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Oocyte euploidy, pronuclear zygote morphology and embryo chromosomal complement

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BACKGROUND: Pronuclear morphology has been proposed as an indicator of embryo development and chromosomal complement. In this study, the morphology of pronuclear zygotes generated from euploid oocytes [diagnosed by first polar body (PB1) analysis] was evaluated and compared with the configurations observed in chromosomally normal embryos (diagnosed by blastomere analysis). MATERIALS AND METHODS: Group 1—238 patients underwent 273 assisted conception cycles in combination with the screening of aneuploidy on PB1 for the chromosomes 13, 15, 16, 18, 21 and 22. Only normal oocytes were inseminated. Group 2—218 patients underwent 318 assisted conception cycles with aneuploidy screening on day 3 embryos. In both groups, oocytes were checked for fertilization and pronuclear morphology at 16 h after insemination. RESULTS: Seventy-three percent of zygotes from Group 1 had the configurations with centralized and juxtaposed pronuclei, large-size aligned or scattered nucleoli and PB located in the longitudinal or perpendicular axis of pronuclei. In Group 2, these configurations corresponded to those with the highest proportion of chromosomally normal embryos. Accordingly, in both groups, these configurations had a higher implantation rate than all the others. CONCLUSIONS: These observations confirm that some patterns of pronuclear morphology are associated with a higher proportion of euploidy and implantation reaffirming the relevance of this scoring system for the prediction of zygote viability.

Key words: aneuploidy/blastomere biopsy/implantation/polar body biopsy/preimplantation genetic diagnosis/zygote morphology

Introduction

The synchronized occurrences of nuclear and cytoplasmic maturation are crucial events that make the oocyte ready for meiosis completion and fertilization. In a competent oocyte, the entry of the fertilizing spermatozoon triggers a series of complex processes that are fundamental with respect to oocyte activation and embryo formation and viability. Spatial or temporal asynchrony in the sequence of these steps could severely affect further development.

One of the earliest transcriptional activities in the fertilized oocyte is represented by the synthesis of pre-rRNA in nuclear precursor bodies (NPBs). Nucleoli within pronuclei are formed by NPB and represent the sites where the synthesis of pre-rRNA takes place (Tesarik and Kopecny, 1990). The production of rRNA is necessary for protein synthesis to occur when the embryonic genome becomes fully active (Braude *et al*., 1988). Concomitantly, the chromatin in each pronucleus and NPB, which are closely associated, undergoes polarization that seems to be a crucial event in the design of the embryonic axis for the subsequent cell determination in the developing embryo (Van Blerkom *et al*., 1995; Edwards and Beard, 1997). Alterations in any of these strictly related events may be associated with an abnormal pattern of embryo growth resulting in uneven cleavage or fragmentation or arrest of development.

Previous studies have demonstrated that some morphological characteristics of pronuclear zygotes are an expression of what is happening in the oocyte. NPB are attached to chromatin and condense into nucleoli as chromatin condenses into the meiotic spindle (Scott *et al*., 2000). The presence of tiny, scattered nucleoli within pronuclei might indicate that chromatin condensation did not occur, signalling a developmental delay due to slow nuclear and/or cytoplasmic maturation. Accordingly, the implantation rate in these cases is extremely low (Scott *et al*., 2000; Wittemer *et al*., 2000). A poor clinical outcome is also observed in zygotes with unequal number or size of nucleoli. This condition could be a marker of developmental asynchrony between the two pronuclei, indicative of chromosomal abnormalities or altered meiotic events (Scott *et al*., 2000; Tesarik *et al*., 2000). Moreover, the evaluation of the angles subtended by pronuclei and polar bodies has suggested that wide angles might reflect a great degree of cytoplasmic turbulence with a consequent abnormal development (Garello *et al*., 1999).

In agreement with these considerations, the evaluation of pronuclear zygote morphology has been demonstrated to be a

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valid scoring system for the prediction of embryo development and implantation (Scott and Smith, 1998; Tesarik and Greco, 1999; Scott *et al*., 2000; Wittemer *et al*., 2000; Montag and van der Ven, 2001; Salumets *et al*., 2001).

As chromosomal abnormalities represent a major cause of developmental arrest resulting in failed implantation and spontaneous abortion, it is not surprising that a close association between pronuclear zygote morphology and chromosomal complement has been documented (Gianaroli *et al*., 2003; Kahraman *et al*., 2002; Balaban *et al*., 2004). Some zygote configurations have been identified which are especially related to a euploid complement, while other configurations are characterized by a significantly higher proportion of complex abnormalities (Gianaroli *et al*., 2003).

