognosis day 6 transfer patients) were transferred per patient, depending on the number and quality of the embryos available and patient preferences. Transfers of more than two blastocysts were conducted at the request of patients only after patients were informed about the potential and risks of multiple pregnancy. Clinical pregnancies, and the numbers of implantations, were determined by detection of fetal heart motion by transvaginal ultrasound examination at 6-8 weeks gestation.

Blastocyst Measurements

Prior to transfer, quantitative measurements of morphological features of all expanded blastocysts were taken at 400x magnification with the use of an ocular micrometer. Blastocyst diameter (from outer zona to outer zona) was recorded, along with the longest length and widest perpendicular width of each inner cell mass (µm). In addition, the number of trophectoderm cells in a cross-sectional circumference of each expanded blastocyst was recorded. The same observer (BSS) made all blastocyst measurements. A single size measurement (µm²) for each ICM was calculated by multiplying the length and width measurements. Inner cell mass shape was quantified by calculating a "Roundness Index" (RI =
length divided by width). Thus, an ICM with an RI of 1 would be perfectly round, while larger RIs would indicate progressively more elongated ICMs.

Statistical Analysis

Statistical analysis of embryonic features and their association with embryonic viability is complicated by the tendency to transfer embryos in groups rather than individually. Multiple embryo transfer is the norm in order to achieve acceptable pregnancy rates, because only one-third or fewer of transferred embryos can ordinarily be expected to implant. Pregnancies often result from the implantation of a subset, rather than all, of the embryos transferred, making unambiguous identification of implanting embryos impossible. Two different methods, the first using only those embryos for which the outcome was known and the second using data from all transferred embryos, were therefore used to examine the relationship between ICM measurements and implantation rates.

Optimal characteristics of blastocysts were identified by examination of the subset of embryos for which success or failure to implant could be determined unambiguously. Unambiguous determinations were possible only for transfers in which either all or none of the embryos transferred implanted. Differences in blastocyst measurements between embryos known to implant and those known not to have implanted were examined by t-test (for blastocyst size, ICM size and trophectoderm cell number) or Mann-Whitney U-test (for ICM shape). Chi-square comparisons of implantation rates were made between blastocysts grouped within discrete ranges of the measured characteristics.
The second method involved the use of multiple linear regression techniques to estimate implantation rates for the optimal and sub-optimal blastocyst grades defined in the previous analysis of known-outcome embryos. Multiple regression analysis allowed for the inclusion of data on all embryos transferred to all patients, enabling a much more comprehensive analysis of available data.

An additional advantage of the multiple regression analysis used here is that it reduces the downward bias in the estimation of embryo implantation potential that results from variations in embryo transfer efficiency and endometrial receptivity. Some proportion of viable embryos fail to implant as a result of transfer inefficiency or because they are transferred to a uterus that is non-receptive. Inclusion of these cycles therefore causes underestimation of embryo implantation potential. The proportion of failures due to inefficient embryo transfer and poor receptivity is unknown and difficult to determine, making this bias difficult to correct. However, all patients with implantation of some, but not all, of the embryos transferred to them clearly received effective transfers and were receptive. Therefore, the addition of these patients to the analysis reduces the proportion of cycles that failed because of factors unrelated to embryo quality and the downward bias in implantation rates caused by inclusion of such cycles.

For multiple linear regression analyses, embryos were grouped into optimal or sub-optimal categories according to the results of analyses of known-outcome embryos. In all multiple regression analyses, blastocysts that were transferred before expanding were included as an additional group, so that all transferred
embryos were accounted for. The numbers of embryos in each category transferred to each patient were used as independent variables, with the number of subsequent implantations being the dependent variable. Intercepts were set at zero because pregnancy could not occur if no embryos were transferred. The resulting partial regression coefficients give the number of units change in the criterion variable (i.e., the number of implanted embryos) given a one unit change in each predictor variable (i.e., the number of embryos transferred in each category). Thus these regression coefficients were estimates of the implantation rates for each group of embryos.

