GENETICS

Unequal Pronuclear Size—A Powerful Predictor of Embryonic Chromosome Anomalies

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Purpose: *Our purpose was to evaluate whether pronuclei of unequal size, observed in 13.7% of zygotes evaluated after in vitro fertilization (IVF), are predictive of chromosome anomalies in the developing embryo.*
Methods: *The ploidy of 38 embryos grown from zygotes with unequal-sized pronuclei was assessed by fluorescent in situ hybridization (FISH). Twenty-six embryos developed after intracytoplasmic injection of sperm (ICSI) and 12 embryos were derived from conventional IVF.*
Results: *Chromosome anomalies were documented in the ICSI and IVF groups in 88.5 and 50% of cases, respectively.*
Conclusions: *We suggest that FISH should be employed to examine the ploidy of zygotes with unequal pronuclei, prior to embryo transfer.*

KEY WORDS: fluorescent in situ hybridization; in vitro fertilization; intracytoplasmic sperm injection; preimplantation diagnosis; pronuclear size.

INTRODUCTION

Microscopic scoring of oocytes, zygotes, and embryos is the only noninvasive way to assess their "normalcy." Oocytes and embryos with "good" scores are generally considered to be "normal" and to confer better chances for implantation and pregnancy. Nevertheless, the correlation between the microscopic morphology of oocytes or embryos and their chromosomal constitution and subsequent development is, in most cases, anecdotal. Harper *et al.* (1) used dual fluorescent in situ hybridization (FISH) to investigate the incidence of chromosome anomalies in 69 normally fertilized embryos with "good" morphology and found 15% abnormalities of the sex chromosomes and 46% autosomal anomalies, many of them being mosaic and representing postfertilization errors. Laverge *et al.* (2) analyzed spare embryos with poor morphological scores and/or multinucleated blastomeres, most of them obtained after intracytoplasmic sperm injection (ICSI), and found that only 33.6% were uniformly diploid. Other examples are large oocytes or embryos with a high percentage of fragmentation, in which chromosomal abnormalities were found in 100 and 80% of cases, respectively (3).

The first evaluation of embryos for normalcy is performed at the pronuclei stage, 17 to 20 hr postinsemination or -injection. The number of pronuclei observed at this stage usually reflects the ploidy of the zygote, with two pronuclei and two polar bodies, suggesting a normal, diploid fertilization. In our laboratory, careful evaluation of the diameter of the two pronuclei ascertained a significant size difference between the two in 13.7% of fertilized oocytes. We employed FISH techniques to analyze embryos that demonstrated pronuclei of unequal size, attempting to correlate the occurrence of unequal pronuclei and that of abnormal ploidy of the embryo.

MATERIALS AND METHODS

Ovarian Stimulation

Ovarian stimulation for in vitro fertilization (IVF) was achieved using pituitary down regulation with

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gonadotropin releasing hormone (GnRH) agonist (Decapeptyl CR, 3.75 mg; Ferring, Malmo, Sweden), followed by daily injection of human menopausal gonadotropins [Pergonal (Teva, Petach Tiqva, Israel) and Puregon (NV Organon, Oss, The Netherlands)]. Oocytes were retrieved 36 hr post-human chorionic gonadotropin (hCG) injection (10,000 IU; Chorigon; Teva) and inseminated 3 to 5 hr later, either by ICSI or by conventional IVF.

Pronuclear Size Measurements

Oocytes were assessed for the presence of pronuclei on an inverted microscope (Zeiss, Germany) 17 to 20 hr after insemination or injection. Pronuclei were measured by trained embryologists with the aid of an eye micrometer. Equal pronuclei were used for standard size and deviation—the diameters measured were 25.0 ± 1.5 and 24.7 ± 1.7 μm (not significant; $P = 0.579$).

Oocytes with pronuclei of unequal size were selected by subjective impression of the embryologist, followed by meticulous measurements of the diameter of pronuclei and oocytes. Each measurement was repeated, 2 hr apart, by different embryologists. When one of the pronuclei was measured to be smaller than the other by two standard deviations (derived from the "normal" group), pronuclei were defined as "of unequal size." These zygotes were scored as 1 + 1 PN, photographed, and held separately in culture, where their development was further evaluated at 40 to 44 hr postinsemination.