The aim of this study was to deepen the knowledge about pronuclear zygote morphology in relation to the chromosomal complement. For this reason, two groups of patients were enrolled. In Group 1, the chromosomal complement was evaluated in oocytes by analysing the first polar body (PB1), and those which were diagnosed as euploid were selected for insemination. In Group 2, blastomere biopsy was performed in preimplantation embryos for aneuploidy testing. In both groups, the morphology of pronuclear zygotes was evaluated with the purpose of verifying whether the configurations obtained after the fertilization of euploid oocytes (Group 1) could be correlated with those observed in chromosomally normal embryos (Group 2). The rationale of the study stemmed from the assumption that errors occurring during oogenesis directly affect oocyte development.

Materials and methods

Patients

Between October 2001 and December 2005, 456 patients underwent 591 assisted conception cycles for infertility. They belonged to two different categories.

Group 1

Two hundred and thirty-eight patients with a mean maternal age of 38.2 ± 3.7 years underwent 273 assisted conception cycles in combination with the screening of aneuploidy on PB1. Indications to aneuploidy screening were (i) maternal age ≥36 years (211 cycles, mean age 39.7 ± 2.6 years and (ii) ≥3 previous IVF failures (62 cycles, mean age 33.1 ± 2.1 years). Fluorescence *in-situ* hybridization (FISH) normal oocytes were selected for insemination, and, according to the national legislation on IVF prohibiting the formation of more than three embryos, a maximum of three oocytes were inseminated per patient (Benagiano and Gianaroli, 2004). The data from this group refer to patients who were treated from March 2004 until December 2005, with the PB1 analysis being the only form of PGD permitted after the introduction of the law on IVF in March 2004 (Benagiano and Gianaroli, 2004; Magli *et al*., in press).

Group 2

About two hundred and eighteen patients with a poor prognosis of term pregnancy underwent 318 assisted conception cycles in combination with the screening of aneuploidy on day 3 *in vitro*-generated embryos. The mean maternal age was 39.0 ± 3.5 years, and the indications for PGD were (i) maternal age ≥36 years (271 cycles, mean age 40.0 ± 2.7 years) and (ii) ≥3 previous IVF failures (47 cycles, maternal age 33.1 ± 1.7 years). The data from this group refer to patients who were treated from October 2001 until February 2004, for which there were no restrictions on the number of oocytes to be inseminated.

The induction of multiple follicular growth was performed by administering exogenous gonadotrophins after a long desensitization protocol with long-acting GnRH analogues (Ferraretti *et al*., 1996, 2004). Oocytes were collected transvaginally via ultrasound guidance at 34–36 h after HCG administration and cultured in a 5% CO_2 humidified gas atmosphere at 37°C. In both groups, oocyte insemination was performed with ICSI, between 5 and 6 h after oocyte collection. For homogeneity reasons, only patients with ejaculated sperm were included in the study.

Assessment of fertilization and embryo development

A fertilization check was performed at 16 h post-insemination at an inverted microscope equipped with Hoffman modulation optics. Presence and morphology of pronuclei and polar bodies was recorded by rolling, if necessary, the oocytes to position them with both pronuclei and polar bodies in focus.

As previously described (Gianaroli *et al*., 2003), pronuclear zygotes were evaluated for the shape and location of pronuclei and nucleoli and for the position of polar bodies. Briefly, five patterns of pronuclear morphology were described: A—juxtaposed and centralized, B—juxtaposed and peripheral, C—centralized and separated, D—unequal in size and E—fragmented. Nucleolar morphology was classified according to their position and size within the pronuclei: 1—large size and aligned; 2—large size, scattered; 3—large size, aligned in one pronucleus and scattered in the other; 4—small size, scattered and 5—any other configuration in which the two pronuclei totally differ between them. The position of polar bodies was described in relation to the longitudinal axis of pronuclei: α—in the longitudinal axis ±30°, β—perpendicular to the longitudinal axis ±30° and γ—in different angles with a rotation >30° off the longitudinal or the perpendicular axis (Garello *et al*., 1999; Scott, 2003). In Group 1, this evaluation was necessarily based only on the position of the second PB (PB2).

