Introductions

The embryologist must rely upon light microscopic features that provide morphological, non-invasive aspects for the assessment of normality when optimizing embryo selection (particularly with the transition toward single-embryo transfer) between the pronuclear and blastocyst stages for patient IVF culture, embryo transfer and cryopreservation.

Morphological characteristics suggested to indicate top quality include the relative number and alignment pattern of nucleolar precursor bodies at the pronuclear stage (Scott and Smith, 1998; Tesarik and Greco, 1999; Coskun et al., 2003) and stage-appropriate cell numbers (Gardener et al., 1998; Alikani et al., 2000; Racowsky et al., 2003) resulting from symmetric cell divisions on day 2 or 3. Asymmetric cell division is considered a negative indicator for implantation and pregnancy, and has been shown to be directly proportional to aneuploidy and multinucleation. (Munné et al., 1994; Hardarson et al., 2001). Certain cytoplasmic fragmentation patterns (non-localized, anucleate fragmentation, for example, Alikani and Cohen, 1995; Antczak and Van Blerkom, 1999; Van Blerkom et al., 1999).
2001) may be evidence that an embryo is arresting or, in some cases, it has been proposed that these embryos may have entered the apoptosis cascade (Jurisicova et al., 1996, 2003). Highly fragmented embryos are clearly detectable in the clinical IVF lab. Assessment of the potential impact on embryo development is associated with measurable degrees and/or identifiable patterns of fragmentation (Alikani and Cohen, 1995; Van Blerkom et al., 2001). However, when used singly or in combination, none of these morphological descriptors is an absolute indicator of embryo competence as demonstrated by normal outcomes from embryos that have been judged to be poor quality (Veeck, 1999).

Multinucleation in one or more blastomeres at the earliest stages of development would seem to be an unambiguous indicator of incompetence and pending demise because this defect should be, and generally is associated with errors in chromosomal replication and segregation. Multinucleation is a relatively common occurrence in cohorts of oocytes fertilized in vitro with reported frequencies (per patient cycle) of between 17% and 74% (Kligman et al., 1996; Van Royen et al., 2003).

While there is plentiful evidence that multinucleated blastomeres occur in clinical IVF (Munné and Cohen, 1993; Kligman et al., 1996; Van Royen et al., 2003), a systematic study in which temporal and developmental aspects of their origin and fate are analysed has not been reported. In particular, the identification of multinucleated blastomeres in early cleavage stage embryos can be problematic as nuclei in both mononucleated and multinucleated blastomeres are transient structures whose detection and characterization require timed studies. Indeed, it is not always apparent whether multinucleated cells contain separate nuclear structures or a single nucleus that undergoes transient changes in shape [Van Blerkom J (Boulder, Colorado), personal communication].

However if they are indeed always separate entities it is possible that replication and chromosomal condensation in separate, closely apposed compartments, could lead to chromosomal abnormality in the daughter cells if nuclear breakdown prior to the second cell division results in separated chromosomal masses attaching to the same mitotic spindle. While it might seem intuitive that severe genetic disorders in affected embryos would preclude implantation and further development, pregnancies with multinucleated embryos have been reported (Balakier and Cadesky, 1997; Pelinck et al., 1998).

Different developmental patterns, outcomes and embryo performance in vitro were noticed between micronucleated embryos and binucleated embryos. In contrast, Staessen and Van Steirteghem (1998) found no difference in the development or genetic constitution of binucleated and multinucleated embryos, and relevantly, did show that blastomeres developing from these embryos were not always abnormal; however, they did not distinguish between micronucleation and multinucleation.

The present study used time-lapse imaging, as well, to examine temporal aspects of the genesis and fate of binucleated (BN) and micronucleated (MN) blastomeres in cultured human embryos derived by intracytoplasmic sperm injection (ICSI). Specifically, the following questions were asked: (i) how do BN and MN embryos compare to each other in growth and development? (ii) are the chromosomal complements in BN and MN embryos different from each other? (iii) what is the fate of multiple nuclei in affected blastomeres (as compared with their mononucleated counterparts)? and (iv) does the presence of these characteristics in a cohort of embryos indicate information regarding total cohort health and or competence?

