Pronuclear and Morphological Features As a Cumulative Score to Select Embryos in ICSI (Intracytoplasmic Sperm Injection) Cycles According to Sperm Origin

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Purpose: To analyze best parameter to select embryos according to sperm origin in ICSI cycles.

Methods: One hundred seventy-two ICSI cycles were divided among three different groups: A (ejaculated spermatozoa from nonmale factor infertility), B (ejaculated spermatozoa from oligospermia), and C (spermatozoa from azoospermia). Embryos were divided on Day 1 into two patterns: S0 (pronuclei (PN) aligned and close with normal arrangement of nucleoli) and S1 (when these characteristics were absent) and also on transfer day according to morphological features.

Results: Relationships of PN patterns related to sperm origin were noted. More S0 embryos were detected with better sperm quality. Higher number of good quality embryos was obtained when male factor was absent. Ejaculated and epididymal spermatozoa provide better quality embryos than do testicular spermatozoa.

Conclusions: PN classification associated with transfer day morphology is valuable additional noninvasive criterion for elective embryo transfer, mainly in the cases with severe male factor.

KEY WORDS: Embryo selection; ICSI; morphological features; PN morphology; sperm origin.

INTRODUCTION

In the era of assisted reproductive technologies, the intracytoplasmic sperm injection (ICSI) has become a powerful therapeutic tool to overcome fertilization failure with various degrees and types of infertility (1). However, the efficiency of assisted reproductive techniques in the human still has been

low with <30% of embryos transferred achieving developmental potential.

It is accepted that successful embryo implantation depends mainly on embryo quality. Nowadays, every possible effort should be made to find the best embryo for uterine transfer with the aim of achieving a maximal chance of pregnancy and, at the same time, avoiding the risk of multiple-child birth. Several parameters are used routinely to evaluate embryo developmental potential before transferring to the uterus, including the timing, morphology, and characteristics of the different stages (2–6).

Recently, it has been shown that pronuclear morphology assessment correlates well with implantation and pregnancy rates (1,4,7–9). This criterion has been reported to be of value as an additional and important tool for prediction of embryo development independently of semen quality, allowing the selection of

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competent and viable embryos at a stage as early as pronuclear development, when paternal and maternal genomes are still physically separated.

Demirel *et al.* (10) have proposed to find out some correlation between the source of spermatozoa in ICSI and the morphological features of pronucleate zygotes, which make up the basis of a pronuclear scoring system for the selection of the most viable embryos to transfer. In that work, ejaculated and testicular spermatozoa were similar in terms of embryo quality and development. However, the real influence of male gamete quality is still unsolved.

The purpose of our study was to find out whether the quality of spermatozoa used for ICSI has an impact on the morphological features of pronuclear zygotes.

Furthermore, on the basis of our own classification score system, we evaluated an elective parameters to select embryos in ICSI cycles, using ejaculated epididymal or testicular spermatozoa.

MATERIALS AND METHODS

The records of 172 ICSI cycles (141 patients) were included in this prospective study from December 1999 to September 2000. Cycles were divided in three groups according to ICSI indication: Group A (ejaculated sperm from nonmale factor, $n = 110$ cycles/84 patients), Group B (ejaculated sperm from oligospermic patients, $n = 32$ cycles/32 patients), and Group C (testicular or epididymal spermatozoa from azoospermic patients, $n = 30$ cycles/25 patients).

Nonmale Factor Indications

In Group A, the use of ICSI includes the following situations: tubal and anovulatory factors, idiopathic or unexplained fertility; generation of embryos for preimplantation genetic diagnosis (PGD) where embryos "clean" from any extraneous contaminating sperm is needed in some cases when the male factor is absent; or, in any case where there is an extreme need to maximize normal fertilization, e.g., when a woman has only a few eggs retrieved.

Oligospermic Patients

This group has included patients with very poor sperm parameters defined as a sperm count of $<1 \times 10^6/\text{mL}$ or a recovery rate of $<0.5 \times 10^6$ motile spermatozoa, and the proportion of morphological

normal spermatozoa according to Kruger's strict criteria being 4% or less. Ejaculated spermatozoa were prepared early on the morning of oocyte retrieval with the discontinuous gradient of density (isolate) method.

Azoospermic Patients

On the day of ovum pickup, percutaneous epididymal sperm aspiration (PESA) or testicular sperm aspiration (TESA) was performed in cases of obstructive and nonobstructive azoospermia, respectively. Patients who have failed to PESA and need to be submitted to TESA procedure were excluded from this study. All procedures were performed in our outpatient procedure room under local anesthesia.

