

Article

Pronuclear morphology predicts embryo development and chromosome constitution



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Abstract

The relationship between pronuclear morphology on the one hand, and embryo development and chromosomal status on the other, was evaluated in 68 couples scheduled to undergo preimplantation genetic diagnosis because of advanced maternal age or recurrent implantation failure. Zygotes were grouped according to their pronuclear (PN) morphology. During the period from fertilization to embryo transfer, cleavage rate, embryo quality, blastocyst formation and results of the chromosomal analysis of 240 embryos were recorded. Both embryo cleavage characteristics and chromosome constitution were related to PN morphology. Embryos developing from zygotes with the normal PN pattern (pattern 0) cleaved faster and formed embryos with better morphology as compared with zygotes with abnormal PN patterns. Aneuploidy rate of embryos derived from zygotes with the normal PN pattern, with a single PN anomaly and with a double PN anomaly was 25.6, 73 and 83% respectively. Chromosomally normal embryos with the normal PN pattern progressed to the blastocyst stage at a higher rate (90%) than chromosomally normal embryos with a single (61%) or a double (40%) PN anomaly. The same relationship applied to chromosomally abnormal embryos. It is concluded that PN morphology predicts both the risk of embryo developmental arrest and that of chromosomal abnormalities.

Keywords: *aneuploidy, blastocyst, embryo development, embryo selection, preimplantation genetic diagnosis, pronuclear morphology*

Introduction

Pronuclear morphology assessment of zygotes has been largely considered as a tool for the prediction of subsequent embryo development (Balaban *et al.*, 1998; Scott and Smith, 1998; Tesarik and Greco, 1999; Scott *et al.*, 2000; Tesarik *et al.*, 2000; Lundqvist *et al.*, 2001; Montag and van der Ven, 2001; Salumets *et al.*, 2001; Scott, 2003). Recent studies have revealed a correlation between polarity or morphological status of two pronuclei and further cleavage of the embryo (Scott and Smith, 1998; Tesarik and Greco, 1999). The pronuclear pattern of the zygote was also shown to be closely related to blastocyst formation and quality, an ideal pronuclear pattern being associated with better quality cleavage stage embryos, higher cleavage rates and a higher incidence of blastocyst formation (Scott *et al.*, 2000; Balaban *et al.*, 2001).

Pronuclear scoring is increasingly used as a single selection criterion in countries in which government legislation does not permit embryo selection (Ludwig *et al.*, 2000; Montag and van der Ven, 2001; Zollner *et al.*, 2002). Moreover, in conjunction with embryo grading on days 2 and 3 after fertilization, it offers embryo selection facilities that are comparable with those provided by extended embryo culture to the blastocyst stage (Rienzi *et al.*, 2002).

It has been estimated that, depending on maternal age, some 40–50% of embryos derived from assisted reproductive techniques have some kind of chromosomal abnormality (Munné and Cohen, 1998; Kuliev *et al.*, 2003), and this proportion is even higher among embryos showing retarded growth and abnormal cellular morphology (Findikli *et al.*, 2004). Elimination of aneuploid embryos after preimplantation

genetic diagnosis (PGD) improves implantation rate in women of advanced reproductive age (Munné *et al.*, 2003). Severe chromosomal abnormalities may compromise embryonic development, and cleavage arrest is often observed in different stages of development. However, progression to the blastocyst stage does not guarantee an embryo with a normal chromosomal constitution (Banerjee *et al.*, 2000; Sandalinas *et al.*, 2001). Simple aneuploidies such as trisomy 21 affect neither the mitotic index nor the morphological grade of the cleaving embryo. The question then arises whether PN morphology can also predict the chromosomal normality of embryos.

Two recent studies have suggested a link between zygote pronuclear morphology and chromosomal status of the embryo (Coskun *et al.*, 2003; Gámiz *et al.*, 2003). The purpose of this study was to further analyse the relationship between pronuclear morphology, embryo cleavage characteristics and the chromosomal status of the preimplantation embryo.

Materials and methods

Design

A retrospective analysis of relationships between pronuclear morphology, embryo development to the blastocyst stage and chromosomal status of cleaving embryos was undertaken in 68 couples who were scheduled to undergo PGD for advanced maternal age or recurrent implantation failure in IVF/intracytoplasmic sperm injection (ICSI). The study was approved by the Institutional Review Board of the American Hospital of Istanbul, and each couple signed individual consent forms.