The findings demonstrate that the origin of BN embryos may largely be oocyte dependent, while MN embryos may result from an inherent biological defect possibly affecting the remaining cohort of mononucleated embryos as well. The results are discussed with respect to the chromosomal normality of BN and MN embryos and results of their sibling embryos.

Materials and methods

Study design

This was a prospective cohort study including all patients (<40 years old) sequentially attending the authors’ infertility centre and undergoing ICSI, with a minimum of one multinucleated embryo in the cohort on day 2 of development.

MN embryos (also referred to as fractured nuclei) were defined as those with one or two large nuclei (with nucleoli) surrounded by one or more smaller nuclei at least one half the diameter of the large one, in one or more blastomeres (Figure 1). ‘Multinucleated’ embryos with same size nuclei were not considered to be micronucleated. This study did not include ‘multinucleated’ embryos since incidence was low for this phenotype in our laboratory. Diameters were measured using OCTAX laser-assisted hatching imaging software (Octax, 1.48 mm diode non-contact laser, Zander Medical, Vero Beach, USA). BN embryos were defined as those with two nuclei of relatively the same size in one or more blastomeres (Figure 2).

IVF-ICSI procedure

All patients were down-regulated (using Leuprolide Acetate; Abbott Pharmaceuticals, Montreal, Canada) using a long agonist protocol (Meriano et al., 2001). The technique for IVF-ICSI has been described previously (Greenblatt et al., 1995). Approximately 18 h after injection, oocytes were assessed for signs of fertilization (two distinct pronuclei and two polar bodies) and pronuclear morphology was evaluated based on characteristics described by Scott and Smith (1998) and Tesarik and Greco (1999). At 40–42 h and 69–71 h incubation, cleaved embryos were identified and graded, based upon blastomere symmetry, degree of fragmentation, and nuclear status.

Patients were divided into three groups for descriptive statistics: group 1 included patients with at least one BN blastomere in the embryo cohort (n = 25); group 2 included patients with MN embryos (n = 26); and group 3 included patients with both morphologies (n = 29). Multinucleation was easily observed using high magnification (200–400x) and Hoffman modulation optics. Photographs were taken if confirmation was required.
Embryos slated for blastocyst culture were assessed on day 3 and placed into fresh equilibrated Blastocyst medium (catalogue no. 99292, Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% (v/v) SSS (Irvine Scientific) and incubated at 37°C in a 5% CO₂, 5% O₂, and 90% N₂ environment until day 5. Patient consent was obtained for biopsy of embryos not slated for transfer. Embryos were biopsied after 3 days’ incubation using an Octax laser (1.48 µm diode non-contact laser, Zander Medical, Vero Beach, USA) mounted on a Zeiss Axiovert 100 system (Carl Zeiss, Toronto, Canada) to breach the zona pellucida for removal of the blastomere. After the biopsy, the embryo was rinsed in two washes of equilibrated human tubal fluid (HTF)/10%SSS and placed back into culture.

In the event that consent was withheld, multinucleated embryos were left in culture with the remaining embryos in the cohort and observed as usual.

**Fluorescence in-situ hybridization (FISH)**

After biopsy, blastomere nuclei were fixed and hybridized as described by Munné et al., 1996.

FISH was performed using Vysis HBYrite with the PGT Multivision probe set for chromosomes 13, 18, 21, X and Y labelled with Spectrum labels Red, Aqua, Green, Blue and Gold respectively (Vysis Inc., Downer’s Grove, IL, USA, distributed by Intermedico, Markham, Ontario, Canada). Chromatin was stained with propidium iodide (1:6 v/v with antifade) for visualization of the nucleus. Normal lymphocyte metaphase and abnormal metaphase control slides were run concurrently with patient samples.

