Embryo evaluation by analysing blastomere nuclei

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BACKGROUND: To create a more effective selection standard for early embryos, we developed a new grading system consisting of conventional morphological evaluation in combination with analysis of blastomere nuclei.

METHODS: A total of 744 embryos used during 459 cycles of embryo transfer on day 2 and blastocyst transfer were subjected to retrospective analysis. The overall implantation rate was 15.5% (115/744). Morphological evaluation of the embryos was performed on day 2 by referring to both the size of blastomere and fragmentation (conventional method) and the nucleic features of the blastomeres—either multinucleated or anucleic (nuclei counting method). The implantation rate for every transferred embryo and blastocyst was examined. RESULTS: Although a high implantation rate was observed with the highest quality embryos as judged by either the conventional method (24.1%; 57/237) or the nuclei counting method (26.1%; 104/399), the nuclei counting method predicted implantation rate better than the conventional method. The embryos that were considered to be high quality according to the conventional method, but low quality according to the nuclei counting method, had a limited implantation success rate of 6.3% (4/66). Also, after blastocyst transfer, implantation occurred most often when high quality embryos evaluated by the nuclei counting method were used (25.5%; 25/98), while the blastocysts from low quality embryos seldom implanted (3.2%; 2/63). CONCLUSIONS: When choosing which embryo to transfer, the normality of blastomere nuclei may be a more important index of quality than standard fragmentation features and/or blastomere uniformity analysis. When choosing among embryos, if nucleic status is identical, then embryos with the least fragmentation should be chosen. Moreover, in blastocyst transfer, a blastocyst whose nuclei were judged normal on day 2 should be selected on day 5 over any other blastocysts.

Key words: implantation rate/IVF/multinucleated blastomere/nuclei counting/selection of embryo

Introduction

Obtaining embryos capable of implantation and conception is critical for successful IVF and embryo transfer. When high quality embryos can be selected and transferred, pregnancy rates increase and multiple pregnancy can be avoided through restrictions on the number of embryos transferred. At present, morphological standards for the evaluation of embryos in IVF are made independently at each reproductive institute, following commonly accepted guidelines. Although the degree of fragmentation, blastomere formation and cell number in early stage embryos are thought to be important markers, these determinations do not perfectly predict whether zygotes will implant or not.

Blastocyst transfer is a useful method for selecting good quality embryos, with the follow-on benefit of fewer transferred embryos and reduced incidence of multiple pregnancies. Gardner et al. (2000) reported pregnancy rates >60% for top-scoring embryos used in blastocyst transfer. However, other studies indicated that the overall pregnancy rates in IVF did not improve with blastocyst transfer because transfer cancellations increased (Coskun et al., 2000; Vlaisavljevic et al., 2001; Karaki et al., 2002; Lundqvist et al., 2002). Additionally, some reports showed an increased incidence of monozygotic twins after blastocyst transfer (Behr et al., 2000; da Costa et al., 2001; Sheiner et al., 2001; Ménézo and Sakkas, 2002; Tarlatzis et al., 2002). Therefore, the risk of multiple pregnancy remains even if blastocyst transfer is performed.

Moreover, Scholtes and Zeilmaker (1998) reported that 381 of 929 candidate embryos for blastocyst transfer (41%) did not reach blastocyst stage. They also indicated that implantation rate per blastocyst was 23% in the first blastocyst transfer cycle, which was similar to regular early stage embryo transfer cycles. Royen et al. (1999) reported that the implantation rate was 49% when two top quality embryos (judged as having four or five blastomeres on day 2 and seven or more on day 3; ≤20% fragmentation on day 3) were transferred on day 3. They indicated that the implantation rate seemed to be similar to the blastocyst transfer rate reported by Gardner et al. (2000),

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implying that early embryo transfer is more useful than blastocyst transfer given the advantages of a shorter culture time and, by extension, less cost.

The common point in the reports of Gardner et al. (1998) and Royen et al. (1999) is that a single embryo transfer using a top quality embryo in an early embryonic stage is beneficial, proving a high implantation rate and avoiding multiple pregnancy risk. When the number of transferred embryos can be reduced to one without lowering the pregnancy rate, embryo transfer cost will decrease, and the incidence of cancellations will be reduced. Moreover, a single embryo transfer will leave a higher number of embryos per cycle available for cryopreservation (Neubourg et al., 2002), thereby potentially increasing the cumulative conception rate per patient.

