The effect of pronuclear morphology on embryo quality parameters and blastocyst transfer outcome

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BACKGROUND: Embryo quality may be accurately assessed as early as the pronuclear zygote phase, as shown in recent studies. However, it is not known whether good quality zygotes are destined to become good quality cleavage stage embryos and blastocysts. METHODS: In this retrospective study, 86 intracytoplasmic sperm injection–embryo transfer cycles were studied where each available embryo was scored from the zygote until the blastocyst stage. Embryonic normality parameters such as pronuclear pattern, early cleavage, cleavage stage embryo grade, the presence of embryos with ≥8 cells on day 3 and blastocyst quality were recorded. Embryo transfer was undertaken at the blastocyst stage and the outcome was studied according to the pronuclear pattern exhibited by the zygotes. RESULTS: Embryos that showed an ideal pronuclear pattern (0 PN pattern) cleaved earlier and faster and resulted in better quality cleavage stage embryos and blastocysts. The incidence of blastocyst formation was 72% in zygotes showing a 0 PN pattern, compared with 12.7% in zygotes with double pronuclear abnormality. Higher implantation and pregnancy rates were obtained when at least one blastocyst derived from a 0 PN pattern zygote was included in the set of embryos to be transferred. CONCLUSIONS: Our results indicate that the pronuclear pattern of the zygote is closely related to blastocyst formation and quality. Blastocysts derived from 0 PN zygotes have a higher potential for implantation.

Key words: blastocyst/embryo quality/embryo transfer/zygote

Introduction

Selection of embryos endowed with the capacity to implant has been a difficult and often imprecise practice in assisted reproduction. Embryos have been selected for transfer based upon gross embryo morphology, rate of cell division, pronuclear (PN) morphology and progression to the blastocyst stage or karyotype normality. Unfortunately, none of the above selection criteria are accurate in terms of predicting pregnancy. Poor quality embryos are known to implant and yield multiple pregnancies, whereas conception might not occur despite the transfer of a batch of good quality embryos.

Tesarik and Greco have suggested that embryo quality can be assessed after fertilization and prior to cleavage using a PN scoring system (Tesarik and Greco, 1999). This is a single observation scoring system, utilizing parameters such as nucleoli size, number and distribution. In a retrospective analysis of their data the authors found a strong correlation between implantation and PN score. The Tesarik and Greco scoring system is more practical compared with the zygote grading system proposed by Scott and Smith (Scott and Smith, 1998). The latter was based on retrospective observations correlated with pregnancy and on previously published observations on zygote morphology (Wright et al., 1990). The authors used the zygote morphology grading system to prospectively select embryos for transfer and cryopreservation.

Embryo grading is based on blastomere morphology, cleavage stage and fragmentation and there appears to be a clear relationship between these parameters and implantation and pregnancy rates after IVF (Zeibe et al., 1997). Faster cleaving embryos tend to implant at a higher rate and selection based on cleavage speed has been shown to increase the efficiency of implantation.

Embryo transfer is performed 2, 3 or 5 days after oocyte retrieval. Delaying the transfer for one more day has been shown to increase implantation and pregnancy rates (Dawson et al., 1995). Prolonging the culture period allows for better selection of more advanced embryos or those not arresting, as laboratory assessment is undertaken after the expression of the embryonic genome. At this stage, paternal genetic factors that influence embryo viability will have also made their impact, which may aid selection of the genetically normal embryo. The efficiency of blastocyst transfer, however, has not been tested in randomized trials involving an unselective patient population.

Based on all of the information available, the clinician is faced with the difficult task of selecting the best embryo at
the most appropriate time to transfer. The correlation between parameters used to assess embryo quality and viability is not clearly established. The aim of the present study was to correlate all reported predictors of embryo quality and selection criteria from PN morphology up to the blastocyst grade.

Materials and Methods

Patients

The study involved couples who had undergone embryo transfer on day 5 and had PN morphology, early cleavage, cleavage stage embryo morphology, the presence of 8-cell cleavage stage embryos on day 3 and blastocyst grading recorded for each embryo. Retrospective analysis of this data was performed for 86 intracytoplasmic sperm injection (ICSI) cycles performed for male factor infertility.

Ovarian stimulation, oocyte retrieval and embryo transfer

Ovarian stimulation was undertaken using a long gonadotrophin-releasing hormone analogue protocol combined with pure or recombinant FSH. Human chorionic gonadotrophin was administered when the leading follicle reached 20 mm with ≥2 follicles larger than 16 mm in mean diameter. Follicular aspiration was performed with transvaginal ultrasound guidance under local anaesthesia and i.v. sedation. ICSI was performed as previously described (Van Steirteghem et al., 1993). Patients who formed the basis for this study had all their embryos cultured up to the blastocyst stage.