Following hybridization, slides were either read immediately or held at −20°C until the next day. Slides were read on a Zeiss fluorescence microscope (Axioplan 2, Carl Zeiss) equipped with appropriate filters. Images were captured using a digital camera utilizing Carl Zeiss Axiovision 3.0 software. Results were reported as normal, aneuploid (one more or less chromosome than normal), or polyploid.

**Time-lapse photography**

BN and MN embryos allotted for time-lapse photography were placed in a warm (37°C) drop of HEPES-buffered HTF/10%SSS on a culture dish lid overlaid with sterile mineral oil. Since the multinucleation has already taken place at observation, the use of HEPES-buffered media was not seen as an external variable to the formation of this anomaly. The dissolution of this anomaly was seen to happen with greater frequency in HEPES-buffered media than PBS, or non-HEPES buffered media (unpublished evaluation of time-lapse method, J Meriano). The use of HEPES was not thought to be detrimental for this study since the certificate of analysis from the manufacturer ensured 80% mouse blastocyst formation and hatching in the HEPES-buffered media.

The dish was placed onto the heated stage of an inverted microscope (Zeiss Axiovert 100 with Hoffman modulated optics) equipped with a digital camera attached to a frame grabber in a computer. A holding pipette on the left manipulator held the embryo steady during filming, and the embryo was positioned touching the bottom of the dish to optimize focus and viewing of the BN or MN nuclei. The time between frame acquisitions was set to 2.5 min (this interval was predetermined after filming tri-pronuclear oocytes). Total filming took place over a 12–18-hour period, and throughout, the microscope was covered to maintain the temperature of the media at 37°C and to minimize vibrations from the airflow system. The heated stage was adjusted to 39°C for the maintenance of a temperature of 37°C as checked by infrared thermometer (Extech instruments, model 42525, Waltham MA, USA), biweekly. Frames were subsequently reviewed independently and as a video. Since the nuclei roll and move within the cytoplasm and nucleoli roll and move within nuclei,
over a period of time, it was necessary to review each frame individually to calculate the time of nuclear dissolution.

**Statistical analysis**

Data were collected regarding patient age, number of days of stimulation, day of human chorionic gonadotrophin (HCG) administration, fertilization rate, maturation rate and clinical pregnancy rate (defined as the visualization by ultrasound of a fetal heart at 7 weeks’ gestation). Day 3 cleavage rate was defined as all embryos that developed to at least six cells 70 h post-insemination.

All analyses were performed using SigmaStat (SPSS Inc., Chicago, IL, USA). Student’s t test, chi-squared and z tests were performed as appropriate and significance was set at \( P < 0.05 \). Confidence intervals (95% CI) and standard error (SE) are included where required; all means are reported as mean ± standard error of mean (SEM).

**Results**

**Frequency of multinucleated embryos**

Of 560 patient cycles, 80 had at least one embryo in their cohort with multinucleation (14.3%). Seven-hundred and seventy embryos were derived from the 80 cycles, and a total of 183 embryos were multinucleated (23.8%) in this study group. BN blastomeres were more common than MN blastomeres (102/183 = 55.7% versus 81/183 = 44.3% respectively).

**Comparison of patient groups (Table 1)**

There was no significant difference between the three groups with regard to age, mean day of HCG administration, fertilization rate, maturation rate, or mean number of days of stimulation. Group 1 (BN phenotype in at least one embryo per cohort) had a significantly lower number of oocytes retrieved than either group 2 (MN phenotype in at least one embryo per cohort) or group 3 (both BN and MN phenotypes in the embryo cohort). No significant differences were seen in the day 3 cleavage rates.

Blastocyst development was significantly better in BN embryos as compared with MN embryos (39/102, 38.2% versus 7/81, 8.6%, \( P = 0.001 \)). There was no significant difference between sibling mononucleated blastocyst development compared with BN blastocyst development [147/356, 41.3% vs 39/102, 38.2%, not significant (NS)] (Table 2).