Based on these analyses, effective morphological evaluation of early stage embryos is critical. Recently, some studies have indicated that analysis of blastomere nuclei can predict embryo quality. In particular, multinucleated blastomeres (MNB) are considered to be abnormal by some reports, because the possibility of chromosomal abnormality of MNB is high (Munne and Cohen, 1993; Pickering et al., 1995; Kligman et al., 1996). Analysis of blastomere nuclei using high-power inverted microscope revealed MNB in 14.5% of 1885 embryos obtained from 338 patients, and that 43% of patients had MNB embryos (Balakier and Cadesky, 1997; Jackson et al., 1998). Although normal childbirth sometimes resulted from embryo transfer with embryos having MNB (Balakier and Cadesky, 1997; Jackson et al., 1998), embryos with MNB may not be the best choice for embryo transfer given the risk of abnormality. Hence we conclude that looking for MNB is an important way to select the highest quality embryos for transfer.

In the present study, to create a more effective selection standard for early embryos, we developed a new embryo grading system, using both conventional morphological evaluation and blastomere nuclei analysis. The implantation rate of each grading method for standard embryo transfer and blastocyst transfer was examined retrospectively.

Materials and methods

Subjects

At our hospitals between August 1998 and December 2001, 611 oocyte retrievals for IVF were performed. In these cases, no oocytes were retrieved in 39 cycles, no fertilized oocytes were obtained in 97 cycles, and all fertilized oocytes were cryopreserved in 79 cycles. As a result, 908 embryos from 532 cycles (712 embryos in 397 embryo transfer cycles on day 2, and 196 embryos for 135 blastocyst transfers on day 5) were subjected to the combined conventional and nuclei counting method. Remaining embryos were cryopreserved on day 2 or cultured in sequential medium until day 5 after retrieval. The embryos were cultured in blastocyst medium (Irvine Scientific) with 10% SSS during day 3–day 5 after oocyte retrieval. One or two blastocyst(s) were selected and transferred in blastocyst transfer. The number of transferred blastocysts was 1.5 ± 0.5, the implantation rate was 16.8%, and the patient’s age was 33.9 ± 4.5 years old. Remaining blastocysts in good condition were cryopreserved on day 5.

Implantation was confirmed by the presence of gestational sac(s) using transvaginal ultrasound imaging. Chemical pregnancies indicating positive urinary hCG (>50 IU/l) alone were not counted as implantation in this study.

All procedures were performed after receiving informed consent from the patients, following the guidelines of the Japan Society of Obstetrics and Gynecology, and had the permission of the ethics committee of the each hospital.

Morphological grading of embryos

Morphological embryo evaluation was performed on day 2 after retrieval (43–48 h after insemination or ICSI) using an inverted microscope (Olympus IX70; Olympus Electric Industry Co., Japan) at ×300–600 magnification (Figure 1). The number of blastomeres in embryos ranged between two and eight. The embryos were classified into four morphological grades in accordance with our conventional criteria (Kondo et al., 1996) consisting of blastomere size and the amount of anucleate fragmentation (conventional method): grade 1 (g1), blastomere uniform in size and shape and little or no fragmentation; grade 2 (g2), blastomeres uneven in size and shape and/or fragmentation <10% of the embryonic surface; grade 3 (g3), fragmentation of 10–30% of the embryonic surface; and grade 4 (g4), fragments >30% of the embryonic surface.

Next, embryos were classified according to the number of nuclei in each blastomere (nuclei counting method): grade A (gA) embryos having only mononucleated blastomeres; grade B (gB) embryos having one or more blastomeres containing no visible nucleus; and grade C (gC) embryos having one or more MNB. To observe the nuclei in each blastomere carefully, the embryo was rotated manually with a glass pipette.

Finally, all of the embryos were classified into 12 groups from g1A to g4C using a combination of the conventional and nuclei counting methods (combination method).
having one or more blastomeres containing no visible nucleus; and

The statistical analysis

day 2 were transferred on day 5.

Results

Conventional method

As shown in Table I, the implantation rate of each grade determined by the conventional method for embryo transfer, blastocyst transfer and both embryo transfer and blastocyst transfer cycles decreased significantly from the best grade (g1) to the worst (g4), except that the implantation of the blastocysts originated from g3 was higher than g2 in blastocyst transfer cycles. There was no significant difference in the patients’ age between the groups (g1: 33.3 ± 6.0; g2: 33.1 ± 4.4; g3: 33.4 ± 5.2; g4: 32.9 ± 5.0).