Assisted reproduction techniques

Ovarian stimulation was undertaken either with a long gonadotrophin-releasing hormone (GnRH) agonist protocol starting in the mid-luteal phase of the previous cycle, or with a microdose flare-up protocol combined with human menopausal gonadotrophin or recombinant follicle stimulating hormone (FSH). Final maturation of the oocytes was triggered with 10,000 IU of human chorionic gonadotrophin (HCG; Profasi, Serono) when the leading follicle reached 20 mm and there were at least two more follicles of ≥ 16 mm in diameter. Oocyte recovery was performed 36 h after HCG administration. IVF or ICSI and in-vitro culture of embryos were undertaken as previously described (Balaban *et al.*, 1998; Gardner *et al.*, 1998). Sequential media system (G1 and G2 media; Vitro Life, IVF Science Scandinavia, Gothenburg, Sweden), designed for embryo culture to the blastocyst stage, was used. Embryos were individually cultured in microdroplets containing G1 medium on days 1 and 2 and G2 medium on days 3–5.

On day 3 after IVF or ICSI, embryos were subjected to biopsy followed by fluorescence in-situ hybridization (FISH) of isolated blastomeres (see below). Those embryos that were found normal for the chromosome studied were cultured until the blastocyst stage and transferred to the patient's uterus on day 5 of development.

Evaluation of PN morphology

PN morphology of zygotes was evaluated 17 h after IVF or ICSI, according to previously described criteria set by Tesarik and Greco (1999). For simplification of data analysis, embryos were allocated to three groups. Group 1 consisted of embryos with the normal PN pattern, corresponding to pattern 0 according to the original classification (**Figure 1**), group 2 accommodated zygotes with a single PN anomaly, corresponding of one of patterns 1–5 of the original classification (**Figure 1**), and group 3 included zygotes with 2 or more PN anomalies. These evaluations concerned two-pronucleated (2PN) zygotes only; one-pronucleated and three-or-more-pronucleated zygotes were not taken into account. Cleavage to the two-cell stage by 27 h after IVF or ICSI is referred to as early cleavage.

Embryo biopsy

Three days after IVF or ICSI, embryos suitable for biopsy were selected according to their blastomere number and quality. Only embryos with five or more blastomeres having <20% fragmentation were included. The zona pellucida was opened using a 1.48 μ m infrared diode laser in computer controlled non-contact mode (IVF Work Station and Zona Laser Treatment System; Hamilton Thorne Instruments, Beverly, MA, USA). The laser system was attached to the objective turret of an Olympus IX-70 inverted microscope. Zona opening was achieved by four or five shots of 25 ms using 55 MW power density. Embryos were placed in 5 μ l droplets of calcium–magnesium-free medium (EB-10; Vitrolife, Gothenburg, Sweden). Following zona opening, a single blastomere containing a clearly visible nucleus was removed from each embryo. The isolated blastomeres were placed in 5 μ l droplets of G1 medium and transported to the PGD laboratory.

FISH

Isolated blastomeres were washed in 0.6% BSA and 1% sodium citrate then fixed on glass slides using droplets of Carnoy's fixative containing methanol and acetic acid in 3:1 ratio. The fixative was slowly added until the cytoplasm was dissolved.

Multicolour FISH analysis was performed with five DNA probes (Vysis, Naperville, IL, USA) for simultaneous detection of chromosomes X, Y, 13, 18 and 21 (Gianaroli *et al.*, 1997). Briefly, the probe mixture consisted of LSI 13 (1314), CEP 18 (alpha satellite D18Z1), LSI 21 (21q22.13–21q22.2), CEP X (alpha satellite DXZ1) and CEP Y (alpha satellite DYZ3). The probes were labelled as follows: chromosome Y with Spectrum Aqua, chromosome 13 with Spectrum Orange, chromosome 18 with Spectrum Orange and Spectrum Aqua (1:1 mixture), chromosome X with Spectrum Orange and Spectrum Green (1:1 mixture), and chromosome 21 with biotin (Dr Weier, Lawrence Berkeley National Laboratory, UCSF, Berkeley, CA, USA). The latter was visualized with fluorescein isothiocyanate-conjugated avidin. The hybridization solution was constituted by mixing 1 μ l of each probe, concentrating the resulting mixture to 3 μ l and adding to 7 μ l of whole chromosome paint hybridization buffer (Vysis). The resulting 10 μ l of hybridization solution was