FISH Analysis

Fixation of embryos and FISH analyses was performed between day 3 and day 5 postinsemination, as described previously by us (4). Briefly, the zona pellucida of the embryos was dissolved using acid Tyrode's (pH 2.4; Sigma Chemical Co., St. Louis, MO). Without separating the blastomeres, each embryo was held in an hypotonic solution of 0.5% sodium citrate in water (Sigma) until swelling of the blastomeres was observed, transferred to a glass slide with a minimal volume of hypotonic solution, and, finally, fixed by dripping a methanol/acetic acid (3:1) mixture until complete dissolution of the cytoplasm. FISH was performed utilizing direct chromosome enumerator probes (CEP; Vysis, Stutgtart, Fasanenhof, Germany) for chromosomes X, Y, and 18, and indirect α satellite probes (Oncor, Gaithersburg, MD) for chromosomes 13 and 21. Whenever an abnormality was verified for chromosomes X, Y, and 18, chromosomes 13 and 21 were not evaluated. The long FISH protocol was applied, with hybridization of slides overnight at 37°C, followed by repeated washings with formamide and standard saline citrate (SSC) solutions (Oncor). FISH signals were analyzed under fluorescence microscopy (Olympus BX 70; Japan).

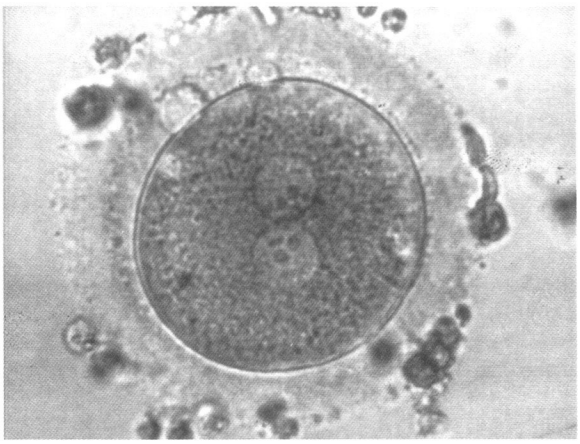

Fig. 1. A zygote with equal pronuclei.

RESULTS

The average diameters, for large and small pronuclei, were 25.2 ± 3.6 and 18.4 ± 3. 9 μm, respectively ($P < 0.0001$). The mean diameter of zygotes showed insignificant differences between the equal and the unequal pronuclei groups: 108.8 ± 3.3 and 112.5 ± 4.7 μm, respectively. The figures demonstrate zygotes with equal (Fig. 1) and unequal (Fig. 2) pronuclei.

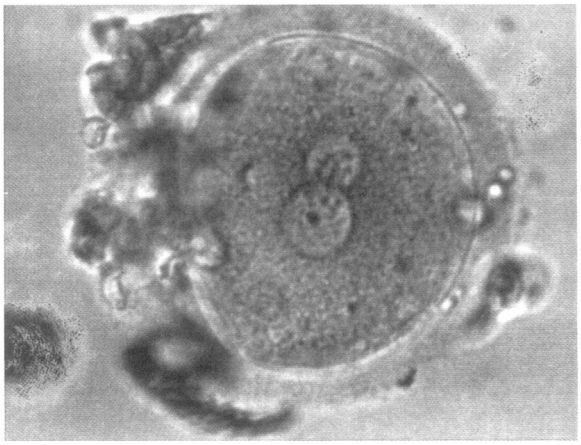

Fig. 2. A zygote with unequal pronuclei.

A total of 38 embryos (34 patients) derived from zygotes with unequal pronuclei was analyzed. Twelve embryos developed after in vitro insemination and 26 embryos were obtained following ICSI. The mean number of oocytes retrieved in the unequal pronuclei group was significantly higher than that obtained in a group of 163 patients with equal pronuclear size treated in the study period (16.3 ± 8.4 vs 12.3 ± 8.2; $P = 0.01$). Up to 32 blastomeres from each embryo derived from zygotes with unequal pronuclei were evaluated. Three blastomeres, each from a different embryo, were lost in the spreading procedure.