In-vitro culture of embryos

In-vitro culture of embryos was undertaken as previously described (Balaban et al., 1998; Gardner et al., 1998). Sequential media system (G1 and G2 media, Vitro Life, IVF Science Scandinavia, Goteborg, Sweden) designed for further embryonic development was used. Embryos were individually cultured in microdroplets containing G1 media on day 1 and 2. After assessment of cell number and morphology, embryos were transferred to G2 medium for further culture up to the blastocyst stage. Laser zona opening was performed on all embryos on day 3.

Zygote and embryo evaluation

During the period from fertilization to blastocyst transfer the following events were recorded: (i) fertilization, (ii) early cleavage, (iii) cleavage stage embryo morphology, (iv) presence of 8-cell embryos on day 3, (v) blastocyst formation on day 5, (vi) presence of hatching blastocysts. All of these were related to PN morphology that had been recorded 14–17 h after ICSI. A ×40 objective on an inverted microscope with Hoffman modulation contrast optics was used for the evaluation of embryo characteristics.

Scoring of zygotes was carried out according to Tesarik and Greco (Tesarik and Greco, 1999) and is summarized as follows: 0 PN pattern: the number of nucleolar precursor bodies (NPB) in both pronuclei never differs by more than three; NPB polarized when <7 and never polarized when ≥7 in at least one pronucleus; the number of NPB in a pronucleus never <3; the distribution of NPB either polarized or non-polarized in both pronuclei. Zygotes that did not conform to this morphological pattern were considered as abnormal and classified into one of the five following patterns: pattern 1 included zygotes that presented a large difference (>3) in the number of NPB in both pronuclei; pattern 2 included zygotes with a small number of NPB (<7) with polarization in at least one pronucleus; pattern 3 included zygotes with a large number of NPB (>7) with polarization in at least one pronucleus; pattern 4 included zygotes that had a very small number of NPB (<3) in at least one pronucleus; pattern 5 presented a polarized distribution of NPB in one pronucleus and non-polarized in the other.

Early cleavage was defined as evidence of cleavage 24 h after insemination or ICSI (Shoukir et al., 1997).

Cleavage stage embryos were graded as follows: grade 1 embryo: no fragmentation with equal sized homogeneous blastomeres, grade 2 embryo: <20% fragmentation with equal sized homogeneous blastomeres, grade 3 embryo: 20–50% fragmentation with equal or unequal sized blastomeres, grade 4 embryo: >50% fragmentation with equal or unequal sized blastomeres.

Blastocyst grading was according to Dokras et al. (Dokras et al., 1993). This grading system has been shown by our group to be clearly related to the success of blastocyst transfer (Balaban et al., 2000). Grade 1 blastocysts were characterized by early cavitation, resulting in the formation of an eccentric and then expanded cavity lined with a distinct inner cell mass region and trophectoderm layer. Grade 2 blastocysts exhibited a transitional phase where single or multiple vacuoles were seen which over subsequent days developed into the typical blastocyst appearance of the grade 1 blastocysts. Grade 3 blastocysts were defined as blastocysts with several degenerative foci in the inner cell mass with cells appearing dark and necrotic.

Statistical analysis of results

One way analysis of variance and $\chi^2$ tests were used to compare the groups. A P value < 0.05 was accepted as significant.

Results

Table I shows the relationship of PN morphology to embryo cleavage, early cleavage, cleavage stage embryo morphology, the rate of ≥8 cell embryos on day 3, arrested embryos, and blastocyst grade and hatching. There appeared to be trend towards worsening embryo quality reflected in all parameters observed when PN morphology deviated from the ideal (0 pattern). To render interpretation of results simpler, PN morphology patterns were sub-grouped into (i) ideal PN pattern, (ii) single PN anomaly, and (iii) double PN anomaly. The results according to this sub-grouping are shown in Table II. As is evident from the table, nearly all parameters of cleavage stage embryo and blastocyst quality appeared to be related to PN morphology. Early cleavage and the incidence of embryos with ≥8 cells on day 3 were significantly higher for zygotes with 0 pattern.

