Journal of Assisted Reproduction and Genetics, Vol. 22, No. 3, March 2005 (© 2005)
DOI: 10.1007/s10815-005-4874-x

Assisted Reproduction

The impact of pronuclear orientation to select chromosomally normal embryos

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Submitted October 1, 2004; accepted October 29, 2004

***Purpose*:** To find an effective method to select embryos from ICSI with better chromosomal status when preimplantation-genetic-diagnosis (PGD) is not applied.
***Methods*:** Several morpholological evaluations were done in same embryos. Embryos from ICSI were classified on day 1 according to pronuclear-nucleoli arrangement. On day 3, classification was done according to number, fragmentation, size and shape of cells. In some patients, embryos exhibiting good quality on day 3 (at least six regular blastomeres with cell fragmentation lower than 20%) were also submitted to PGD, irrespective to pronuclear-nucleoli morphology.
***Results*:** Forty-two per cent of normally fertilized embryos showed pronuclear-nucleoli-good-morphology; from those, 86% were also classified as good quality on day 3. Good-quality embryos submitted to PGD have shown lower chromosomal abnormality rates when also classified as pronuclear-nucleoli-good-morphology.
***Conclusions*:** Pronuclear-nucleoli morphology seems to be correlated with PGD results. This criterion may prove useful for pre-select embryos with normal chromosomal package when PGD is not applied.

KEY WORDS: Embryo selection; ICSI; PN morphology; preimplantation diagnosis.

INTRODUCTION

Current techniques used in human assisted reproduction to identify better-quality embryos may result in doubtful response. Successful pregnancy cases include at least one "really good-quality embryo" selected. Embryo quality has been assessed in different aspects, with the same purpose: the selection of those with highest implantation potential.

IVF laboratories currently perform embryo transfer on day 2 or 3 after fertilization. From all available embryos, a substantial number of morphologically suboptimal pre-embryos are discarded. The decision to discard the pre-embryos is commonly based for existing non-invasive markers performed under low magnification at laboratories.

Several non-invasive strategies can be used to improve end-results: pronuclei disposition, nucleolar organization (1–3), assessment of cell number, the morphological appearance at the time of transfer, and identification of normal-cleaving embryos with only mononucleate blastomeres without fragmentation (4–9).

Morphological and developmental features in human embryos have generally been associated with chromosomal abnormalities as well as with embryo

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failure, although normal embryos showing may be genetically abnormal.

This assumption is partially based on the fact that at 4–8 cell pre-embryo stage is just beginning to make use of its own genomic expression (10,11) and aneuploid or suboptimal pre-embryos might not be detected. In spite of this, differential morphology of human 1-cell pronuclear oocytes in terms of nuclei morphology and the cytoplasmic movements have been strongly correlated with implantation and development to the blastocyst stage (1,12).

Prolonged culture systems should be an alternative to emphasize the assumption that culturing pre-embryos to blastocyst stage will favor normal pre-embryos with higher potential of implantation—thus allowing self-selection of those embryos capable of proceeding to blastulation—and exclusion of those less viable (13,14). Most of embryos from IVF patients (40–60%) are genetically abnormal (15), and as many as 40% of blastocysts exhibit some degree of chromosomal mosaicism in inner cell mass (16). The high rate of genetic dysfunction correlates with previously reported blastocyst conversion rates of 50% (17), as well as with our laboratory findings, that almost half embryos on day 3 seems will not survive until day 5. Since absolute correlations are seldom seen, embryo selection remains one of the most difficult endeavors in assisted reproduction.

Although it is an invasive method, pre-implantation genetic diagnosis (PGD)—followed by fluorescence in situ hybridization (FISH)—may be performed to aneuploidy screening at 8-cell stage (15,18,19). While PGD has the advantage of selecting numerically normal embryos for some chromosomes, this technique is only indicated for patients reporting the following conditions: advanced maternal age, recurrent miscarriage, severe male factor, and genetic diseases.

Based on our own studies and literature findings, we have been tried to establish a correlation between embryos quality, resulting from intracytoplasmic sperm injection (ICSI) cycles, in accordance with zygote and day 3 morphology. In addition, another comparison procedure was carried out between good-quality embryos in both classifications, pronuclei (PN) and day 3, compared with PGD results. At the same time, we also have tried to find an effective non-invasive method to select embryos with better chromosomal status following ICSI in cases without PGD indications.