Regularly fertilized oocytes were cultured individually in microdroplets under oil and scored daily at regular time intervals. Number and morphology of nuclei and blastomeres and the percentage of fragments in the perivitelline space were evaluated.

PB biopsy and FISH

Approximately 1 h after collection, oocytes were denuded by hyaluronidase treatment (40 IU/ml, Medicult, Jyllinge, Denmark), and PB biopsy on metaphase II (MII) oocytes was started immediately (Magli *et al*., in press). For the biopsy, each oocyte was manipulated individually in HEPES-buffered medium supplemented with 10% human serum albumin (HSA, SAGE Biopharma, Trumbull CT, USA) in 0.1 M sucrose, overlaid with pre-equilibrated mineral oil. A slit of 20–25 μm was opened mechanically in the zona pellucida, and the PB was aspirated with a polished glass pipette (12 μm inner diameter) (Cieslak *et al*., 1999). After biopsy, the oocyte was incubated until the time of insemination, while PB1 was processed in the cytogenetic laboratory for the chromosomes 13, 16, 18, 21 and 22 (Multivision PB Panel, Vysis Inc., Downers Grove, IL, USA). Since February 2005, the probe for chromosome 15 was also included (CEP 15 alpha satellite, Spectrum Orange, Vysis) resulting in 622 oocytes screened for six chromosomes. As PB1 is the mirror image of the oocyte, the presence of double-dotted signals (one dot per chromatid) indicates that no errors occurred at meiosis I. Alternatively, the presence or absence of two additional dots implies that the oocyte is nullisomic or disomic respectively, while the presence or absence of a single-dotted signal indicates the occurrence of a meiotic error due to the predivision of chromatids (Angell, 1991). Chromosomally normal oocytes were inseminated by ICSI up to a maximum of three oocytes per cycle, at

5–6 h after oocyte collection (~4 h after PB biopsy) (Benagiano and Gianaroli, 2004; Magli *et al*., in press). Embryo transfer was performed on day 2 or day 3. However, embryo culture was extended to the blastocyst stage when three fertilized oocytes grew regularly to favour selection in culture. This was done with the purpose of avoiding the transfer of three embryos, embryo cryopreservation being currently forbidden and there being an obligation by law to transfer all the generated viable embryos.

Embryo biopsy and FISH

Blastomere biopsy was performed on day 3 embryos presenting with at least four regular blastomeres and a percentage of fragmentation not >40%. Embryos were manipulated individually in HEPES-buffered medium supplemented with 10% HSA overlaid with pre-equilibrated mineral oil. A breach of ~20–25 μm was opened in the zona pellucida with acidic Tyrode's solution, and one nucleated cell was aspirated by using a polished glass needle. The biopsied embryo was incubated until the time of transfer, while the corresponding cell was processed and hybridized with the probes specific for the chromosomes XY, 13, 15, 16, 18, 21, and 22 in a two-step protocol (Magli *et al*., 2001a). Embryo transfer was performed on day 4 (Gianaroli *et al*., 1999), and only embryos diagnosed as chromosomally normal were selected for transfer.

Clinical outcome

Clinical pregnancies were defined by the presence of gestational sacs with fetal heartbeat detected at ultrasound analysis. The implantation rate represents the ratio between the number of gestational sacs with fetal heartbeat and the total number of embryos transferred.

The chromosomal diagnosis for oocytes and embryos was performed by technicians blinded to zygote morphology.

Statistical analysis

Data were analysed by Fisher's exact test and chi-square analysis applying the Yates' correction, 2×2 contingency tables. Z-test was applied for the analysis of non-pre-ordinate paired sets of data according to the formula $z = x - np/[np\ (1 - p)]$ where x is the first discriminant value, n is the sum of discriminant values and p is the theoretical value of H_0 hypothesis for which discriminant data are expected as equals. Z follows a chi-square distribution (Camussi *et al*., 1995).

Results

A total of 2535 pronuclear zygotes were screened, belonging respectively 562 to Group 1 and 1973 to Group 2. The great majority of zygotes had centralized and juxtaposed pronuclei (pattern A, 78%) and polar bodies located either in the longitudinal axis of pronuclei (pattern α, 46%) or at 90° with respect to the longitudinal axis (pattern β, 42%) (Table I). The frequency of nucleolar morphology was equally represented by patterns 1 and 2 (28% each), 3 and 4 (19 and 18% respectively) while only 7% were originated from pattern 5.

The analysis of FISH results in relation to pronuclear zygote morphology was conducted separately for Group 1 and Group 2 patients.