Pregnancy and multiple pregnancy rates were calculated among patients grouped according to optimal or sub-optimal characteristics of transferred embryos. Pregnancy and multiple pregnancy rates were compared between groups by chi-square analysis.

RESULTS

Data from 112 IVF patients undergoing transfer of expanded blastocysts on day 5 (26 using donated oocytes) were analyzed. The mean (± SD) age of patients using their own oocytes was 33.1 ± 4.3 years (range = 24 to 41). Patients using donated oocytes were aged 39.3 ± 4.7 years (range = 28 to 46). A total of 270 blastocysts were transferred (mean = 2.4 per patient). Sixty-five pregnancies (58% of patients) resulted from the implantation of 107 embryos (39.6% of embryos transferred). Pregnancy rates per transfer were higher among the patients using donated oocytes, but not significantly so (69% vs. 55%, p = 0.19).
All except 18 of the blastocysts transferred on day 5 were expanded at the time of transfer. Expanded blastocyst diameter ranged from 155 to 265 µm (mean ± SD = 194 ± 18). The number of trophectoderm cells in a cross-sectional circumference ranged from 4 to 20 (mean ± SD = 10.8 ± 2.6). Inner cell mass size, calculated as the product of the longest length and widest perpendicular width, ranged from 1,050 to 15,000 µm² (Fig. 1A, mean ± SD = 4,458 ± 1,667). Most ICMs were between 2,000 and 6,000 µm². Inner cell mass shape, as quantified by the “Roundness Index” (RI = length divided by width) ranged from 1 to 2.24 (median = 1.19), with most being below 1.4 (Fig. 1B).

The few blastocysts grown in P-1/Blastocyst media were morphologically similar to blastocysts cultured in IVC-1/IVC-3 media. Blastocysts cultured from donated oocytes were morphologically similar to those cultured from the oocytes of patients themselves. There were no significant differences in blastocyst diameter, trophectoderm cell number, ICM size or ICM shape between these different groups of embryos (Table 5-1). Embryos from all sources were therefore pooled for the remainder of the analyses.
Table 5-1. Morphological features of expanded blastocysts transferred on day 5, compared between media types and between patients who did and did not use donated oocytes.

<table>
<thead>
<tr>
<th>Sequential media type</th>
<th>Source of oocytes</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICV-1/IVC-3</td>
<td>P-1/Blastocyst</td>
<td>P-value</td>
<td>Donor</td>
<td>Patient</td>
<td>P-value</td>
</tr>
<tr>
<td>Number of blastocysts</td>
<td>238</td>
<td>14</td>
<td></td>
<td>64</td>
<td>188</td>
<td>0.59</td>
</tr>
<tr>
<td>Blastocyst diameter (µm)²</td>
<td>194 ± 18</td>
<td>193 ± 16</td>
<td>0.79</td>
<td>193 ± 17</td>
<td>194 ± 18</td>
<td>0.59</td>
</tr>
<tr>
<td>Trophoderm cell number²</td>
<td>10.8 ± 2.6</td>
<td>10.2 ± 1.6</td>
<td>0.60</td>
<td>11.1 ± 2.4</td>
<td>10.6 ± 2.6</td>
<td>0.22</td>
</tr>
<tr>
<td>ICM size (µm²)²</td>
<td>4,454 ± 1,653</td>
<td>4,532 ± 1,960</td>
<td>0.86</td>
<td>4,293 ± 1,319</td>
<td>4,514 ± 1,769</td>
<td>0.36</td>
</tr>
<tr>
<td>ICM shape (RI)²</td>
<td>1.19</td>
<td>1.22</td>
<td>0.84</td>
<td>1.16</td>
<td>1.20</td>
<td>0.26</td>
</tr>
</tbody>
</table>

² Values are means plus or minus one standard deviation
b Values are medians

Analysis of Known-Outcome Embryos (Day 5 Transfers)

The fate of 149 blastocysts transferred on day 5 could be determined with certainty, because they were transferred either to patients for whom all embryos implanted (n = 48 embryos (1 unexpanded) to 20 patients), or to patients for whom no embryos implanted (n = 101 embryos (9 unexpanded) to 47 patients).