MATERIAL AND METHODS

This prospective study was conducted at the Fertility-Assisted Reproduction Center, in São Paulo, Brazil. Patients and embryos under this study were in compliance with guidelines approved by Internal Review Board, which included informed written consent. It was assessed that 2,978 embryos from 579 unselected and consecutive ICSI cycles is performed in 470 patients.

Ovary Stimulation and Oocyte Retrieval

Baseline characteristics of women randomized to receive ICSI were similar among the groups, as well as stimulation period, gonadotropin use, and oocyte retrieval.

Briefly, stimulation protocol was started by administering GnRH agonist (leuprolide acetate - Reliser, Serono Laboratórios, São Paulo, Brazil) close to 14 days. After ovarian suppression, superovulation was stimulated with recombinant FSH (Gonal-F®, Serono Laboratórios São Paulo, Brazil) in a step-down protocol. When at least two follicles were 18 mm in mean diameter, hCG 10.000 IU (Profasi®; Serono Laboratórios, São Paulo, Brazil) was administered, and 35–36 h later, follicular puncture was performed under transvaginal ultrasonography.

Oocytes retrieved were incubated in human tube fluid medium (HTF - Irvine Scientific # 90125, USA), supplemented for 10% serum substitute supplement (SSS - Irvine Scientific # 99193, USA) for 3–5 h before cumulus cell removal. After that, oocytes were placed in 80 IU/ml hyaluronidase (Irvine Scientific # 90101, USA) for 30–60 s. Oocytes were transferred to fresh medium and—with the use of a pulled pipette—the corona cells remained was removed by gently pipetting in and out.

ICSI Procedure, Culture and Embryo Classification

ICSI procedure was performed as described by Palermo *et al.* (20). The oocytes used to procedure were in metaphase II.

Seventeen hours after ICSI, oocytes were checked for fertilization, transferred to IVF medium and covered with mineral oil. A zygote scoring procedure was performed in all embryos with 2 pronuclei (PN). Abnormally fertilized or unfertilized oocytes were removed from the dish and were not taken into account. Oocytes containing two PN were cultured separately in droplets of 100 μL of HTF medium

supplemented with 10% HSA (Irvine Scientific # 9988, USA), covered with mineral oil in a dish and were kept until transfer.

On zygote morphological analysis, we evaluated the PN arrangement as well as the distribution and number of nucleolar precursor bodies (NPB). The relative size of both PN (equal or unequal) and their position (aligned or not) were noted. The distribution of NPB was considered polarized when all NPB present in PN were in the hemisphere whose pole was the point of contact with the other PN. The NPB number was also assessed. A range of 3–7 NPB was considered normal. This morphological description of the PN stage pre-embryos can be translated into a scoring system which may be evaluated by an experienced embryologist in no later than 15 s. Based on this, it was considered normal pronuclear pattern (S0), embryos with aligned or aligning PN and no discrepant number of NPB when it was compared male and female PN (Fig. 1). Abnormal pattern (S1) of embryos had shown another condition of PN and NPB (Table I).

On day 3 (66–70 h), morphological analysis was recorded by number, size and shape of cells, fragmentation and the morphological condition of cleaving embryos, according to established protocol (Table II). "Good quality embryos" had shown at least six regular blastomeres and maximum of 20% fragmentation on that day. This criterion was used to select embryos to be submitted to PGD, irrespective of PN and cleavage classification on day.

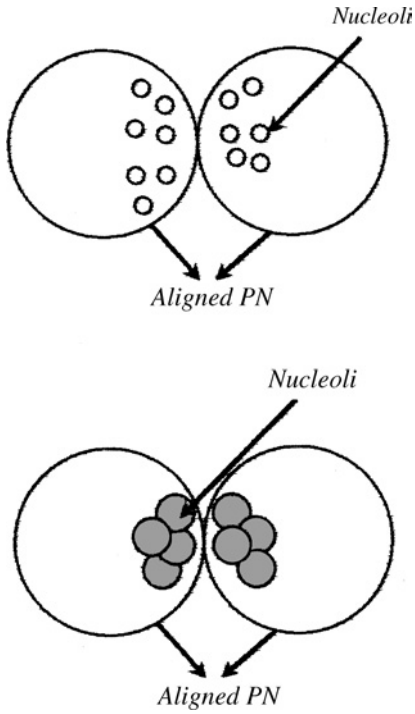

Fig. 1. Schematic representation of pronuclear morphology (PN) corresponding to normal zygote morphology (S0).