Group 1

As the selection of oocytes for insemination was based on PB1 FISH results, all the zygotes obtained from Group 1 were generated from oocytes that had been diagnosed as chromosomally normal at the first mitotic division for the six tested chromosomes.

Following PB biopsy (Table II), the most frequent configuration in the 562 zygotes was that with centralized, juxtaposed pronuclei (pattern A, 86%) compared to all the others (6%, pattern B; 3%, pattern C and D and 2%, pattern E; $P < 0.001$). As most transfers were performed on day 2, embryo development was

Table I. Distribution of 2535 two-pronuclear oocytes from Groups 1 and 2, according to pronuclear morphology, nucleolar morphology and polar body orientation

	Pattern				
Pronucleolar morphology	A	B	C	D	E
Number of two-pronuclear oocytes (%)	1984 (78)	261 (10)	71 (3)	158 (6)	61 (3)
Nucleolar morphology	1	2	3	4	5
Number of two-pronuclear oocytes (%)	720 (28)	702 (28)	494 (19)	452 (18)	167 (7)
Polar body orientation	α	β	γ	–	–
Number of two-pronuclear oocytes (%)	1172 (46)	1064 (42)	299 (12)	–	–

Table II. Distribution of 562 two-pronuclear oocytes generated by the insemination of chromosomally normal oocytes in Group 1 patients

	Pattern					
Pronuclear morphology	A	B	C	D	E	*P*
Number of two-pronuclear oocytes (%)	484 (86)	32 (6)	15 (3)	19 (3)	12 (2)	A versus B,C,D,E; <0.001
Number of 4-cell embryos on day 2 (%)	169 (35)	9 (28)	1 (7)	4 (21)	3 (25)	A versus B + C + D + E; <0.01
Nucleolar morphology	1	2	3	4	5	P
Number of two-pronuclear oocytes (%)	187 (33)	210 (37)	121 (22)	25 (4)	19 (4)	1 + 2 versus 3; 3 versus 4 + 5; <0.001
Number of 4-cell embryos on day 2 (%)	81 (43)	61 (29)	37 (31)	5 (20)	2 (10)	1 + 2+ 3 versus 4 + 5; <0.025
Polar body orientation	α	β	γ	–	–	*P*
Number of two-pronuclear oocytes (%)	306 (54)	244 (44)	12 (2)	–	–	α versus β, γ <0.001, β versus γ <0.001
Number of 4-cell embryos on day 2 (%)	103 (34)	80 (33)	3 (25)			

evaluated at 40-h post-insemination, and good-quality embryos were considered those having four cells with no fragmentation. The proportion of good-quality embryos was significantly higher in pattern A (35%) compared with all the other patterns ($P < 0.01$).

Regarding nucleolar morphology, large-size nucleoli aligned or scattered in both pronuclei were observed in 33 and 37% of zygotes (patterns 1 and 2) compared with 22% in pattern 3 ($P < 0.001$); 4% had small-size, scattered nucleoli (pattern 4), and the remaining 4% contained nucleoli of different number and size (pattern 4+5; $P < 0.001$ versus pattern 3). The highest proportion of good-quality embryos was generated by zygotes with patterns 1, 2 and 3 (43, 29 and 31% respectively) followed by patterns 4 and 5 (20% and 10% respectively, $P < 0.025$).

When considering the effect of PB orientation in relation to the axis of pronuclei, the most frequent configuration was represented by that where polar bodies were located in the longitudinal axis of pronuclei (pattern α, 54%) followed by that in which polar bodies were located perpendicularly to the longitudinal axis (pattern β, 44%; $P < 0.001$); only in 2% of zygotes, the PB2 was located at a different angle (pattern γ; $P < 0.001$). Good-quality embryo development was 34% in pattern α, 33% in pattern β and 25% in pattern γ.

As shown in Figure 1, the results reported above were combined to have an evaluation of zygote morphology that was comprehensive of the single aspects which had been previously considered. Due to the small number in the other categories, only pronuclear oocytes developed from centralized, juxtaposed pronuclei (pattern A) were examined in combination with patterns 1–5 for nucleolar morphology and patterns α–γ for the orientation of polar bodies. According to the results, 43% of zygotes belonged to the configurations A1α and A2α, 30% to the configurations A1β and A2β, 22% to A3β and A3α and the remaining 5% to A4α, A4β, A5α and A2γ.