The mean diameter of expanded blastocysts was nearly identical between implanting and non-implanting embryos (195 µm vs. 194 µm, p = 0.81). The number of trophoderm cells in a cross-sectional circumference was also nearly identical between implanting and non-implanting embryos (11.0 vs. 10.8, p = 0.64). Examination of implantation rates for embryos grouped according to either expanded blastocyst diameter or trophoderm cell numbers revealed no discernable patterns associated with either of these two variables.
The ICMs of implanting embryos were significantly larger than those of embryos failing to implant (5,023 µm² vs. 4,312 µm², p = 0.008). Logistic regression analysis revealed an approximately linear positive relationship between ICM size and implantation rates (Figure 2, p = 0.01). Implantation rates ranged from approximately 20% for 2,000 µm² ICMs to near 50% for ICMs 6,000 µm² or greater. A continuous model such as this is likely to be a more accurate description of the relationship between ICM size and implantation potential. However, for simplification we divided embryos into “large” and “small” ICM categories using a breakpoint which maximized the difference between these two groups.

Implantation rates were significantly higher among blastocysts with ICM measurements greater than 4,500 µm² compared to those with smaller ICMs (Figure 3A, 45% vs. 23%, p = 0.006). Optimal ICM size was therefore defined as “large”, measuring greater than 4,500 µm². Inner cell masses measuring less than 3,800 µm² were associated with especially low implantation rates (18%, p = 0.0028 vs. large ICMs). Blastocysts with ICMs falling between these “large” and “small” size ranges implanted at an intermediate rate of 32%, which was statistically indistinguishable from the rates for either the smaller or larger ICMs (p = 0.18 and p = 0.26, respectively).

A Mann-Whitney U test failed to detect any difference in ICM shape between implanting and non-implanting embryos (median RI = 1.26 vs. 1.29, p = 0.37). However, examination of implantation rates for embryos grouped according to
ICM shape revealed that implantation rates were highest for blastocysts with slightly oval-shaped ICMs (Figure 3B). Embryos with ICM RIs ranging from 1.04 and 1.20 implanted at a significantly higher rate than embryos with either rounder (RI < 1.04) ICMs or more elongated (RI > 1.20) ICMs (49% vs. 13% (p = 0.012) and 25% (p = 0.006)). Optimal ICM shape was therefore defined as "slightly oval", with an RI between 1.04 and 1.20, inclusive.

These "known-outcome" embryos were then grouped according to whether their ICMs fell within the optimal size and/or shape ranges (Figure 3C). "Top-quality" blastocysts with ICMs within both the optimal size and optimal shape ranges (OptS&S) implanted at a rate of 60%. Implantation rates were much lower for embryos with ICMs that were optimally sized (OptSize) only (29%, p = 0.011), optimally shaped (OptShape) only (32%, p = 0.038), or sub-optimally (SubOpt) sized and shaped (19%, p = 0.0001). The implantation rate of 10% for the blastocysts that were unexpanded at the time of transfer was also significantly lower than that of the top-quality embryos (p = 0.0053).

### Analysis of All Embryos Transferred on Day 5

More comprehensive multiple linear regression models, using data from all 270 embryos transferred to all 112 day 5 transfer patients, were used to calculate more accurate estimates of implantation rates for grades of blastocysts defined by the analysis of known-outcome embryos (Fig. 4). This procedure allowed the addition of 45 more patients, each pregnant by a subset of the embryos transferred to them (59 implantations from 121 transferred embryos). As discussed in Methods, multiple regression analysis reduces the downward bias in
estimated implantation rates caused by factors unrelated to embryo quality, resulting in a more accurate estimate of implantation potential.