Table I. Definition of Presumptive Normal and Abnormal Patterns of Pronuclear Stage Morphology

Pattern	Description
S0	Polarized PN without discrepant number and size of NPB in both pronuclei
S1	Non-polarized PN Discrepant size between two PN Large difference (>3) in the number of NPB in both pronuclei Small number (<7) of NPB without polarization in at least one PN Very small number (<3) of NPB in at least one PN Large number (>7) of NPB with polarization in at least one PN Polarized distribution of NPB in one PN and non-polarized in the other

Genetic Analysis

From total of ICSI cycles, 47 cycles has provided embryos that must be submitted to PGD. It was by advanced maternal age, genetic diseases, severe male factor and/or recurrent miscarriage.

On day 3 classification, "good quality embryos" were selected for chromosomal analysis. Each embryo was biopsied in HEPES-buffered medium (Irvine Scientific # 90126, USA) after zona pellucida have been breached with 1.48 μ Diode laser. The biopsied blastomere was placed in hypotonic solution (1% sodium citrate) and fixed on glass slide by fixative (acetic acid/methanol), which was added until cytoplasm had been dissolved. Dehydration in ethanol was then carried out (70, 85, and 100%; 2 min each) (21).

Table II. Embryo Classification Grading on Day Transfer (Day 3)

Pattern	Description
Even blastomere (I)	(A) Without fragmentation (B) Fragmentation lower than 10% (C) Fragmentation: 11–20% (D) Fragmentation: 21–35% (E) Fragmentation higher than 35%
Uneven blastomere (II)	(A) without fragmentation (B) Fragmentation lower than 10% (C) Fragmentation: 11–20% (D) Fragmentation: 21–35% (E) Fragmentation higher than 35%

FISH Procedure and Interpretation of Fluorescence Signals

For the multicolor fluorescent in situ hybridization (FISH) analysis, five DNA probes were used for the simultaneous detection of chromosomes X, Y, 21, 13 and 18 (AneuVysion® EC Assay Kit Components 5 DNA Probes).

Briefly, the hybridization mixture was added to the fixed nucleus and denatured at 78°C for 3 min. After 4 hour of incubation at 37°C, the slide was washed in 0.4× sodium chloride/sodium citrate at 72.5°C for 2 min. Counterstaining in 4,6 diamidino-2 phenylindole in antifade solution was added and the slide was observed under a fluorescence microscope equipped with a triple band pass filter. FISH normal embryos were compared with morphological classification.

Statistical Analysis

Statistical significance was determined with the use of Student's *t*-test, χ^2 test combined with Yates' correction, and Fisher's exact test, as appropriate. Statistically significant differences were determined at the $p < 0.05$ level.

RESULTS

Five hundred seventy-nine ICSI cycles (579) performed on 470 patients were included in this study. Mean maternal age (SD) was 35.5 (5.7) years. The patients have provided 4,378 viable oocytes after ICSI and 81.3% (3,560 of 4,378) became embryos. Abnormal fertilization (1PN or 3PN) occurred in 582 embryos. Those embryos had shown normal fertilization (2PN) (2978 embryos of 3560–83.6%) were included in zygote classification.

After embryos classification into two scores (S0 or S1), it was also divided according to morphological classification on day 3. The number of S0 embryos also classified as "good quality embryos" on transfer day were assessed (Table III). No statistical correlation was found between PN classification and day 3 morphology; i.e., good embryos in PN stage could or could not be classified as "good quality embryos" on day 3.

Later on, 47 ICSI cycles and 37 patients reporting a small proportion of "good quality embryos" (161 in 2,978) were submitted to PGD analysis due to classical advanced maternal age (74.5%–35 cycles), genetic diseases (21.3%–10 cycles), severe male factor (2.1%–1 cycle), recurrent miscarriage (2.1%–1 cycle). Mean maternal age was 38.0 ± 3.2 years. Three cycles (3 patients/23 embryos) were excluded of this study because DNA probes were used for the detection of only three chromosomes (X, Y and 21).

One hundred sixty-five cells were biopsied and 143 (86.7%) exhibited PGD results. Out of those, despite of good morphology on day 3, 84 embryos (58.7%) were classified as S0 in PN stage. Embryos classified as S0 and "good quality" on day 3 showed lower chromosomal abnormalities after PGD results. However, embryos classified as "good quality" on day 3 but exhibited abnormal PN classification showed higher rate of chromosomal abnormalities (Table IV).