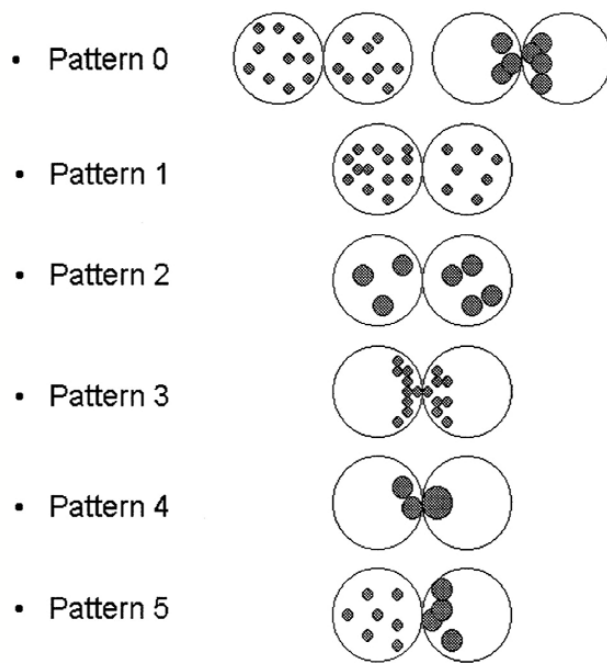


Figure 1. Schematic representation of PN morphology scoring system used in this study. Pattern 0 corresponds to normal zygotes patterns 1–5 represent PN irregularities. Reprinted with permission from Tesarik and Greco, The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology, *Human Reproduction*, 1999, **14**, 1318–1323, European Society of Human Reproduction and Embryology. Reproduced by permission of Oxford University Press/*Human Reproduction*.

added to the fixed blastomeres on a glass slide, the blastomeres were covered with a coverslip, and DNA was denatured at 78°C for 3 min. The slide was then left for 1–4 h at 37°C in a dark moist chamber to allow hybridization. After washing in 0.43 M sodium chloride/sodium citrate at 72°C for 2 min, fluorescein isothiocyanate-labelled avidin followed by anti-avidin was added. The slides were counterstained with 4',6-diamidino-2-phenylindole in antifade solution (Vysis, Downer's Grove, IL, USA) and observed in a fluorescence microscope (Olympus BX60) equipped with a triple band-pass filter set for simultaneous observation of Spectrum Orange, Green and Aqua.

Data analysis and statistics

During the period from fertilization to embryo transfer, the progression of cleavage, embryo quality, blastocyst formation and FISH results of the isolated blastomeres were evaluated. All these parameters were related to PN morphology. One-way analysis of variance and chi-squared tests were used to compare the groups. A P -value <0.05 was considered as statistically significant.

Results

A total of 642 oocytes were retrieved (9.4 oocytes/cycle; 1–21) of which 457 (71.1%) were in metaphase II stage. Following ICSI, 320 oocytes (70%) were fertilized. Eleven of these oocytes showed abnormal fertilization patterns (1 or >2 PN). The representation of individual PN patterns among the 309 normally fertilized 2 PN zygotes is shown in **Table 1**. The development of 11 of these zygotes arrested spontaneously before the first cleavage division, 15 cleaving embryos arrested with <3 blastomeres on day 2 after IVF or ICSI, and 16 embryos arrested with <5 blastomeres on day 3, resulting in 267 embryos cleaving through day 3 of incubation and thus available for biopsy (**Table 2**).

In 27 of the 267 embryos subjected to biopsy, FISH analysis failed to reveal satisfactory results (10%). Of the 240 remaining embryos, 74 (31%) were found to be normal and 166 (69%) abnormal for the five chromosomes evaluated (**Table 2**). Most of the abnormal embryos showed aneuploidy (68%). Other abnormalities were polyploidy (21%) and haploidy (11%).

All embryos developing from pattern 0 (normal PN pattern) zygotes cleaved, and 75% of them showed good morphology (grade I or grade II). The detection of a single PN anomaly was not associated with a reduction of cleavage rate, but fewer of these zygotes developed to good morphology embryos as compared with the normal PN pattern (53 versus 75%; $P < 0.05$) (**Table 3**). The presence of double PN anomalies was associated with a significant decrease in both cleavage rate (76 versus 100%; $P < 0.05$) and the percentage of good morphology embryos (39 versus 75%; $P < 0.05$) as compared with pattern 0 zygotes (**Table 3**). The percentage of embryos showing early cleavage also significantly correlated with the presence and severity of PN anomalies (**Table 3**). The percentage of zygotes that cleaved by 27 h after IVF or ICSI was lower when a single PN anomaly was detected as compared with the normal PN pattern (8 versus 25%; $P < 0.01$). In the case of a double PN anomaly, this percentage was further decreased as compared with zygotes with a single PN anomaly (0 versus 8%; $P < 0.01$) (**Table 3**).