Micronucleated embryos were more frequently derived from embryos with poor pronuclear morphology (56.76%), than were BN embryos (7.7%). Those embryos derived from zygotes with pronuclei of different sizes and/or nucleolus precursor amounts that were different between the two nuclei (\( P = 0.001, 95\% \text{ CI} = 0.227–0.539 \)) (Table 3).

No multinucleated embryos were transferred to patients in this study. The implantation rate in group 1 was higher than in group 2 (\( P = 0.038, 95\% \text{ CI} 0.02–0.276 \)) although the clinical pregnancy rates from transferred siblings mononucleated embryos were not statistically different between the three groups; group 1 did, however, show a trend towards a higher rate than group 2. There was a trend toward an increased spontaneous pregnancy loss rate in group 2, but the sample sizes were too small for the results to reach statistical significance. There was however, a significant increase in the ongoing pregnancy rate (pregnancies that progressed beyond the first trimester) in group 1 compared with group 2 (48% versus 15.4%, \( P = 0.031, 95\% \text{ CI} 0.064–0.575 \)) (Table 1).

**FISH analysis**

Seventeen patients consented to embryo biopsy. A total of 69 blastomeres from 23 embryos were analysed by FISH and 55 biopsied blastomeres (79.7%) yielded results, 28 from BN embryos and 27 from MN embryos. FISH analysis was not effective for 14 out of 69 blastomeres, either due to signal clumping, no signal, or no nucleus retained on the slide. An observed BN or MN embryo was allowed to divide to day 3 before biopsy. Blastomeres at this time were all observed to be mononucleated. Table 4 shows the distribution data for blastomeres from each morphological characteristic studied. There was evidence of aneuploidy, polyploidy and sex chromosome monosomy (XO) among the different blastomeres. Twenty-six of 27 blastomeres from MN embryos (96.3%) were chromosomally abnormal compared with 19/28 blastomeres from BN embryos (68.0%) (\( P = 0.01, 95\% \text{ CI} 0.0773–0.487 \)). Therefore, BN-derived blastomeres were significantly more frequently normal than MN-derived blastomeres (\( P = 0.016, 9/28 = 32.1\% \)).

**Time-lapse photography**

Five BN and six MN blastomeres were viewed by time-lapse photography. Of the BN blastomeres, four showed independent dissolution of their nuclear membranes; three showed cleavage as well. Time elapsed from dissolution of the first nucleus to dissolution of the second ranged from 15 min (for a 3-cell embryo) to 32.5 and 102 min (for 2 × 4-cell embryos respectively). Figures 3 and 4 denote micronucleated embryos during time-lapse imaging. The first image in Figure 3 shows five micronuclei in the optical left blastomere at the beginning of imaging, and the bottom image shows the nuclei disappearing near end of imaging. Figure 4 is a composite of time-lapse images of the same embryo as in Figure 3 showing the various stages of dissolution of the nuclei from start to finish, where the first three dissolve independently and the last two dissolve seemingly simultaneously.

Six MN embryos were filmed, and four showed dissolution of the nuclei; two also showed cleavage. Time elapsed for the complete dissolution of all MN ranged from 21 to 49 min; large nuclei tended to dissolve before the smaller ones.