Group 2

As summarized in Table III, 76% of the 1973 zygotes had centralized, juxtaposed pronuclei (pattern A), 12% had peripheral, juxtaposed pronuclei (pattern B), 3% had pronuclei centralized

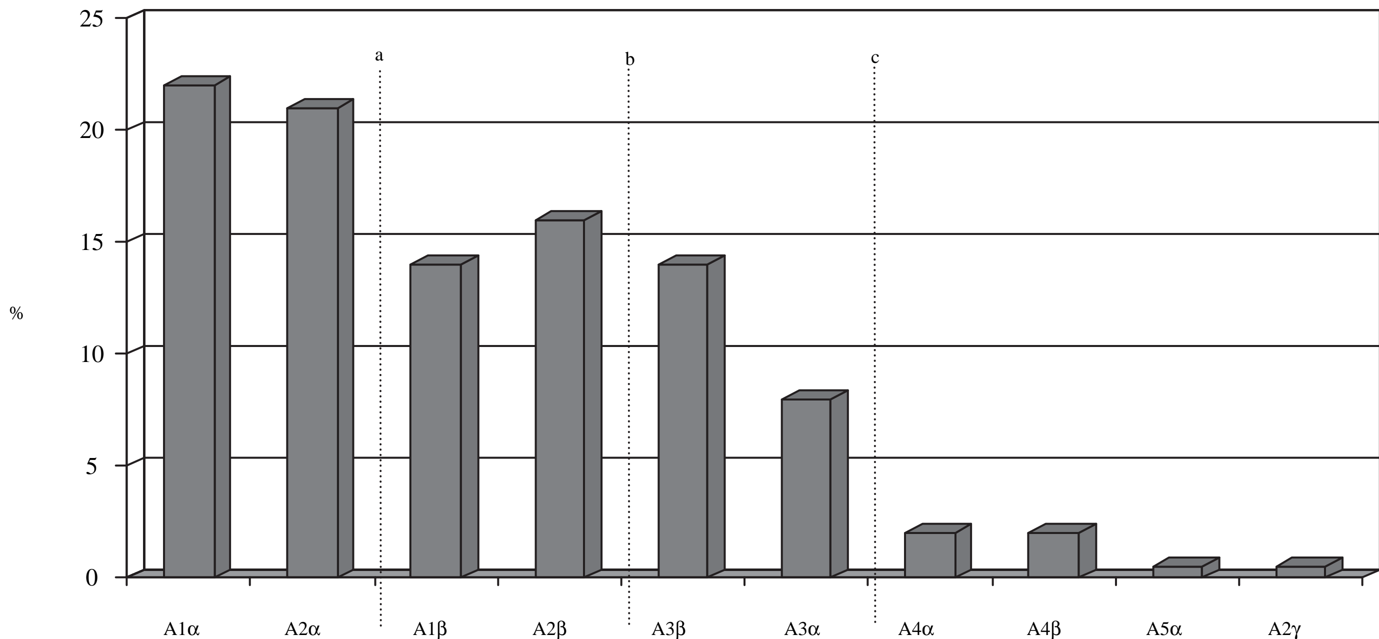

Figure 1. Distribution of two-pronuclear zygotes generated by euploid oocytes in relation to their morphology. [a]A1α, A2α versus A1β and A2β, $P < 0.001$; [b]A1β and A2β versus A3β and A3α, $P < 0.005$; A3β, A3α versus A4α, A4β,A5α,A2γ, $P < 0.001$.

Table III. Distribution of 1973 two-pronuclear oocytes and fluorescence *in-situ* hybridization (FISH) results according to pronuclear morphology in patients from Group 2

	Pattern				
	A	B	C	D	E
Number of two-pronuclear oocytes (%)	1500 (76)	229 (12)	56 (3)	139 (7)	49 (2)
Number of FISH analyzed embryos (%)	1279 (85)[abcd]	169 (74)[a]	13 (23)[b]	60 (43)[c]	30 (61)[d]
Number of FISH normal (%)	443 (35)	60 (35)	0	3 (5)	0
Number of monosomy + trisomy (%)	418 (33)	66 (39)	3 (23)	12 (20)	3 (10)
Number of haploidy + polyploidy (%)	52 (4)	8 (5)	0	6 (10)	7 (23)
Number of complex abnormalities (%)	366 (29)[efg]	35 (21)[hij]	10 (77)[eh]	39 (65)[fi]	20 (67)[gj]