By this method, implantation rates were estimated to be 55% for the optimally sized large ICMs, compared to 31% for smaller ICMs (Figure 4A). Implantation rates were 58% for optimally shaped ICMs compared to 7% for rounder ICMs and 33% for more elongated ICMs (Figure 4B). Blastocysts with OptS&S ICMs, within both the optimal size and optimal shape ranges, implanted at a rate of 71%, compared to 37% for OptSize ICMs, 45% for OptShape ICMs, and 22% for SubOpt ICMs (Figure 4C). The estimated implantation rate for unexpanded blastocysts was 15-22%.

A chi-square analysis revealed a weak but significant positive association between the development of optimal ICM size and optimal ICM shape among the 252 expanded blastocysts transferred on day 5 ($p = 0.039$). Embryos developing both optimal ICM size and optimal ICM shape, and embryos with neither optimal size nor shape, were both slightly more common than would be expected by chance if there was no link between size and shape (24.6% (n = 62) observed vs. 21.4% expected, and 32.1% (n = 81) vs. 28.9%, respectively). Conversely, embryos with optimal ICM size but not shape, and those with optimal ICM shape but not size, were both slightly less common than would be expected by chance if there was no link between size and shape (21.8% (n = 55) vs. 25.0% and 21.4% (n = 54) vs. 24.6%, respectively).
Pregnancy and multiple pregnancy rates also differed according to ICM grades (Table 5-2). Pregnancy rates were highest (79%) for patients receiving transfers including at least one blastocyst having an OptS&S ICM. Pregnancy rates were also very high for transfers including at least one OptShape ICM but no ICMs within the optimal size range (68%), and for transfers including one OptSize ICM and one OptShape ICM but no OptS&S ICMs (71%). Rates were much lower for transfers including OptSize ICMs but no ICMs within the optimal shape range (33%), and for transfers of all SubOpt ICMs (17%). Triples were significantly more common among patients receiving an OptS&S ICM, with all but one triplet pregnancy (90%) resulting from such transfers. One-third (9/27) of the three-embryo transfers including at least one OptS&S ICM resulted in triplicates.

Inner cell mass grades were not significantly related to the age of patients from whom oocytes were retrieved. Compared to other patients, mean patient age was slightly, but not significantly, lower for patients with at least one optimally sized ICM (33.0 vs. 33.3 years, \( p = 0.76 \)), one optimally shaped ICM (32.8 vs. 33.6, \( p = 0.44 \)), or one optimally sized and shaped ICM (32.2 vs. 33.8, \( p = 0.094 \)).
### Table 5-2. Pregnancy rates and numbers of embryos, according to the inner cell mass grades in cohorts transferred on day 5.

<table>
<thead>
<tr>
<th>Transfer type: ICM grades in cohorts of transferred blastocysts</th>
<th>Preganacies per transfer</th>
<th>Multiples per pregnancy</th>
<th>Triplets per pregnancy</th>
<th>Number of embryos per transfer (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: At least one OptS&amp;S</td>
<td>37/47 (78.7%)</td>
<td>19/37 (51.4%)</td>
<td>9/37 (24.3%)</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>B: No OptS&amp;S, but one OptSize and one OptShape</td>
<td>5/7 (71.4%)</td>
<td>4/5 (80.0%)</td>
<td>0/5 (0%)</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>C: At least one OptShape, no OptS&amp;S or OptSize</td>
<td>13/19 (68.4%)</td>
<td>5/13 (38.5%)</td>
<td>0/13 (0%)</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>D: At least one OptSize, no OptS&amp;S or OptShape</td>
<td>7/21 (33.3%)&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>3/7 (42.8%)</td>
<td>1/7 (14.3%)</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>E: All SubOpt</td>
<td>3/18 (16.7%)&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>1/3 (33.3%)</td>
<td>0/3 (0%)</td>
<td>1.7 ± 0.8&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.0005 vs. transfer type A  
<sup>b</sup> P = 0.078 vs. transfer type B  
<sup>c</sup> P < 0.03 vs. transfer type C  
<sup>d</sup> P = 0.0084 vs. transfer type B  
<sup>e</sup> P = 0.022 vs. pooled transfer types B, C, D and E  
<sup>f</sup> P < 0.0001 vs. pooled transfer types A, B, C and D