DISCUSSION

Attributes of normal development, such as holoblastic cleavage, absence of fragmentation and blebbing, and short intercleavage interval remain the main markers for embryo selection (22–24). The core objective of research on embryo selection, therefore, is to develop an objective, noninvasive, accurate, and simple method to choose right embryo.

In most centers, embryos are scored according to the number of blastomeres and grade of fragmentation, but it is still under investigation how this grading can be refined and whether additional testing could improve selection. A report prepared by Land and Evers (25) agreed that embryo morphology scoring procedures are insufficiently standardized, being relevant factors: cell number, lack of fragmentation and multinucleation, and equal cell size.

According to Van Royen *et al.* (26) and Neubourg *et al.* (27), embryos with four or five blastomeres on day 2 and seven or more cells on day 3, with no

Table III. PN Classification from Total Number of Embryos Exhibiting Normal Fertilization

Variables	Score 0	Score 1	Total	*p* value
2PN	1,251 (42.0%)	1,727 (58.0%)	2,978	
"Good quality embryos"	1,087/1,251 (86.9%)	1,470/1,727 (85.1%)	2,557/2,978 (85.8%)	0.188
Embryos selected for transfer	724/1,087 (66.6%)	952 / 1470 (64.8%)	1676/2,557 (65.6%)	0.979
Embryos selected to cryopreservation	380/1,087 (33.4%)	518/1,470 (35.2%)	881/2,557 (34.5%)	0.353

Table IV. Outcome of Embryos from Patients Submitted to PN Classification, Day 3 Morphology Classifications and PGD

	Score 0	Score 1	*p* value
Number of cells with PGD results	84	59	
Abnormal results on PGD	25/84 (29.8%)	33/59 (55.9%)	0.003
Haploidy	8/25 (32%)	11/33 (33%)	0.861
Triploidy	5/25 (20%)	11/33 (33%)	0.407
Polyploidy	1/25 (4%)	4/33 (12%)	0.535
Monosomy	5/25 (20%)	2/33 (6%)	0.227
Trisomy	6/25 (24%)	5/33 (15%)	0.607

more than 20% fragmentation and absence of multinucleated blastomeres during the whole observation, can show embryos with higher implantation potential. However, in other studies (5,28,29) traditional criteria for embryo selection on day 3 have seemed to be relatively inefficient.

While investigating the relative value of embryo selection on day 3, Graham *et al.* (29) did a retrospective comparison with day 5/6, and found a lack of embryo quality correlation between stages. Half of embryos chosen for use on day 3 were chosen again on day 5/6 (28,29). Furthermore, a major problem confounding these studies is the impossibility of knowing which embryo led to pregnancy, once multiple embryos are transferred.

Other groups (1–3,12,30–33) have been attributed high relevance to PN morphology, demonstrating strong relationship between zygote morphology and the viability of future embryos. Thus, those aspects have been observed through non-invasive techniques and it seems to play a key role in evaluation of embryo quality. Our group has already suggested PN elective criterion on selection of embryos.

Our PN scoring is a mix of two other PN scores described in scientific literature (1,2), and we already have studied the impact of our score in previous studies (unpublished data). In these studies, we have been classified embryos in accordance with Scott and Smith (1) (group 1), Tesarik and Grecco (2) (group 2) and our modified classification (group 3). No statistical difference was gotten in embryo development, pregnancy and implantation rates among these three classifications; however, we believe that our protocol was easier and faster than another.

We have already been suggested that PN classification associated with day transfer morphology is a valuable and additional non-invasive criterion for elective embryos transfer. In accordance with this criterion, over 80% of total pregnancies had, at least, one good quality embryo replaced to uterus. It seems to be effective mainly in ICSI cases, because our previous studies had a strong impact on morphological features of zygotes according to sperm quality and source (34,35).

In the present study, we have compared a possible correlation between PN and day transfer morphology. No correlation was found, i.e., no significant differences were obtained between embryo quality and cleavage status, in different patterns of embryo in zygote stage. A previous study that had included 294 ICSI cycles, with clinical pregnancy at 81% of treatment cycles, have been showed similar results. Furthermore, good pronuclei and nucleoli morphology of embryos are likely to show better embryos on day 3, based on the high pregnancy percentages when at least one S0 embryo was transferred (36). Such data have contributed for inclusion of PN classification in our clinical routine for embryo selection.