Time-lapse photography clearly showed nuclei from both BN and MN embryos dissolving separately, and there was a trend indicating that MN nuclei tended to dissolve faster that BN nuclei, although too few were photographed to determine whether this difference was statistically significant. More data will be added to this aspect of the study.
Table 1. Descriptive data for three groups (mean ± SEM).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1 binucleated</th>
<th>Group 2 micronucleated</th>
<th>Group 3 binucleated and mononucleated</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n)</td>
<td>25</td>
<td>26</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>34.8 ± 0.88</td>
<td>34.1 ± 0.78</td>
<td>35.1 ± 0.62</td>
<td>NS</td>
</tr>
<tr>
<td>Mean oestradiol (pmol/l) on day of HCG</td>
<td>8882 ± 1270</td>
<td>10136 ± 1096</td>
<td>8244 ± 597.6</td>
<td>NS</td>
</tr>
<tr>
<td>Mean day of HCG administration</td>
<td>11.3 ± 0.3</td>
<td>11.0 ± 0.27</td>
<td>11.4 ± 0.25</td>
<td>NS</td>
</tr>
<tr>
<td>Mean oocytes retrieved</td>
<td>14.07 ± 1.15</td>
<td>18.2 ± 1.13a</td>
<td>18.1 ± 1.99a</td>
<td>0.015a</td>
</tr>
<tr>
<td>Mean no. of metaphase II</td>
<td>11.4 ± 0.67</td>
<td>13.2 ± 0.97</td>
<td>13.9 ± 1.52</td>
<td>NS</td>
</tr>
<tr>
<td>Mean maturation rate (%)</td>
<td>86.9 ± 2.4</td>
<td>74.6 ± 3.7</td>
<td>81.7 ± 2.7</td>
<td>NS</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>75.5 ± 4.2</td>
<td>71.1 ± 3.79</td>
<td>80.3 ± 1.91</td>
<td>NS</td>
</tr>
<tr>
<td>Cleavage at 72 h (%)</td>
<td>64.9 ± 5</td>
<td>75.5 ± 4.7</td>
<td>62.5 ± 4.8</td>
<td>NS</td>
</tr>
<tr>
<td>Mean no. of days of stimulation</td>
<td>10.3 ± 0.4</td>
<td>10.3 ± 0.4</td>
<td>10.5 ± 0.26</td>
<td>NS</td>
</tr>
<tr>
<td>Pregnancy rate of mononucleated sibling embryos n (%)</td>
<td>13/25 (52.0)</td>
<td>7/26 (26.9)</td>
<td>13/29 (44.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Spontaneous pregnancy loss rate n (%)</td>
<td>1/13 (7.7)</td>
<td>3/7 (42.9)</td>
<td>4/13 (30.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Implantation rates n (%)</td>
<td>17/68 (25.0)</td>
<td>7/69 (10.1)a</td>
<td>15/81 (18.5)</td>
<td>0.038</td>
</tr>
<tr>
<td>Mean no. of embryos transferred</td>
<td>2.43 ± 0.14</td>
<td>2.88 ± 0.16</td>
<td>2.79 ± 0.19</td>
<td>NS</td>
</tr>
<tr>
<td>Ongoing pregnancy rates mononucleated sibling embryos n (%)</td>
<td>12/25 (48.0)</td>
<td>4/26 (15.4)a</td>
<td>9/29 (31.0)%</td>
<td>0.031a</td>
</tr>
</tbody>
</table>

aStatistically different from group 1; NS = no statistical difference (t-test and z-test, Sigma Stat Statistical software – Jandel Scientific).

Table 2. Comparative blastocyst development rates between morphological phenotypes.

<table>
<thead>
<tr>
<th></th>
<th>Binucleated n (%)</th>
<th>Micronucleated n (%)</th>
<th>Mononucleated n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastocyst rate</td>
<td>39/102 (38.2)a</td>
<td>7/81 (8.6)a,b</td>
<td>147/356 (41.3)b</td>
</tr>
</tbody>
</table>

a,bValues with the same superscript letter were significantly different (P = 0.001).
Table 3. Pronuclear morphology and multinucleate phenotypes.