Nuclei counting method

In the nuclei counting method (Table II), the implantation rate in the gA was significantly higher than those in the gB and gC in embryo transfer, blastocyst transfer, and both embryo transfer and blastocyst transfer cycles. Successful implantation following blastocyst transfer occurred most often in the embryos from the gA group, while the blastocysts from the gB and gC groups seldom implanted. The age of the gC group (34.2 ± 5.1 years old) was significantly higher ($P < 0.01$) than that of the gA (32.9 ± 4.5) and gB (32.8 ± 4.6) groups.

Blastocysts that had been classified by the combination method on day 2 were transferred on day 5.

Statistical analysis

The $\chi^2$-test was used to compare implantation and blastulation rates. Student’s $t$-test was used to compare the ages between the groups. The statistical significance was defined as $P < 0.05$.

Table I. Implantation rate (%) of embryos evaluated by the conventional method

<table>
<thead>
<tr>
<th>Grade</th>
<th>ET cycles</th>
<th>BT cycles</th>
<th>Both ET and BT cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>g1</td>
<td>23.6 (43/182)*</td>
<td>25.5 (14/55)</td>
<td>34.1 (57/237)*</td>
</tr>
<tr>
<td>g2</td>
<td>16.0 (31/194)**</td>
<td>11.9 (7/59)</td>
<td>15.0 (38/253)**</td>
</tr>
<tr>
<td>g3</td>
<td>10.2 (12/118)**</td>
<td>15.6 (6/38)</td>
<td>11.5 (18/156)**</td>
</tr>
<tr>
<td>g4</td>
<td>2.2 (2/89)</td>
<td>0 (0/9)</td>
<td>2.0 (2/98)</td>
</tr>
</tbody>
</table>

Total 15.1 (88/583) 16.8 (27/161) 15.5 (115/744)

* $P < 0.01$ versus g3 and g4.
** $P < 0.01$ versus g4.
*** $P < 0.05$ versus g4.
$\dagger P < 0.05$ versus g2, and $P < 0.01$ versus g3 and g4.

ET = embryo transfer; BT = blastocyst transfer; g = embryo grade.

Table II. Implantation rate (%) of embryos evaluated by the nuclei counting method

<table>
<thead>
<tr>
<th>Grade</th>
<th>ET cycles</th>
<th>BT cycles</th>
<th>Both ET and BT cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>gA</td>
<td>26.2 (79/301)*</td>
<td>25.5 (25/98)*</td>
<td>26.1 (104/399)*</td>
</tr>
<tr>
<td>gB</td>
<td>3.9 (8/206)</td>
<td>4.8 (2/42)</td>
<td>4.0 (10/248)</td>
</tr>
<tr>
<td>gC</td>
<td>1.3 (1/76)</td>
<td>0 (0/21)</td>
<td>1.0 (1/97)</td>
</tr>
</tbody>
</table>

* $P < 0.01$ versus gB and gC.

ET = embryo transfer; BT = blastocyst transfer; g = embryo grade.

Blastulation rate

The relationship between grading by the nuclei counting method and blastulation rate was investigated using the embryos cultured to day 5 (Figure 2). The blastulation rate in gA (56.2%) was significantly higher than that in gB (33.9%) or gC (17.4%). Also, a significant difference in the arrest rate was found between the gA (21.9%) and the gB (48.2%) or gC (71.1%).

Combination method

The implantation rates of each grade in the combination method at embryo transfer on day 2 decreased in order from g1A to g4C along the evaluation with nuclei counting (g1A, g2A, g3A, g4A, g1B, ..., g3C, g4C), not along the conventional method (g1A, g1B, g1C, g2A, ..., g4B, g4C), except the implantation rate in g1C was better (but not statistically significant) than gB group (Table III). By comparing with the g1A, the implantation rates in the g1B and g1C, which are considered to be ‘high quality’ according to the conventional method but ‘low quality’ according to the nuclei counting method, had limited implantation success rates of 5.0% ($P < 0.01$) and 7.1% ($P = 0.059$) respectively. The same as in the g1 group, g2A had a significantly higher implantation rate than g2B ($P < 0.01$) and g2C ($P = 0.015$). Similarly, the implantation rate of 3A was higher than that of g3B ($P = 0.019$) or g3C ($P = 0.027$). On the contrary, within the gA group, no significant differences in the implantation rate were observed between the embryos evaluated as ‘high quality’ by the conventional method (g1A) and those as ‘lower quality’ by the conventional method (g2A ($P = 0.417$) or g3A ($P = 0.133$), except g4A ($P = 0.029$). Also within the gB or gC groups, no significant differences among the grades judged by the conventional method were found.