Abnormal chromosomal signals were found in 61.6% of all the embryos in the study group. An abnormal chromosomal complement was observed in 50% of the embryos developing from zygotes with unequal pronuclei after in vitro insemination and in 88.5% of the embryos derived from ICSI ($P < 0.0001$). Moreover, in many cases each blastomere analyzed had a different chromosomal constitution and aberrations, a situation defined as "chaos" (Table I). No correlation was found between the grade of size difference of the pronuclei and the chromosomal normalcy of the embryos: in some cases a large difference in pronuclear size resulted in normal embryos, while in other cases a very small difference was linked with abnormal ploidy.

Eight embryos, all derived after ICSI, stopped developing at the two-cell stage and one embryo remained one cell. In the one-cell embryo, two pronuclei appeared after 18 hr, but the FISH signals demonstrated only one set of chromosomes, suggesting activation of the oocyte, and not true fertilization. Embryo 33, obtained after ICSI, divided to the stage of six cells. FISH analysis demonstrated activation of the oocyte with one set of chromosomes.

DISCUSSION

Our results show that despite abnormal ploidy of embryos derived from zygotes with unequal pronuclei, many of them continue to divide and may be transferred to the uterus—in fact, arrest of development was observed in only 9 (23.7%) of the embryos in the study group. Since all the embryos with arrested development at the one-cell or two-cell stage in the study group were derived from ICSI, the corrected fraction of embryos selected for elimination prior to embryo transfer is 34.6%. This implies that even if we choose for transfer only actively dividing embryos derived from zygotes with unequal pronuclei, some 50 to 65% of them will have severe chromosome anomalies. A recent study by Sadowy *et al.* (5) also reported that some 64% of embryos derived from zygotes with dysmorphic pronuclei had abnormal chromosomes on FISH analysis.

Pronuclear formation starts with decondensation and replication of DNA, followed by depolarization of the membranes and alignment of both maternal and paternal pronuclei in a specific orientation. The maternal pronucleus remains near the pericortical cytoplasm, while the male pronucleus migrates toward the maternal pronucleus until singamy occurs (6). Any failure in the described sequence of events can result in abnormal fertilization and arrest of embryonic development. As detailed in Table I, Y signals were demonstrated in only five embryos with unequal pronuclei. Assuming equal segregation of the X and Y chromosomes, this would mean that normal fertilization occurred in only 10 (26.3%) of the embryos studied. Thus, in most embryos, unequal pronuclei suggest developmental failure of the male pronucleus and parthenogenetic activation of the oocyte. These findings are even more pronounced in the ICSI group, where 88.5% of the zygotes with unequal-sized pronuclei led to chromosomally abnormal embryos. A comparable situation is found with single pronucleus (1 PN) formation—in the ICSI group, 1 PN indicates, in most cases, activation of the oocyte. In contrast, more than half (61.9%) of the 1 PN embryos developed after conventional in vitro insemination were found to be diploid and to develop normally (7). The ICSI procedure by itself can explain parthenogenetic activation (7).

Our results also point to an association between a higher number of oocytes retrieved and an increased frequency of unequal pronuclei after fertilization. This effect of aggressive induction of ovulation on the condition and maturity of the oocyte cytoplasm has been reported by Sadowy *et al.* (5) to be more common in regular IVF than in association with ICSI. In our patients, the frequency of unequal pronuclei was 12.7% after in vitro insemination and 16.4% after ICSI (NS).

We found that the number of cells and of nuclei was different in half of the embryos evaluated, while the other 19 embryos had the same numbers of blastomeres and nuclei. It has been shown previously by Munné *et al.* (8), that some blastomeres do not contain nuclei, while in others more than one nucleus exists. Other studies (2, 9) showed that the presence of one or more multinucleated blastomere during early embryonic development is associated with a high percentage of numerical chromosome anomalies. The presence of variable chromosome anomalies caused by uncontrolled mitotic divisions in different blasto-