<table>
<thead>
<tr>
<th>Pronuclear score</th>
<th>Micronucleated, n (%)</th>
<th>Binucleated, n (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>15/81 (18.5)</td>
<td>53/102 (52.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>N3</td>
<td>20/81 (25.0)</td>
<td>41/102 (40.2)</td>
<td></td>
</tr>
<tr>
<td>Good morphology, N1 and N3</td>
<td>35/81 (43.2)</td>
<td>94/102 (92.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>N4</td>
<td>30/81 (37.0)</td>
<td>8/102 (7.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>N5</td>
<td>3/81 (3.7)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>N6</td>
<td>7/81 (8.6)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>N8</td>
<td>1/81 (1.2)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Combination of negative indicator pronuclear morphology, N4–N8</td>
<td>41/81 (50.6)</td>
<td>8/102 (7.8)</td>
<td>0.001^a</td>
</tr>
</tbody>
</table>

^a Z-test with Yates correction applied, SEM = 0.0667, 95% CI 0.277–0.539 (Sigma stat software, Jandel Scientific, San Rafael, CA, USA).

Table 4. Distribution of results for blastomeres with FISH signal.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Aneuploid n (%)</th>
<th>Polyploid n (%)</th>
<th>Normal n (%)</th>
<th>(Sex) XO n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastomeres from micronucleated embryos</td>
<td>18/27 (66.7)</td>
<td>4/27 (14.8)</td>
<td>1/27 (3.7)^a</td>
<td>4/27 (14.8)</td>
</tr>
<tr>
<td>Blastomeres from binucleated embryos</td>
<td>16/28 (57.1)</td>
<td>3/28 (10.7)</td>
<td>9/28 (32.1)^a</td>
<td>0</td>
</tr>
</tbody>
</table>

^a Z-test, power α = 0.005, 0.692; values are significantly different (P = 0.016).

Figure 3. (a) First picture of series in Figure 4 clearly showing five micronuclei in the left blastomere and (b) the second to last picture in the series showing the two remaining nuclei dissolving together but after the first three have dissolved independently.
Figure 4. Micronuclei time-lapse, each frame 2.5 min. Five micronuclei in the left blastomere: rotation of nuclei made it difficult to observe, however through repetitive observations frame per frame one could visualize the independent dissolution of the nuclei. The smaller nuclei dissolved first and larger ones last. The top nucleus dissolved first and the bottom two, together. A total time of 47 minutes elapsed from start to finish.
Discussion

The incidence of multinucleation in patient embryos derived from assisted reproductive techniques, ranges from 17–74% (Kligman et al., 1996; Van Royen et al., 2003). The finding of 23.7% of embryos with multinucleation in the present study cohort is within that range. However, only 14.3% of the patients had multinucleated embryos which is much lower than the frequencies reported by Van Royen et al. (2003) and Jackson et al. (1998) (79.4% and 74% respectively). A number of factors may account for this difference: multinucleation was noted on day 2 compared with day 2 and 3 for Van Royen, and also timing of the observation may be crucial, as not all embryos develop at the same relative rate. As well, the fact that fertilization timing and the timing of observations differs from clinic to clinic may also be a factor.

Embryos with multinucleated blastomeres have been shown to be associated with abnormal events in IVF culture that may affect embryo development. Severe hypoxia of the developing follicle during stimulation is associated with MNB in that, greater than 90% of MNB, were derived from oocytes whose follicular environment was severely hypoxic during in-vivo maturation (Van Blerekom et al., 1997). Secondly, an association was shown between vascular endothelial growth factor (VEGF) and intrafollicular oxygen levels and between the competence of the corresponding oocytes and the embryos derived from oocytes resulting from hypoxic follicles. Jackson et al., (1998) found a correlation between MN embryos and rate of aggressiveness of ovarian stimulation, suggesting that follicle growth may be too rapid for proper vascularization (in some patients) resulting in impaired oxygenation of the oocyte, in some cases. Secondly, the stimulation of many oocytes may in some patients yield a high diversity of quality and competence of oocytes. Since it has been shown that up to 25% of oocytes in a cohort may be abnormal (Zenzes and Casper, 1992) at the outset (oocyte retrieval), it is possible that the more aggressive the stimulation protocol, the more heterogeneous the population of follicles stimulated and the higher the possibility of recovering abnormally developing oocytes.