Ovary Stimulation and Oocyte Retrieval

The baseline characteristics of women randomized to receive ICSI were similar among the groups, as were the duration of stimulation, gonadotrophin use, and oocyte retrieval.

Ovulatory induction was performed through ovarian blockage with GnRH agonist (leuprolide acetate) for at least 14 days. Superovulation was made with recombinant FSH (Gonal-F[®], Serono Laboratórios, São Paulo, Brazil) in a step-down protocol, until a minimum of two follicles reach an average diameter of 18 mm. Follicular puncture was performed 35–36 h after the administration of hCG 10,000 IU (Profasi[®], Serono Laboratórios) under transvaginal ultrasonography. Oocytes were recovered and incubated in human tube fluid (HTF) medium supplemented 7.5% SSS for approximately 3–5 h before cumulus cell removal. Cumulus cells were removed from the oocytes by placing them in hyaluronidase at a concentration of 80 IU/mL for approximately 30–60 s. Oocytes were transferred to fresh medium and, with the use of a pulled pipette, the corona cells were removed by gently pipetting in and out.

ICSI Procedure, Culture, and Embryo Classification

ICSI procedure was performed with oocytes in metaphase II according to the technique described by Palermo (11).

Seventeen hours after ICSI, fertilization was assessed in oocytes in IVF medium and covered with mineral oil. A zygote scoring procedure in all 2PN embryos was performed. Abnormally fertilized or unfertilized oocytes were removed from the dish and

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

were not taken into account. Oocytes containing two pronuclei were cultured separately in droplets of 30 μ L of Gamete medium covered with mineral oil in a dish and were kept until transfer.

For the morphological analyses on zygote stage, we evaluated in this study the pronuclear arrangement (PN) as well as the distribution and number of nucleolar precursor bodies (NPBs). The relative sizes of both pronuclei (equal or unequal) and their position (aligned or not) were noted. The distribution of NPBs was considered polarized when all NPBs present in the pronucleus were in the hemisphere whose pole was the point of contact with the other pronucleus. The number of NPBs was also assessed. A range of 3–7 NPBs was considered normal. On the basis of this, normal pronuclear pattern (S0) was attributed to those embryos that have aligned or been aligning PN and no discrepant number of NPBs compared between male and female PN (Fig. 1). An embryo showing another conditions (S1) was rated as abnormal pattern (Table I).

Twenty-four hours later, cleavage of fertilized oocytes was assessed. On Day 3, a morphological classification comprised grades according to number, size, and shape of cells; fragmentation and the morphological condition of cleaving embryos was assessed on Day 3 after ICSI procedure according to established protocol (Table II).

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

Pattern	Description
S0	Polarized PN with no discrepant number and size of NPBs in both PN
S1	Nonpolarized PN Discrepant size between two PN Big difference (>3) in the number of NPBs in both PN Small number (<7) of NPBs without polarization in at least one PN Very small number (<3) of NPBs in at least one PN Large number (>7) of NPBs with polarization in at least one PN Polarized distribution of NPBs in one PN and nonpolarized in the other

Note. PN-Pronuclei, NPBs Nuclear polar bodies (nucleoli).

Embryos that had shown S0 classification on zygote stage and, at least, six cells on Day +3 (transfer day) and the maximum of 20% of fragmentation were considered “good quality embryos.” However, the morphology on transfer was mandatory to embryo selection, regardless the PN morphology. Selected embryos were transferred into 15 μ L of IVF medium using Edwards Wallace catheter of 23 cm (Simcare Manufacturing Ltd., United Kingdom). Luteal phase support was done by daily administration of 600 mg of vaginal progesterone (Utrogestan[®], Laboratories, Besins-Iskovesco, Paris, France). Serum β -hCG levels were measured 12 days after the embryo transfer.

Main Outcome Measured

Laboratory and clinical data were analyzed. Normal fertilization rates (2 PN), percentage of S0 embryos in each group, percentage of S0 embryos that was also classified as a good embryo on day transfer, number of S0 embryos transferred, quality and

Table II. Embryo Classification Grading on Day Transfer

Pattern	Description
Even blastomere (I)	(A) Without fragmentation (B) With less than 10% of fragmentation (C) With 11–20% of fragmentation (D) With 20–50% of fragmentation (E) With more than 50% of fragmentation
Uneven blastomere (II)	(A) Without fragmentation (B) With less than 10% of fragmentation (C) With 11–20% of fragmentation (D) With 20–50% of fragmentation (E) With more than 50% of fragmentation

percentage of embryo transfer on Day +3 as well as the clinical pregnancy, implantation, and miscarriage rates were measured.