Table I. Chromosome Analysis of Embryos with Unequal Pronuclei

Case No.	No. of cells/nuclei	No. of abnormal cells	Protocol	FISH analysis
1	4/4		IVG	
2	7/7	4	IVF	−X,−18//−X,+18//−X,−18//−18
3	9/9	4	IVF	+X,+18//+X,+18//+18//−X,−18
4	9/9		IVF	
5	4/4[a]	3	ICSI	−X,−18//+X,//+X,+18
6	2/2[a]	2	ICSI	−X,−18//−X,−2x18
7	2/1[a]	1	ICSI	−X
8	2/3[a]	3	ICSI	−X//−2xX,−2x18//−2xX,−2x18
9	5/5	2	IVF	+X,+2x18,+2x(13–21)//+X,+18,+2x(13–21)
10	5/4	2	ICSI	−X,−18//+2x18
11	10/7	4	ICSI	+X//−18//−18//−X
12	2/2[a]	1	ICSI	+18
13	4/3		ICSI	
14	6/5	5	ICSI	All −X,−18
15	8/7		IVF	
16	5/2	2	IVF	−X,−18//−X,−18
17	1/1[a]	1	ICSI	−X,−18
18	4/3		IVF	
19	4/6	4	ICSI	+X,+Y,+2X18,+2x(13–21)//+X,+Y,+2x18,+(13–21)//+18//−(13–21)
20	6/6	5	ICSI	+18//+X,+18//+X,+2x18,+(13–21)//+18//+X,−(13–21)
21	2/3[a]		ICSI	
22	8/5	5	ICSI	All −X,−18,+(13–21)
23	4/4	1	IVF	+(13–21)
24	8/6	6	ICSI	
25	3/3	2	ICSI	−Y,+2x18//−Y,−18
26	4/6	5	ICSI	+X,+18//+X,+18//+X,−18//−X,−18//−2xX
27	32/32		IVF	
28	4/3	2	ICSI	−Y//−18
29	2/2[a]	2	ICSI	+2xX,+2x18//no signals
30	2/2[a]	2	ICSI	−X,−18//−X,−18
31	7/4	2	IVF	−Y,+18,−(13–21)//+y//
32	6/5	4	ICSI	+Y,−(13–21)//−18//−Y,−18,−(13–21)//−Y,−18,−(13–21)
33	6/5	5	ICSI	All −X,−18
34	6/5		IVF	
35	8/8	5	ICSI	+18//−X,−18//−18//+X,−18//+X
36	6/6		ICSI	
37	12/12		ICSI	
38	8/8	6	ICSI	+Y,+2x18//+Y//+Y,−X,+18//+X,−18//+X,−18//+X,+Y

[a] Stopped developing.

meres of the same embryo, a situation termed "chaotic" by Harper *et al.* (1), is also particularly frequent in multinucleated blastomeres (2). Recently, Delhanty *et al.* (10) noted that some patients had "chaotic" embryos in repeated cycles, whereas other patients were completely free of this type of anomaly. Chaotic embryos are unlikely to progress beyond implantation and may be the etiology of some cases diagnosed as unexplained infertility.

Advanced maternal age is associated with an increased risk of aneuploidy in liveborns. The effect of maternal age on embryonic chromosome anomalies has been reported by Munné *et al.* (8), in embryos with good morphology, the rate of nonmosaic chromosome anomalies increased from 16% at maternal age 20 to 34 years to 36.5% at 35 to 39 years and 52.7% after 40 years of age. The mean maternal age in our study was 32.2 years. Thus, controlled for maternal age, the rate of chromosomally abnormal embryos in our study was twice higher than that reported by Munné (8): the relative risk can be calculated as 1.5 in the conventional IVF group and 2.6 in the ICSI embryos. Laverge *et al.* (2) analyzed 116 embryos with poor morphological score and/or multinucleated blastomeres, most of them obtained after ICSI, and found that only 33.6% of them were uniformly diploid. This result is almost 3 times higher than the rate of normal embryos in the ICSI group in our study, emphasizing that uneven pronuclei

are apparently a more powerful predictor of embryonic chromosome anomalies than abnormal morphology or multinucleation.

In conclusion, despite apparently normal morphology, a very high percentage (50% to 88%) of embryos developing from zygotes containing pronuclei of unequal size are chromosomally abnormal. Preimplantation genetic evaluation prior to embryo transfer is strongly recommended in these cases.

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