Mononucleated, sibling embryos where multinucleation was present in the cohort have also been described to have reduced developmental potential (Jackson et al., 1998). Although implantation rates of normal sibling embryos were similar to control embryos, the chance of spontaneous abortion was markedly increased. This suggests the possibility that the cohort may have an underlying pathology for which multinucleation may be one of the most evident phenotypes. The present study observed this finding for cohorts containing micronucleated embryos, but it was not observed in the BN group.

In-vitro maturation of cumulus-denuded oocytes affects normal cytokinesis. Nogueira et al., (2000) matured denuded prophase I oocytes to metaphase II in vitro for ICSI and found that the level of nucleation after normal fertilization in resulting embryos was as follows: 30% binucleated, 43% multinucleated, and 56% were anucleate. Arrested cleavage at 24 h post-fertilization was also more prominent in the embryos derived from the in vitro-matured oocytes, suggesting that the development is indeed affected. Key modifications of the cytoplasm and nucleus are paramount to the maintenance of viable oocytes, during maturation in vivo or in vitro. Interruptions or interference of such intracellular restructuring (i.e. microtubular formation, mitochondrial relocation) may affect development of the future embryo (Albertini et al., 2003). In a similar study, (Balakier et al., 2004) metaphase I oocytes were matured in vitro for ICSI (also denuded) to metaphase II and then injected with sperm at different time intervals after first polar body extrusion. Although fertilization rates increased with time before injection after polar body extrusion, the overall observed multinucleation rate in resulting embryos was found to be 23% ranging from 36% with 1–1.5 h wait to ICSI after first polar body extrusion, to 17% in the 2–2.5-h group. These percentages differed significantly from the control group where multinucleation was only 13% per embryo.

These two studies suggest that if nuclear maturity and cytoplasmic remodelling are not complete before fertilization, it is likely that an embryo’s developmental ability is decreased. Nuclear maturity or remodelling performed in vivo in the follicle involves imprinting of maternal alleles (Albertini et al., 2003), an important step in the natural preparation of the oocyte for fertilization, as well as the structural and molecular remodelling of the cytoplasm in preparation for the energy requirements and physical changes involved in the resumption of meiosis, fertilization and embryo development and cleavage. Multinucleation may be a result of an abnormal event causing spindle complex malfunction and/or division abnormalities.

The blastocyst rate for embryos with BN was higher than for those embryos with MN, and similar to the rates observed with mononucleated embryos. The mononucleated sibling embryos in the BN group (group 1), had implantation rates and ongoing pregnancy rates that were significantly higher than for MN sibling embryos (group 2). Group 3, with embryos of both phenotypes, exhibited rates ‘in-between’ groups 1 and 2, indicating a possible underlying defect of MN embryos that may have been present in all or most embryos in the cohort, even those that appeared normal. In contrast, sibling embryos in the cohorts with BN embryos appeared unaffected, suggesting that binucleation is an independent variable restricted to that particular oocyte or embryo, whereas micromucleation may be indicative of overall cohort health. Previous reports have noted that multinucleated embryos most often derive from embryos with poor pronuclear morphology (Tesarak et al., 1987; Balaban et al., 2001), but they did not differentiate between BN and MN embryos.