FISH analysis revealed a 26% aneuploidy rate in embryos derived from pattern 0 zygotes. In contrast, 73 and 83% of the embryos derived from zygotes with, respectively, a single or a double PN anomaly were found to be chromosomally

Table 1. Overall distribution of pronuclear patterns of the zygotes.

PN pattern	n	%
0	28	9.0
1	50	16.1
2	41	13.2
3	46	14.8
4	52	16.8
5	51	16.5
1 + 2	19	6.1
2 + 4	22	7.1

Table 2. Overall results of preimplantation genetic diagnosis in 68 couples with advanced maternal age or recurrent implantation failure.

	n	%
Total no. cleaved embryos	283	100
Biopsied embryos	267	94.3
FISH failure	27	10.1
Normal embryos	74	30.8
Abnormal embryos	166	69.2
Aneuploidy	112	67.5
Monosomy	26	23.2
Trisomy	35	31.3
Nullisomy	10	8.9
Complex aneuploidy	41	36.6
Polyplody	35	21.1
Haploidy	19	11.4

Table 3. Relationship between pronuclear pattern, embryo cleavage and morphology.

	Normal PN pattern (pattern 0) (%)	Single PN anomaly (%)	Double PN anomaly (%)
No. pre-embryos	28 (100)	240 (100)	41 (100)
Cleaving embryos	28 (100) ^a	224 (93) ^a	31 (76) ^b
Grade I + II embryos	21 (75) ^a	126 (53) ^b	16 (39) ^b
Early cleavage	7 (25) ^d	18 (8) ^e	0 (0) ^f
>8 cell embryos on day 3	13 (46) ^a	56 (23) ^b	3 (7) ^c

a versus b versus c: $P < 0.05$.d versus e versus f: $P < 0.01$.

Successive data in columns are given as percentages of the top row.

Table 4. Relationship between pronuclear pattern, chromosomal status and blastocyst development.

	Normal PN pattern (pattern 0) (%)	Single PN anomaly (%)	Double PN anomaly (%)
Embryos analysed by FISH	27 (100)	183 (100)	30 (100)
Euploid embryos	20 (74) ^a	49 (27) ^b	5 (17) ^c
Aneuploid embryos	7 (26) ^a	134 (73) ^b	25 (83) ^b
Blastocysts from euploid embryos	18 (90) ^a	30 (61) ^b	2 (40) ^c
Blastocysts from aneuploid embryos	2 (29) ^a	14 (10) ^b	1 (4) ^c

a versus b versus c: $P < 0.05$.

Successive data in columns are given as percentages of the top row.

abnormal (**Table 4**). Blastocyst formation also correlated with PN morphology and chromosomal constitution of the embryo. The percentage of embryos reaching the blastocyst stage by day 5 of in-vitro culture was significantly compromised by the presence of a single PN anomaly and further reduced by the presence of a double PN anomaly (**Table 4**). However, some aneuploid embryos did develop into blastocysts, albeit at a lower rate than chromosomally normal embryos, irrespective of the previously detected PN pattern (**Table 4**).

All of the 68 couples enrolled in this study had an embryo transfer, leading to 15 pregnancies (pregnancy rate, 22.1%), three of which were lost before the detection of heartbeat. Altogether, 74 embryos were transferred. Twelve embryonic sacs with heartbeat were detected, giving an implantation rate of 16.2%.

Discussion

The results of this study show that pronuclear morphology can predict not only the risk of developmental arrest and abnormal cleavage of preimplantation human embryos, but also shows a clear relationship with the incidence of chromosomal abnormalities. This latter finding confirms and extends the observations published in another recent study (Coskun *et al.*, 2003). However, another study suggests that this relationship may not be present in embryos derived in older women (Gámiz *et al.*, 2003). The data are also in agreement with previous studies showing a correlation between PN morphology and different parameters of preimplantation embryo development (Balaban *et al.*, 1998; Scott and Smith, 1998; Tesarik and Greco, 1999; Scott *et al.*, 2000; Tesarik *et al.*, 2000; Lundqvist *et al.*, 2001; Montag and van der Ven, 2001; Scott, 2003).

It has previously been shown that there is a good relationship between markers of embryo normality/quality starting from the zygote up to the blastocyst stage (Balaban *et al.*, 2001). Similar to Scott *et al.*, this study has further simplified the Tesarik system by gathering six PN patterns into three groups (Scott *et al.*, 2000). Zygotes showing an ideal PN pattern developed embryos that cleaved earlier and faster, had better cleavage stage embryo 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 implantation and clinical pregnancy rates (Ludwig *et al.*, 2000; Scott *et al.*, 2000; Balaban *et al.*, 2001; Montag and van der Ven, 2001).