Embryo transfers were grouped as indicated in Table III. Patients in group 1 had one blastocyst that was derived from a zygote with a 0 PN pattern transferred. Patients in group 2 had >1 blastocyst derived from a zygote with a 0 PN pattern transferred. None of the transferred blastocysts in group 3 patients were derived from 0 PN pattern zygotes. In group 4 patients, all blastocysts transferred were derived from zygotes with a 0 PN pattern. Mean female age, mean number of oocytes retrieved and mean number of embryos transferred were similar in all groups. Implantation rate per embryo was significantly higher in group 4 than in all other groups. The lowest implantation rates were obtained in the group where none of the transferred blastocysts originated from zygotes showing a 0 PN pattern. It is of interest to note that the percentage of grade 1 and 2 blastocysts transferred were similar in all groups.

Table IV shows the outcome of homogeneous blastocyst transfers. Implantation rate per embryo was significantly higher (27.2%) in the group of patients who had only grade 1 and 2
Table I. Cleavage stage embryo quality, early cleavage, 8-cell embryos and blastocyst quality according to PN pattern

<table>
<thead>
<tr>
<th>PN pattern</th>
<th>Equal sized 2PN</th>
<th>Cleavage cleavage on D3</th>
<th>G1+G2 embryo</th>
<th>G3+G4 formation</th>
<th>Early Embryo</th>
<th>≥8 cell blastocyst</th>
<th>% Blastocyst blastocyst</th>
<th>Arrested blastocyst</th>
<th>BG1+BG2 morula</th>
<th>BG3 MNB</th>
<th>Hatching</th>
<th>Expanded</th>
<th>Early</th>
<th>Cavitating</th>
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MNB = multinucleated blastomeres; BG = blastocyst grade; PN = pronucleus/i.

Table II. Cleavage stage embryo quality, early cleavage, 8-cell embryos and blastocyst quality according to PN pattern. PN pattern has been subgrouped under: ideal PN pattern, single PN anomaly, and double PN anomaly

<table>
<thead>
<tr>
<th>PN pattern</th>
<th>Equal sized 2PN</th>
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<th>Early</th>
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<td>78</td>
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<tr>
<td>Single PN</td>
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<td>51</td>
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<td>Anomaly</td>
<td>70.5%</td>
<td>94.1%</td>
<td>59.2%</td>
<td>40.8%</td>
<td>8.6%</td>
<td>30.9%</td>
<td>35.7%</td>
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<td>49.3%</td>
<td>50.7%</td>
<td>11.8%</td>
<td>31.8%</td>
<td>30</td>
<td>26.2%</td>
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<tr>
<td>Double PN</td>
<td>13.4%</td>
<td>73.3%</td>
<td>54.5%</td>
<td>45.4%</td>
<td>4.5%</td>
<td>16.7%</td>
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<td>77.3%</td>
<td>46.7%</td>
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<td>6.7%</td>
<td>46.7%</td>
<td>33.3%</td>
<td>13.3%</td>
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</tbody>
</table>

MNB = multinucleated blastomeres; BG = blastocyst grade; PN = pronucleus/i.
Multiple pregnancy rate: 2/4 (50%) 0 0 0
Clinical pregnancy/transfer: 2 4/7 (57.1%) 2/6 (33.3%) 1/4 (25%) 0
Implantation rate per embryo: 1 6/22 (27.2%) 2/18 (11.1%) 1/16 (6.2%) 0
Mean number of blastocysts transferred: 3.1 3.0 4.0 4.3

blastocysts replaced that were derived from 0 PN pattern zygotes compared with grade 1 and 2 blastocysts that were derived from zygotes with abnormal PN patterns (11.1%). None of the patients conceived when poor quality blastocysts that were derived from zygotes with abnormal PN patterns were transferred. The incidence of multiple gestations appeared to be very high (50%) in the group that had good quality blastocysts transferred derived from 0 PN pattern zygotes.

There were freezable blastocysts in the group who had >1 blastocyst with a 0 PN pattern and the group who had all blastocysts with a 0 PN pattern. A total of eight couples had their blastocysts cryopreserved. Six of these conceived in their fresh cycle. One couple requested their blastocysts to be thawed but unfortunately did not conceive.

Discussion
The results of this study are in line with other studies in the literature and suggest that there is a good relationship between markers of embryo normality/quality starting from the zygote stage up to the blastocyst stage. Zygotes showing a 0 PN pattern became embryos that cleaved earlier and faster, had better cleavage stage morphology, reached the blastocyst stage more often and were better grade blastocysts. Furthermore, blastocysts arising from zygotes with an ideal PN pattern implanted more efficiently resulting in higher clinical pregnancy rates. Grade 1 and 2 blastocysts derived from 0 pattern zygotes had the highest implantation potential. This group may be a candidate for elective single embryo transfer (eSET). Grade 1 and 2 blastocysts derived from zygotes with abnormal PN patterns also implanted, albeit at a lower rate. High implantation rates are to be expected of grade 1 and 2 blastocysts. This was clearly shown in a recent study by our group (Balaban et al., 2000). The current findings further elaborate on the results of the previous study.