Mechanisms of multinucleation include fragmentation of the nucleus (Pickering et al., 1995) and flawed migration of chromosomes at anaphase of the first mitotic division. Considering the fragility of the meiotic spindle of the metaphase II oocyte, it has been shown in animal and human studies that the spindle is temperature sensitive and the microtubules will depolymerize when stressed with negative temperature changes (Pickering et al., 1990, Boiso et al., 2002.), causing stray chromosomes, chromosomal scattering and eventually chromosomally abnormal embryos. Mechanisms that cause multinucleation may also be initiated by external variation in temperature, pH or inadequate culture media conditions (Munné and Cohen, 1993; Winston et al.,
1993; Pickering et al., 1990, 1995). Developmental abnormality in the oocyte, spermatozoon or resulting embryo may also affect embryo capacity and may be related to MNB, no matter what the cause given the variety of situations in which MNB occur. The literature describing multinucleation in embryos is extensive; however no definitive reason for formation has ever been described. The level of abnormality and apparent multifactor causes is generally accepted. Others include inherent abnormalities like karyokinesis without cytokinesis, a mechanism suggested by Pickering et al (1995) that indicates a possible fault in the oocyte spindle or division of the developing embryo. Intracellular restructuring, remodelling, and imprinting are of paramount importance to the developing oocyte and eventual embryo, and any interruptions of these delicate processes may cause errors in division resulting in abnormal embryos with the potential to express multinucleate phenotypes.

FISH analysis provided additional support for the concept that BN embryos have fewer chromosomal abnormalities than MN embryos. From the limited data obtained by time-lapse photography, it was observed that nuclei do break down independently, confirming that each set of chromosomal material will be distinct and attached to separate mitotic spindles, resulting in the continuous development of abnormal daughter cells if there are pre-existing chromosomal abnormalities. Staessen and Van Steirteghem (1998) noted that the chromosomal complement was not always abnormal in multinucleated embryos. This group distinguished multinucleated embryos from binucleated and found that 30.4% were uniformly diploid, similar to the percentage of normal embryo found in the present study for the binucleated embryos (32% normal) but not the micronucleated ones. Eviskiv and Verlinsky (2001) proposed that at a certain threshold of aneuploidy, embryos may initiate the apoptosis cascade; others may continue to develop through compaction and zygotic genomic activation. The former may be the case with MN embryos, whereas the latter may apply to BN embryos. Given that BN embryos can contain normal blastomeres, it is not unreasonable to assume that some may continue to develop, and this would explain the very limited potential of BN embryos to result in pregnancy (Balakier and Cadesky, 1997; Pelinck et al., 1998).

The small sample sizes of the groups in the present study limited the statistical significance of some of the conclusions, particularly regarding the detrimental effect of MN embryos on their transferred sibling embryos and the resultant increased incidence of pregnancy loss. However, other measures of difference between BN and MN embryos and their corresponding sibling embryos (implantation and ongoing pregnancy rates, for example Table 1) did attain statistical significance despite the sample sizes.

Although performed time-lapse photography was performed on only a few embryos, it was clear that all nuclei dissolved independently. Future studies will allow determination of whether the observed trend of MN nuclei dissolving faster than BN nuclei is significant, and whether the speed of dissolution has an impact on the possible ploidy of the resulting embryo. The methodology of the time-lapse portion also needs to be refined.

It has been shown that multinucleation in embryos derived from ICSI-IVF is easily observed and that it is a good indication of the health and developmental potential of the remainder of the embryo cohort. Differences were also found between BN and MN phenotypes: BN embryos are similar to mononucleated embryos with regard to blastocyst development, and there are higher rates of ongoing pregnancy and lower rates of spontaneous pregnancy loss in BN sibling embryos than in MN sibling embryos. It was confirmed that if MN embryos are present, the remainder of the cohort is negatively affected (and repair is unlikely) whereas the presence of BN embryos has no impact on their siblings. Poor pronuclear morphology was more often associated with MN blastomeres that BN blastomeres and FISH analysis clearly demonstrated that BN blastomeres were more likely to have a normal chromosomal complement, based on the results obtained in this study with five probes. There are clearly two diverse morphologies with distinct causal mechanisms that are under investigation.

In conclusion, observation of multinucleation should be part of all routine embryo assessments in conjunction with other non-invasive morphological inspections including determination of fragmentation, blastomere symmetry, pronuclear and oocyte grading, blastocyst culture, and energy consumption of cells, in order to optimize embryo selection. Exclusion of multinucleated embryos from transfer remains prudent practice. The presence of certain morphologies, in this case micronucleation, may indicate the health of the entire cohort as well.

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