### Analysis of Embryos Transferred on Day 6

Sixty-two patients (mean age = 33.5 ± 4.7 years) received transfers of blastocysts that expanded six days after oocyte retrieval. These patients received 2.3 ± 0.9 blastocysts per transfer (four embryos each to four patients). The pregnancy rate for day 6 transfers was 39% (24/62), with implantation of 22% (32/142) of all transferred embryos.

Comparisons between blastocysts that expanded on day 5 and those that expanded on day 6 revealed significant differences in ICM morphology. Compared to ICMs of blastocysts that expanded on day 5, ICMs of blastocysts that expanded on day 6 were smaller (4,458 µm² vs. 3,891 µm², p = 0.0016) and more elongated (RI = 1.19 vs. 1.27, p = 0.0045).
Examination of implantation rates among categorized known-outcome embryos (n = 11 implanting and 75 non-implanting) suggested that a lower cutoff for differentiating between optimal and sub-optimal size might be appropriate for blastocysts expanding on day 6. The difference was clearest using a cutoff value of 3,800 µm². Blastocysts with ICMs larger than 3,800 µm² implanted more often than blastocysts with smaller ICMs (20% vs. 5%, p = 0.044). Sample sizes were too small to distinguish clear trends in implantation rates according to ICM shape.

The more comprehensive and accurate estimates of implantation potential based on multiple regression analysis of all embryos transferred on day 6 indicated implantation rates near 30% for day-6 blastocysts with ICMs larger than 3,800 µm² or with ICM RIs between 1.04 and 1.20. Blastocysts with smaller or more elongated ICMs implanted at rates of 21% and 23% respectively. Unexpanded blastocysts and those with ICM RIs below 1.04 did not significantly contribute to implantation rates. (Table 5-3)

Pregnancy rates for day 6 transfers were highest when embryo cohorts included at least one ICM of both optimal size and shape, or one optimally sized ICM and one optimally shaped ICM. The lowest pregnancy rates occurred among patients receiving cohorts of embryos without any optimally sized or shaped ICMs. (Table 5-4)
Table 5-3. Estimated implantation rates of blastocysts transferred on day 6 (based on multiple regression analysis) according to ICM size or shape grades.

<table>
<thead>
<tr>
<th>ICM grade</th>
<th>Number of embryos</th>
<th>Implantation rate (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large (optimal size), &gt; 3,800 µm²</td>
<td>64</td>
<td>29% (0.0005)</td>
</tr>
<tr>
<td>Small (sub-optimal size), &lt; 3,800 µm²</td>
<td>68</td>
<td>21% (0.0026)</td>
</tr>
<tr>
<td>Slightly oval (optimal shape), RI = 1.04 to 1.20</td>
<td>51</td>
<td>28% (0.0007)</td>
</tr>
<tr>
<td>Too oval (sub-optimal shape), RI &gt; 1.20</td>
<td>77</td>
<td>23% (0.0003)</td>
</tr>
<tr>
<td>Too round (sub-optimal shape), RI &lt; 1.04</td>
<td>4</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Unexpanded blastocysts</td>
<td>10</td>
<td>Not Significant</td>
</tr>
</tbody>
</table>

Table 5-4. Pregnancy rates according to inner cell mass grades in cohorts transferred on day 6.