Assessment of the zygote and the cleavage stage embryo is a crude science. This makes it difficult for the clinician to select the most appropriate embryo/s for transfer. Tesarik and Greco defined a set of criteria for PN morphology and related this to other parameters of embryo normality or quality such as the occurrence of cleavage arrest, the incidence of blastomere multinucleation and cleavage stage embryo morphology (Tesarik and Greco, 1999). In this study they showed that when compared with the whole unselected group of embryos, those arising from pattern 0 zygotes resulted in better quality cleavage stage embryos and were less likely to arrest at the cleavage stage. This assessment system may enable the embryologist to select the embryo with the highest implantation probability.
potential as early as possible. Furthermore, ethical concerns regarding selection of embryos for fresh transfer versus cryopreservation may be obviated.

Several other criteria of embryo quality such as early cleavage, strict cleavage stage embryo morphology assessment, and the presence of \( \geq 8 \) cell embryos on day 3 may be combined with PN morphology to make a more accurate selection. Correlation between PN morphology and other reported markers of embryo normality have been recently studied. Wittemer et al. studied embryo development to day 3 and correlated different embryological parameters with PN scoring (Wittemer et al., 2000). They concluded that pattern 0 zygotes lead to more good quality embryos with a higher implantation potential than embryos developing from less favourable zygote patterns.

Scott et al. used a previously defined PN scoring system that they revisited during the course of their study and calculated a zygote score based on zygote grade, day 3 embryo morphology and the ability of embryos to reach the blastocyst stage (Scott et al., 2000). Cleaving embryos that were selected initially by zygote morphology and secondarily by embryo morphology on the third day implanted at a higher rate compared with those selected by morphology alone (31 versus 19%). Zygote scored blastocyst transfer cycles resulted in higher implantation rates compared with non-scored cycles (58 versus 39%). The authors concluded that zygote scoring increased implantation and pregnancy rates both on day 3 and day 5. Zygote scoring showed a strong correlation between both embryo morphology on day 3 and the ability of the individual embryo to reach blastocyst stage on day 5 or 6.

Racowsky et al. retrospectively analysed the outcomes of day 3 versus day 5 transfers (Racowsky et al., 2000). They showed that the presence of 8-cell embryos on day 3 strongly predicted the success of embryo transfer whether the transfer was performed on day 3 or day 5. None of the embryos that had \(< 8\) blastomeres on day 3 implanted when they were transferred on day 5.

Alikani et al. showed that the degree and pattern of fragmentation significantly impacted pregnancy and implantation (Alikani et al., 1999). In a more recent study, the same group examined the relationship between morphological anomalies of cleavage stage embryos and their ability to form normal blastocysts in vitro (Alikani et al., 2000). A normal cleavage rate (7–9 cells on day 3) was associated with a higher progression rate to the blastocyst stage, whereas excessive embryo fragmentation (\(> 15\%\)) had a negative impact.

Ludwig et al. defined a PN score where they graded zygotes 16–18 h post ICSI according the position of the pronuclei, the alignment of the nucleoli at the junction of the two pronuclei, and the appearance of the cytoplasm (Ludwig et al., 2000). In this study, embryo selection was performed solely by PN scoring. A threshold value of 13 for zygote score was accepted as a cut off as this appeared to allow prediction of the establishment of pregnancy. Cumulative embryo score on the day of transfer was similar in patients with zygote scores of \(> 13\) and \(< 13\). PN score did not differ in those cycles with and without male factor. Pregnancy rates were 4% and 22% when the PN score was \(< 13\) and \(> 13\) respectively.

It appears from the analysis of results of several studies in the literature that PN scoring can successfully be incorporated into the practice of embryo evaluation for prediction of implantation potential of the individual embryo. Markers of embryo normality so far defined appear to have a strong relationship with each other. Our study indicates that the ‘good’ embryo can be determined at the onset of embryonic development as early as the zygote stage. Embryos preselected for transfer at this stage appear to be those that cleave faster, have a better cleavage stage morphology, reach the blastocyst stage more often and give rise to better quality and hatching blastocysts. It remains to be determined in a randomized trial whether embryo selection using different well-defined criteria gives rise to similar pregnancy rates. Most ethical concerns can undoubtedly be solved and work load on the laboratory personnel reduced if the embryo endowed with the potential to implant can be identified as early as possible during the period of embryonic development.

References


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