Figure 1. Embryos were classified into 12 groups from grade 1A to grade 4C using criteria consisting of the size of blastomeres, the amount of anucleate fragmentation and the nuclei number of blastomeres. The numbers and letters represent the following: (1) blastomere uniform in size and shape and little or no fragmentation; (2) blastomeres uneven in size and shape and/or fragmentation <10% of the embryonic surface; (3) fragmentation 10–30% of the embryonic surface; (4) fragments >30% of the embryonic surface; (A) embryos having only mononucleated blastomeres; (B) embryos having one or more blastomeres containing no visible nucleus; and (C) embryos having one or more multinucleated blastomeres.
These features of the implantation rates in the embryo transfer cycles were observed similarly when the blastocyst transfer cycle data were attached (Table III).

These findings indicate that the nuclei counting method may be more suitable for the evaluation of embryo quality than the conventional method.

Discussion

Although several reports indicate that MNB in early embryos is abnormal (Munne and Cohen, 1993; Pickering et al., 1995; Kligman et al., 1996), the clinical significance of MNB has not been fully assessed yet. In the present clinical study, we examined the implantation rate of embryos evaluated morphologically on day 2 in embryo transfer or blastocyst transfer cycles.

Our results show that the implantation rate decreased sequentially from g1 that is considered to be good using the conventional method, which reconfirms that the degree of fragmentation and blastomere uniformity are important markers for morphological evaluation of early stage embryos. On the other hand, in the nuclei counting method, the implantation rate of gA was high and those of gB and gC were very low. Thus, which evaluation method should be applied dominantly to select the embryos to transfer? To clarify this point, we developed and tested a new grading system where the two methods were combined (combination method). Using this method, we found that the implantation rates for each grade decreased in order from g1 that is considered to be good using the conventional method of morphological analysis (Table III). The embryos in g1B and g1C, which are considered to be ‘high quality’ according to the conventional method but ‘low quality’ according to the nuclei counting method, showed a low implantation rate. Moreover, the implantation of blastocysts rarely occurred outside the gA group (Table II), indicating that the rates of blastocyst formation and implantation in the gB and gC groups could be remarkably low even when these embryos are cultured longer. These results indicate that observations of nuclear features in the blastomeres of day 2 embryos can be important criteria for selecting the best embryos suitable to transfer.

Although the reason for the low implantation rate of embryos in the gC can be explained by the MNB presence, the low implantation rate of embryos in the gB which contained blastomeres with no visible nucleus is more noteworthy. Usually, when MNB is not observed, the embryo is judged to be normal, which means that gB embryos are considered to be normal. What, then, is the clinical significance of the anucleated blastomere? It is interesting that Palmstierna et al. (1998) reported that visible mononucleated blastomeres and zona pellucida thickness variation seemed to be strong predictors of pregnancy. Given that the development of blastomere nuclei involves distinct phases during which the nuclei are more or less visible, it is critical that observers using the nuclei counting method evaluate gB embryos often at consistent time intervals. When a nucleus is still unobservable,
the embryo may contain an anucleated blastomere. Further investigation is required to clarify the importance of cell cycle synchrony when mononucleated blastomeres are observed simultaneously.

Although it is well known that pregnancy rates in older women are low (Feldberg et al., 1990; Stolwijk et al., 1997), the causes of embryo quality deterioration are not fully documented. In the present study, older patients did not have much fragmentation in their embryos but had more multi-nucleation. These results suggest that an increase in MNB in the embryos may be a cause of deceased implantation rates in older women.

Choosing an embryo with the highest implantation potential is the key to both successful single embryo transfer and the prevention of multiple pregnancies. Based on this study, we found that when choosing an embryo for transfer, the normality of blastomere nuclei may be more important than conventional measures of fragmentation and/or blastomere uniformity. If blastomere nuclei normality is identical, then embryos with less fragmentation should be chosen. In cases of blastocyst transfer, a blastocyst whose nucleus was evaluated as normal on day 2 should be selected for transfer at day 5. Hence we recommended that all reproductive clinics consider using our combined evaluation method to increase their chances for success during IVF.

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References

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