<table>
<thead>
<tr>
<th>Transfer type: ICM grades in cohorts of transferred blastocysts</th>
<th>Pregnancies per transfer(^a)</th>
<th>Number of twins</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: At least one OptS&amp;S</td>
<td>11/25 (44%)</td>
<td>3</td>
</tr>
<tr>
<td>B: No OptS&amp;S, but one OptSize and one OptShape</td>
<td>5/6 (83%)</td>
<td>3</td>
</tr>
<tr>
<td>C: At least one OptShape, no OptS&amp;S or OptSize</td>
<td>1/4 (25%)</td>
<td>0</td>
</tr>
<tr>
<td>D: At least one OptSize, no Opt S&amp;S or OptShape</td>
<td>6/17 (35%)</td>
<td>1</td>
</tr>
<tr>
<td>E: All SubOpt</td>
<td>1/10 (10%)(^b)</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) Pooled transfer types A and B greater than others, \(p = 0.037\)

\(^b\) Less than all other transfer types pooled, \(p = 0.042\)
FIG. 5-1 Frequency histograms of ICM dimensions among blastocysts transferred on day 5: Size (A), calculated as the product of longest length and widest perpendicular width, in intervals of 1,000 µm²; and Shape (B), according to the Roundness Index (RI), calculated as length divided by width, in intervals of 0.1. Dark shading indicates embryos derived from patients’ oocytes, light shading indicates embryos derived from donated oocytes.
FIG. 5-2 Implantation rates among known-outcome blastocysts transferred on day 5, grouped into 500 µm² ICM size intervals (or 1,000 intervals below 2,000 µm² and above 7,000 µm²) plotted against the mean ICM size of the embryos in each group. Point size is proportional to the number of embryos represented. The trend line was determined by logistic regression analysis (p = 0.01).
FIG. 5-3 Implantation rates among known-outcome blastocysts transferred on day 5, grouped by ICM size (A), ICM shape (B), or both ICM size and shape (C). (A) ICM size: less than or equal to 4,500 µm² (n = 70) or greater than 4,500 µm² (n = 69). P = 0.006 for difference. (B) ICM shape: RI less than 1.04 (n = 15), RI from 1.04 to 1.20 (n = 57), or RI greater than 1.20 (n = 67). Implantation rates were significantly higher for slightly oval ICMs compared to either rounder ICMs (p = 0.012) or more elongated ICMs (p = 0.006). (C) ICM size and shape: optimal size and shape (OptS&S, n = 35), optimal size only (OptSize, n = 34), optimal shape only (OptShape, n = 22), or neither optimal size nor shape (SubOpt, n = 48). Implantation rates were significantly higher for OptS&S embryos compared to OptSize embryos (p = 0.011), OptShape embryos (p = 0.038) or SubOpt embryos (p = 0.0001).
FIG. 5-4 Estimated implantation rates of embryos transferred on day 5 (based on multiple linear regression analysis, with 95% confidence intervals) according to ICM size and/or shape categories: (A) Size groupings only: less than or equal to 4,500 µm² (n = 136) or greater than 4,500 µm² (n = 116); (B) Shape groupings only: RI less than 1.04 (n = 21), RI from 1.04 to 1.20 (n = 117) or RI greater than 1.20 (n = 114); or (C) Combined size and shape groupings: OptS&S (n = 62), OptSize (n = 54), OptShape (n = 55), or SubOpt (n = 81).
FIG. 5-5 Graphic representation of the length-to-width proportions associated with representative values of RI. Dark shading indicates "optimal" shape, and light shading "sub-optimal" shape.
This study is the first to our knowledge to examine the relationship between quantitative measurements of human blastocyst morphology and an embryo’s potential for implantation after transfer. The results demonstrate a strong relationship between the size and shape of the inner cell mass of a human blastocyst and its viability.