Statistical Analyses

For statistical analyses, the chi-square and Student's *t* test were used. A *p* value of <0.05 was considered to be significant.

RESULTS

General Variables

A total of 1039 embryos were included in this study. Of these, 852 2PN embryos (82.0%) could be classified on the basis of both pronuclear scoring and morphological features on day transfer. Normal fertilization rates (2PN) had shown no differences when the three groups were compared. Difference was noted between maternal age (mean $\pm$ SD) among the groups (Group A > Groups B and C), however, without statistical significance (Table III).

Embryo Classification According to Sperm Origin

Group A has provided more S0 embryos than have Groups B and C (*p* < 0.05). In all, 528 of 852 classified embryos (62%) were transferred according to the mandatory parameter (day transfer morphology, irrespective the score on zygote stage). Of these, 342 were classified as S0 embryos in all groups (64.8%). Division per group is shown in Table IV. There was a positive correlation between "S" scoring and morphological features on transfer day among the groups. However, characteristics from cleavage process have shown no correlation with pronuclear classification using the same comparisons. Nonejaculated spermatozoa were studied in separated groups (C1: ICSI with epididymal spermatozoa and C2: ICSI

Table IV. Outcomes in Treatment ICSI Cycles

Variables	Group A	Group B	Group C	Total study	<i>p</i> value
2PN embryos	553	194	105	852	
S0 embryos	301 (54.4) ^a	71 (36.6)	41 (39.1)	413	0.033
Transferred embryos	337 (60.9)	115 (59.3)	76 (72.4)	528	0.071
Good quality embryos	234 of 337 (69.4) ^a	69 of 115 (60.0)	39 of 76 (51.3)	342	0.047
transferred					
S0 embryos showing normal cleavage status (%)	28.9	29.4	21.5		0.082

^a Group C is compared with Groups A and B.

* *p* < 0.05.

with testicular spermatozoa). More S0 embryos were gotten in Group C1 compared to C2 (71.4% vs. 28.6%, *p* = 0.033, respectively). In these subgroups we could also note the same positive correlation with good PN embryos and good morphological embryos on day transfer (100% vs. 93.3% of embryos classified as "good quality embryos," Groups C1 and C2, respectively). Cleavage status has shown higher relationship with PN morphology in Group C1 than in C2 (58.3% vs. 35.7%, C1 and C2, *p* = 0.025, respectively).

Pregnancy, Miscarriage, and Implantation Rates

Higher pregnancy and implantation rates as well as lower miscarriage rates were detected when more good quality embryos were transferred. Sperm from azoospermic patients have provided lower pregnancy and implantation rates than have other groups (Group A > B > C; *p* < 0.05). This also was noted comparing epididymal and testicular spermatozoa (pregnancy rate per patient: 21.9% vs. 13.3%, respectively, *p* = 0.026, and miscarriage rate: 16.1% vs. 39.4%, respectively, *p* = 0.001).

Epididymal and testicular sperm were compared to those ejaculated on the basis of clinical data from Groups A and B and no statistical difference between Groups A, B, and C1 (pregnancy rate per patient: 27.2, 22.2, and 21.9%, respectively, *p* = 0.178; and miscarriage rate: 17.4, 14.3, and 16.1%, *p* = 0.225, respectively). When these groups were compared with Group C2, we could find significant difference (*p* = 0.002).

It is interesting to note that more than 80% of total pregnancy number had, at least, one good quality embryo replaced to the uterus (Table V).

Table III. Outcomes in Treatment ICSI Cycles

Variables	Group A	Group B	Group C	Total study	<i>p</i> value
Cycles/patients	110/84	32/32	30/25	172/141	
Mean maternal age (SD)	36.6 (4.1)	31.9 (5.0)	33.3 (5.9)	33.9 (5)	0.052
Total embryo number	661	233	145	1039	
2PN embryos (%)	553 (83.7)	194 (83.3)	105 (72.4)	852 (82)	0.061

Note. 2PN: Normal fertilization.

Table V. Pregnancy, Miscarriage, and Implantation Rates in the Three Groups Studied

Variables	Group A	Group B	Group C	<i>p</i> value
Pregnancy rate per patient (%)	27.2	22.2	17.1 ^a	0.026*
Miscarriage rate (%)	17.4	16.3	29.3 ^a	0.001*
Implantation rate (%)	19.2	14.4	8.2 ^a	0.003*
Pregnancies with at least one Z0 embryo transferred (%)	94.7	83.3	0	

^a Group C is compared with Groups A and B.* *p* < 0.05.