[abcdefghij]$P < 0.001$.
Numbers with same superscripts are significantly different.

but not juxtaposed (pattern C), 7% had pronuclei of different size (pattern D) and 2% had fragmented pronuclei (pattern E). The development to good-quality embryos, expressed as those that were selected for embryo biopsy ($n = 1551$), was dependent upon the pattern of pronuclei and was significantly higher for zygotes with centralized, juxtaposed pronuclei (pattern A, 85%) compared with all the other patterns ($P < 0.001$). According to the FISH results, chromosomally normal embryos were mainly detected in the patterns with centralized, juxtaposed pronuclei (pattern A, 35%) and peripheral, juxtaposed pronuclei (pattern B, 35%), whereas the three other patterns gave rise to embryos carrying complex abnormalities in very high proportions (77, 65 and 67% for patterns C, D and E respectively).

Table IV summarizes the results in relation to nucleolar morphology. Large-size nucleoli aligned or scattered in both pronuclei or in one pronucleus were observed in 27, 25 and 19% respectively of zygotes (pattern 1–3); 22% had small-size, scattered nucleoli (pattern 4), and the remaining 7% contained nucleoli of different number and size (pattern 5). The highest proportion of good-quality embryos was generated by zygotes with patterns 1 and 2 (87 and 80% respectively), followed by patterns 3 and 4 (78 and 74% respectively, $P < 0.001$), while the lowest proportion was found in pattern 5 (57%, $P < 0.001$). The incidence of euploidy varied accordingly, ranging from 52% in pattern 1 and 45% in pattern 2 to 7% in pattern 5 ($P < 0.001$). Monosomy and trisomy were more common in patterns 1–3 (32, 36 and 46% respectively) whereas complex abnormalities showed the opposite progression, being 65 and 63% in patterns 4 and 5 respectively, 28% in pattern 3, 14 and 17% in patterns 1 and 2 ($P < 0.001$).

The effect of PB orientation in relation to the longitudinal axis of pronuclei is reported in Table V. The most frequent configurations were represented by those where polar bodies were located in either the longitudinal axis of pronuclei (pattern α, 44%) or perpendicular to the longitudinal axis (pattern β, 42%); in the remaining 14% of zygotes, polar bodies had different orientations (pattern γ). Embryo development was significantly higher in patterns α and β (84 and 82% respectively) compared with pattern γ for which only 54% ($P < 0.001$) had a regular development on day 3. In this last configuration, the lowest proportion of euploid embryos (3 versus 40% in pattern α and 32% in pattern β, $P < 0.001$) and the highest rate of complex abnormalities (75 versus 23 and 28% in patterns α and β respectively, $P < 0.001$) were detected.

As for Group 1, the combination of the different patterns only included zygotes from pattern A (Figure 2). The proportion of chromosomally normal embryos was significantly higher in the configurations A1α and A2α (59 and 52%) compared with A1β and A2β (49 and 44% respectively, $P < 0.025$). In turn, A1β and A2β had more euploid embryos than A3β and A3α had (25 and 20%, $P < 0.001$), and these had more euploid embryos than the remaining configurations A4α, A4β, A5α, A2γ, A1γ, A5β, A4γ, A3γ and A5γ ($P < 0.001$). The incidence of complex abnormalities had an opposite trend being higher in this last group of configurations in comparison with the other configurations ($P < 0.001$). In A1α and A2α, the proportion of chromosomally normal embryos (59 and 52%) was significantly higher than the proportion of monosomic–trisomic embryos (28% $P < 0.001$ and 36% $P < 0.01$).

Implantation

Following the transfer of 812 embryos in 473 cycles, 117 pregnancies were generated (50 from Group 1 and 67 from Group 2) yielding 127 gestational sacs (53 from Group 1 and 74 from Group 2). The clinical pregnancy rate calculated per oocyte retrieval was similar in the two groups (18% in Group 1 and 21% in Group 2), whereas it was significantly higher in Group 2 (29%) than in Group 1 (21%, $P < 0.05$) when calculated per transferred cycle or per patient (31% in Group 2 versus 21% in Group 1, $P < 0.05$). A correlation between implantation and pronuclear configuration was possible for 53 gestational sacs. As shown in Figure 3, 33 gestational sacs developed from zygotes that had the configurations A1α and A2α (62%), 15 had the configurations A1β and A2β (28%) and the 5 remaining sacs developed from zygotes of the type A3α, B2α, B2β (2 sacs) and B5β (10%). These proportions were similar when comparing Group 1 and Group 2, for which 13 and 40 gestational sacs respectively derived from known pronuclear zygote configurations.