The relationship between ICM dimensions and viability is not surprising, given that the ICM represents the group of cells destined to grow into the fetus. We expected above average viability among embryos with larger ICMs, as large ICM size is indicative of vigorous growth. There was a nearly linear increase in implantation rates with increasing ICM size. For grading purposes, the relationship was simplified using a breakpoint to define optimal "large" ICMs and sub-optimal "small" ICMs. Blastocysts with ICM length*width measurements greater than 4,500 µm² implanted at nearly twice the rate of embryos with smaller ICMs. This relationship is consistent with a study of mouse embryos that showed a positive relationship between the number of ICM cells and the rate of embryo implantation (41).

The importance of subtle differences in ICM shape (Fig. 5) is less intuitive, and very intriguing. Blastocysts with slightly oval ICMs (RI = 1.04 to 1.20) implanted at approximately twice the rate of blastocysts with ICMs outside this optimal range. Apparently, even in the earliest stages of human development, shape is a highly constrained characteristic of the inner cell mass. At only five days old, at the first visible signs of cell differentiation, embryonic cells destined to grow into a fetus
(and eventually an adult human) have already begun to show signs of the elongated (although only very slightly at this point) bilaterally symmetrical form into which they will develop. On a practical level, the very subtle difference between an ICM with optimal shape and one with sub-optimal shape (Fig. 5) emphasizes the importance of careful quantitative, rather than just qualitative, observations of embryos in the IVF lab. Given the surprisingly good predictive value of a simple two-dimensional measure of shape demonstrated here, emerging technologies in three-dimensional imaging and measurement may prove to be valuable tools for evaluating blastocyst quality in the future.

The other blastocyst characteristics that were measured did not appear to be related to implantation rates. Blastocyst diameter did not differ between implanting and non-implanting embryos. Trophectoderm cell numbers were also similar for implanting and non-implanting blastocysts. The latter result is consistent with a study of mouse embryos showing no relationship between the number of trophectoderm cells and implantation rates (41). This lack of association between trophectoderm cell numbers and implantation rates casts doubt on the appropriateness of inclusion of the trophectoderm grade in a recently reported multi-factor blastocyst scoring system (45).

The lack of association between trophectoderm measurements and implantation is surprising given that it is the trophectoderm that forms the initial connection to the uterine wall and develops into the placenta and associated tissues supporting embryonic development. It may be that the sample sizes used here were insufficient to detect existing relationships. If there is a relationship between
trophectoderm cell numbers and implantation potential it is weak, and not likely to be clinically significant. Although cell number does not appear to be a critical feature of the trophectoderm layer, it is possible that some as yet undetermined characteristic of the trophectoderm layer could be indicative of blastocyst viability and implantation potential. Such possibilities require further study.

There was a significant positive association between the development of optimal ICM size and optimal ICM shape, suggesting that these two features of the inner cell mass are (loosely) linked. Embryos with one of these features, either optimal ICM shape or size, are more likely to have the other, although the association is very weak. Many blastocysts develop ICMs of either optimal size or optimal shape, but not both.

Blastocyst grades based on a combination of the two features shown here to be associated with blastocyst viability, ICM size and shape, revealed that "top-quality" blastocysts (those expanding on day 5 and having ICMs within both the optimal size and optimal shape categories) implanted at a significantly higher rate than all other categories of embryos. Multiple regression analysis including all patients receiving day 5 transfers indicated implantation rates of approximately 71% for these top-quality embryos. Although we could not confirm the significance of differences in implantation rates among other categories of embryos, it seems reasonable to expect embryos with either optimal ICM size or optimal ICM shape to implant at a higher rate than embryos with sub-optimal ICM size and shape. Implantation rates for day 5 expanded blastocysts with optimal ICM size only were estimated to be 37%. Implantation rates for day 5 expanded blastocysts with optimal ICM shape only were estimated to be 45%.
Day 5 expanded blastocysts having ICMs sub-optimal for both ICM size and shape were estimated to implant at a rate of 22%. Estimated implantation rates for blastocysts that had not expanded by transfer on day 5 were also relatively low (22%). This result is consistent with a variety of studies indicating that the rate of development to the expanded blastocyst stage is indicative of viability (1, 30-36).