However, morphologically and developmentally normal embryos can still be genetically abnormal; therefore, they have been correlated with abnormal embryo development. Scientific literature has shown that 16% of morphologically normal embryos on day transfer has exhibited chromosomal abnormalities in patients who are 20–34 years old, 37% in patients who are 35–39 years old, and over 50% when the maternal age is 40–45 years (18,37,38). Special situations required other methods of embryos assessment, such as PGD.

Since the introduction of clinical PGD by Handyside group in 1990 (39), embryos with best implanting and developing potential can be selected to provide healthy babies in special groups of patients (recurrent miscarriage, multiple IVF failure, advanced maternal age, and family genetic diseases). In our center, PGD has already been carried out in 74 patients (104 cycles) due to advanced maternal age (71.3%); severe male factor (18.1%), heritance chromosomal disease (7.4%) and recurrent miscarriage (3.2%). Our results showed numerical alterations in 44.5% of those embryos selected to be transferred by normal morphological appearance on day 3 (40). The high percentage of chromosomal abnormalities even in morphologically normal embryos, make difficult to choose the best embryos with normal chromosomal status. The remaining question is: how can we select chromosomally normal embryos for patients without PGD indications?

The main parameters to select embryos PGD on day 3 were cleavage stage and morphology; embryos showing compromised morphology were not selected. However, selection based on morphology

is highly controversial in literature, where some of them say that embryos with a compromised morphology still give viable pregnancies (28,41).

With improved culture media systems, it is possible to extend culture until blastocyst and some groups have believed in down-selection through that (17,42–44). This process enables to choose embryos with higher viability, although day 3 morphology seems not to be predictive for further blastocyst development (45). On the other hand, PN scoring allows preselection of embryos developing into blastocyst stage (3). Besides these chromosomally abnormal embryos can also develop into blastocyst stage (46).

The present study has shown close correlation between PN morphology and PGD results irrespective of day 3 morphology. All embryos submitted to PGD were classified according to day 3 morphology as "good quality embryos." Embryos classified as S0 and also good quality on day 3 showed lower chromosomal abnormalities after PGD results. However, when embryos were only classified as good quality on day 3 and exhibited abnormal PN classification (SI), the rate of chromosomal abnormalities was much higher.

PN morphology seems to exhibit relevant information on chromosomal status of embryos. We believe that those embryos carrying normal chromosomal conditions allow normal pronuclear development, such as the normal pronuclear apposition and growth, to become compatible with further normal development of zygote. The abnormal pronuclear stage indicates the failure of one or more fertilization events, probably due to some structure impairment to form normal pronuclei arrangement, and consequently embryos with limitless implantation potential. Such data have been contributed for the inclusion of this criterion in our IVF laboratory routine.

Gianarolli *et al.* (47) studied patients with advanced maternal age or repeated IVF failures, who known to have predisposition to generate a high proportion to chromosomally abnormal embryos. They validated a scoring system for embryos selection generated by PGD patients, and have showed that morphological analyses performed at pronuclear stage can help select embryos to be transferred. Although a correlation between morphology, developmental competence and chromosomal abnormalities is established, the absolute correlations are rare and embryo selection remains one of the most arduous tasks in assisted reproduction.

As other groups (6,7,48,49), we have proposed a modified cumulative embryo score (35,50) from PN evaluation until day transfer. Following such criterion the embryo selected to be transferred (top quality embryo) must feature good PN morphology (S0), two cells at 26 h after ICSI, and also be classified as "good quality" on day 3. Nevertheless, the embryos exhibiting such characteristics account for only 3.1% of total embryos (unpublished data), and this can probably exhibit embryos with higher implantation potential.

In conclusion, data presented show that human zygote morphology is a valuable, additional, non-invasive criterion that can be useful to pre-select embryos with normal chromosomal package when PGD is not indicated. Morphological characteristics on PN stage pre-embryos can be translated into a scoring system in less than 15 s, so that it can be integrated in the routine work, without detrimental effect on the quality of the embryos. Zygote scoring associated with other morphological features allows selection of the best embryos, especially without PGD indications. Cleavage status evaluation can be considered a complementary parameter but should not be taken into account alone to decide which embryo should be transferred.

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