The question of whether the relationship between PN morphology and embryo chromosomal constitution is affected by female age is an interesting one. However, the number of patients involved in the present study and the age differences between individual women were too small to compare groups of women of different age with sufficient power.

The ultimate goal in assisted reproductive techniques is to obtain embryos with the optimal implantation potential. High

implantation rates were reported for embryo transfer at the blastocyst stage (Gardner *et al.*, 1998). Although development to the blastocyst stage cannot guarantee chromosomal normality, the majority of the embryos failing to continue to develop in extended culture show multiple aneuploidy for chromosomes X, Y, 16, 18 and 21 (Jones and Trounson, 1999). Prolonging the culture period thus appears to eliminate some of the chromosomally abnormal embryos. The results are in agreement with this presumption. Studies using FISH analysis of day 3 human embryos have shown that significantly more euploid embryos reach the blastocyst stage compared with their aneuploid counterparts, although this selection is not reliable and chromosomally abnormal embryos can also make very nice blastocysts (Magli *et al.*, 2000; Sandalinas *et al.*, 2001). Therefore, if used alone, extended culture to day 5 or 6 is not a reliable tool for the selection against clinically relevant chromosome abnormalities.

Even though meiotic spindle anomalies in the first and, to a lesser extent, in the second meiotic division are known to be the main source of embryo aneuploidy (Steuerwald *et al.*, 2001; Kuliev *et al.*, 2003), this condition may also be of sperm or post-zygotic origin (Findikli *et al.*, 2004) and may also be conditioned by epigenetic mechanisms acting after fertilization (Ménézo *et al.*, 1997). It has been shown recently that paternal effects on the early development of the human embryo become manifest as early as the pronuclear stage (Tesarik *et al.*, 2002). The higher incidence of chromosomal abnormalities in embryos developing from zygotes with aberrant PN morphology may thus also be attributed to a variety of nuclear, mitochondrial or cytoplasmic dysfunctions in both the spermatozoon and the oocyte resulting from a different kinds of intrinsic and extrinsic factors, including genetic abnormalities, hypoxia or oxidative stress (Van Blerkom *et al.*, 1995).

It is not clear how the pronuclear morphology is affected by the genetic construction of the zygote or how disturbances in pronuclei formation lead to failure in the gathering of the chromosomal contents of two different gametes. It has been proposed that major disturbances in pronuclear development are incompatible with further embryonic growth and adversely affect embryo implantation potential (Tesarik and Greco, 1999). The polarization of chromatin and nucleoli may represent an early step of the formation of embryonic axis, which will determine subsequent developmental capacity of embryos (Edwards and Beard, 1997). The pattern of embryonic nucleogenesis and distribution and composition of nucleoli are well programmed in a time-dependent manner (Tesarik and Kopecny, 1989; Payne *et al.*, 1997). Timing of growth and fusion of nucleolar precursor bodies were dependent on an early wave of PN transcriptional activity (Tesarik and Kopecny, 1990), and any failure in these steps may affect the development of embryos (Wittemer *et al.*, 2000).

Asynchrony or inequality between pronuclei might be markers for abnormalities in the genetic programming of the preimplantation development. It has been shown that large differences in the sizes of pronuclei were associated with chromosomal defects like aneuploidy (Munné and Cohen, 1998; Sadowy *et al.*, 1998). Different morphological patterns in PN may reflect the abnormal results of aberrant meiotic or

mitotic events such as aneuploidies or polyploidies. Replicative or transcriptional disturbances may present as failure or delay in the formation or polarization of nucleoli or pronuclei.

According to the present data, several conclusions can be reached. Firstly, zygotes with normal PN patterns more often give rise to genetically normal embryos compared with zygotes with abnormal PN patterns. Secondly, more than 80% of zygotes with multiple PN abnormalities result in aneuploid embryos. Thirdly, a high proportion (90%) of euploid embryos derived from normal zygotes reach the blastocyst stage. Interestingly, however, blastocyst stage was also reached by 29% of aneuploid embryos derived from zygotes with the normal PN pattern. On the other hand, very few aneuploid embryos that were derived from zygotes with abnormal PN patterns reached the blastocyst stage. These data confirm the idea that transfer of embryos at the blastocyst stage does not universally protect against aneuploidy. However, combination of prolonged culture with an early evaluation of embryos at the PN stage can be expected to improve the accuracy of embryo selection.

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