DISCUSSION AND CONCLUSIONS

In assisted reproductive technologies, the selection of preembryos for uterine transfer is still based predominantly on morphological appearance according to various scoring system including blastomere uniformity, fragmentation, and cytoplasmic appearance (5,12–15). It is a consensus that better selection criteria in order to improve the implantation and pregnancy rates, allowing to choose embryos with high implantation potential, must be detected. Today, a majority of embryo transfers are still performed at the cleavage stage, i.e., at Day 2 or 3 after ICSI was employed in this study.

Therefore, many factors can be detrimental impairing the embryo development in ICSI cycles. A strong correlation has been established between advanced maternal age and ICSI outcomes (16–19). Female fertility declines with advancing age and this age-related decline in female fertility is attributable to oocyte quality. It appears that bad oocyte quality has a negative effect over the possibility of achieving pregnancy (20,21), but the results are still contradictory.

Prior to the use of ICSI for the treatment of male factor infertility, several authors have already reported that suboptimal semen parameters such as poor morphology and/or poor motility led to decreased success rates (22,23). Even though ICSI was first introduced to treat severe male factor infertility, the quality of injected sperm seems to impair the good quality embryos formation (11).

Our group (24) has suggested that there is evidence linking oligoasthenozoospermia with poor embryo quality and an increased incidence of miscarriage rates in patients who underwent ICSI cycles. It appears that some paternally derived factors could play an important role in the chromosomal package of new embryo.

Analysis of a single parameter is very valuable when trying to understand better which parameters

are most important in an embryo scoring system that includes multiple factors (8,25–28).

The effect of the source of ICSI spermatozoa and the type of male infertility is an open question. Using an ICSI model, we evaluated whether the source of sperm can interfere on embryo morphology according to pronuclear features. A high incidence of S0 embryos obtained in the cycles of patients with nonmale factors suggests that the sperm quality may negatively interfere with pronuclear morphology. In more than 50% of embryos, irrespective the group, the zygote morphology was correlated to transfer day morphology in terms of blastomere number, regularity, and size as well as the percentage of fragmentation. However, this does not occur when zygote morphology and cleavage status (normal, fast, and slow) was compared. More good quality embryos were transferred when male factor is absent suggesting that this correlation is more evident in these cases. Sperm obtained from different sites (ejaculate, epididymis, testicles) exhibited significant differences in pronuclei formation percentages. Mainly in those azoospermic patients, sperm from epididymis and testicles seem to be different in terms of pronuclear morphological features at 17 h after ICSI, differing from a previous report by Demirel *et al.* (10). Ejaculated and epididymal spermatozoa have provided better quality embryos than have testicular spermatozoa. In our previous study (29) no differences were seen in the fertilization rates and embryo transfer regardless the etiology (obstructive vs. nonobstructive) and type of spermatozoa (epididymal vs. testicular). Testicular sperm retrieval results in lower fertilization and pregnancy rates as well as higher abortion rates compared to epididymal sperm retrieval.

It has been suspected for a long time that major abnormalities of pronuclear development, such as the lack of pronuclear apposition or the failure of pronuclear growth, are incompatible with further normal development of the zygote. The former abnormality is now known from an abnormal function of sperm-derived centrioli and the MTOC (microtubule organizing center) (30,31).

Failure to have progressed in pronuclear stage is indicative of failure of one or some fertilization events, probably due to the impairing in some structures to form normal pronucleous arrangement. Spermatozoa retrieved from men with severe male factor may show some abnormalities in terms of MTOC or centrioles, on the basis of the fact that zygote was an abnormal morphology (32,33), rarely progress well, and rarely form blastocysts (9).

Despite this, in our previous work (34,35) we compared PN morphology, transfer day morphology, and PGD results for chromosomes X, Y, 13, 18, and 21. Our results have showed that preembryos with close or aligned PN exhibit higher percentage of normal PGD results for the analyzed chromosomes (56%) compared to preembryos with unequal or separated PN (45.5%). This was also noted when nucleoli distribution was analyzed. These data have contributed to the inclusion of this criterion in our IVF laboratory.

In conclusion, these data show that the morphology of the human zygote is a valuable additional noninvasive criterion and can be used to preselect which embryos will have the highest potential for implantation. Zygote scoring associated with morphological features allows the selection of embryos mainly in those cases when severe male factor is present. The kinetic evaluation according to cleavage status can be considered as a complementary parameter to decide which embryo may be transferred.

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