Comparisons of pregnancy rates according to the ICM size/shape grades of embryos transferred on day 5 revealed that the highest pregnancy rates (79%) occurred when at least one top-quality embryo (with optimal ICM size and shape) was transferred. Pregnancy rates were also very high (69%), and not significantly different from those resulting from transfers of top-quality embryos, when transfers included at least one embryo with optimal ICM shape. Pregnancy rates were significantly lower (33%) for transfers including blastocysts with optimal ICM size but without any embryos having optimal ICM shape, suggesting that ICM shape may be a more important indicator of blastocyst viability than ICM size. Pregnancy rates were especially low (17%) among patients receiving no embryos with either optimal ICM size or shape.

Top quality ICMs, as defined here, were not uncommon among the group of IVF patients examined in this study. Twenty-five percent of all expanded blastocysts transferred on day 5 had optimally sized and shaped ICMs. Forty-two percent of the patients undergoing day 5 embryo transfers received at least one of these top-quality embryos.
Sample sizes and implantation rates of blastocysts expanding on day 6 after oocyte retrieval were relatively low, limiting the potential for detecting differences in implantation rates associated with morphology. However, preliminary analysis of implantation rates for these embryos, in the context of the optimal morphology characteristics defined for blastocysts expanding on day 5, suggest that similar patterns may exist for day 6 expanded blastocysts. Day 6 expanded blastocysts with larger ICMs implanted at a significantly higher rate than those with smaller ICMs. Blastocysts with ICMs within the defined optimal ICM shape range of 1.04 to 1.20 implanted at a higher rate than other embryos, although they could not clearly be statistically distinguished from other embryos.

We have previously reported that blastocysts not expanding until day 6 after oocyte retrieval implant at approximately half the rate of blastocysts expanding on day 5. The current study revealed significant differences in blastocyst morphology between those expanding on day 5 and those expanding on day 6. The ICMs of day 6 expanded blastocysts were significantly smaller and more elongated (with a median outside the optimal shape range) compared to those of day 5 expanded blastocysts. These differences suggest that the reduced viability of day 6 expanded blastocysts is reflected not only in their slower rate of development, but also in poorer morphology at the expanded blastocyst stage.

Interestingly, there appears to be little if any decline in inner cell mass quality (as determined by size and shape) with age. The very slight tendency toward younger ages among the patients producing embryos with optimally sized and/or shaped ICMs was not significant. This suggests that a decline in the quality of the
inner cell mass may not be one of the components contributing to the decline in fertility with age.

The relationship between the inner cell mass dimensions and implantation potential will enable physicians to more accurately predict the implantation potential of individual IVF embryos before they are transferred, and adjust the number of embryos transferred accordingly. The high rate of implantation associated with the transfer of blastocysts having both optimal ICM size and optimal ICM shape could help physicians realize their long sought-after goal of achieving high pregnancy rates with single embryo transfers, at least for those patients producing such embryos. Transferring more than two embryos to such patients introduces a high risk of high-order multiples. Single embryo transfers could eliminate the increasingly common problem of multiple pregnancies that has resulted from the growing use of assisted reproduction. Failure of embryos with top-quality ICMs to implant may also have diagnostic value, in that it may indicate uterine factors or transfer inefficiency preventing pregnancy.

In conclusion, this study provides the first evidence of significant relationships between both inner cell mass size and inner cell mass shape prior to transfer and the implantation potential of blastocyst-stage human embryos. Inner cell mass size and shape are therefore important new variables to be considered when determining which and how many blastocyst-stage embryos to transfer in an effort to maximize pregnancy rates while minimizing the potential for multiples. As more independent indicators of blastocyst viability are identified, these can be incorporated into progressively more complex and effective grading systems.
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