In addition, 162 embryos were transferred in pregnant patients and were possibly involved in implantation. Seventy-one percentage derived from the configurations A1α, A2α, A1β and A2β, and, more specifically, 78 (48%) belonged to the configurations A1α and A2α, and 37 (23%) to A1β and A2β.

Finally, the implantation rate for the different configurations was calculated by considering the pregnant patients for whom each gestational sac derived from known configurations (as

Table IV. Distribution of 1973 two-pronuclear oocytes and fluorescence *in-situ* hybridization (FISH) results according to nucleolar morphology in patients from Group 2

	Pattern				
	1	2	3	4	5
Number of two-pronuclear oocytes (%)	533 (27)	492 (25)	373 (19)	427 (22)	148 (7)
Number of FISH analyzed embryos (%)	463 (87)^{ace}	396 (80)^{bdf}	291 (78)^{abg}	316 (74)^{cdh}	85 (57)^{efg}
Number of FISH normal (%)	239 (52)^{hjm}	177 (45)^{ikn}	60 (21)^{hilo}	24 (8)^{jkl}	6 (7)^{mno}
Number of monosomy + trisomy (%)	150 (32)^{ps}	144 (36)^{qt}	133 (46)^{ru}	56 (18)^{pqr}	19 (22)^{stu}
Number of haploidy + polyploidy (%)	11 (2)	9 (2)	17 (6)	30 (9)	6 (7)
Number of complex abnormalities (%)	63 (14)^{vy}	66 (17)^{xz}	81 (28)$^{w\alpha}$	206 (65)^{vxw}	54 (63)$^{yz\alpha}$

$^{abcdefgijklmnopqrstuvxywz\alpha}P < 0.001$.

Numbers with same superscripts are significantly different.

Table V. Distribution of 1973 two-pronuclear oocytes and fluorescence *in-situ* hybridization (FISH) results according to the orientation of polar bodies in Group 2 patients

	Pattern		
	α	β	γ
Number of two-pronuclear oocytes (%)	866 (44)	820 (42)	287 (14)
Number of FISH analyzed embryos (%)	726 (84)[a]	669 (82)[b]	156 (54)[ab]
Number of FISH normal (%)	288 (40)[c]	214 (32)[d]	4 (3)[cd]
Number of monosomy + trisomy (%)	244 (34)[e]	236 (35)[f]	22 (14)[ef]
Number of haploidy + polyploidy (%)	30 (4)	30 (5)	18 (8)
Number of complex abnormalities (%)	164 (23)[g]	189 (28)[h]	117 (75)[gh]

[abcdefgh]$P<0.001$.
Numbers with same superscripts are significantly different.

reported in Figure 3) and those who failed to achieve a pregnancy. The highest implantation rate was detected in the configurations A1α and A2α (14.8%) when compared with A1β and A2β (7.6%, $P < 0.05$) and to all the remaining the configurations (3.1%, $P < 0.001$).

Discussion

The data from stimulation cycles have demonstrated that few oocytes actually have the potential to implant. This seems to be the case even for embryos presenting with a regular development, as proven by the implantation rate that reaches a maximum of 40–60% in highly selected group of patients in which the transfer is performed with top quality embryos (Jones *et al*., 1998; Gardner *et al*., 2000; Schoolcraft and Gardner, 2000; Gerris *et al*., 2001). The detection of aneuploidy in regularly developing blastocysts might account for the lack of viability irrespective of normal growth, questioning the validity of blastocyst culture as the panacea for IVF transfer (Magli *et al*., 2000, 2001b; Sandalinas *et al*., 2001; Kolibianakis *et al*., 2004).

After entry of the fertilizing spermatozoon, the male pronucleus forms and chromatin starts to decondense in the side facing the oolemma. Concomitantly, the female pronucleus undergoes decondensation on the side facing the centre of the oocyte. For a correct positioning of the chromatin, the male pronucleus rotates onto the female pronucleus, while the sperm centrosome forms the aster that directs microtubule formation (Van Blerkom *et al*., 1995; Payne *et al*., 1997). All these steps are essential to a proper alignment of the two pronuclei onto the polar axis for the second meiotic division to be completed and the first cleavage to occur (Edwards and Beard, 1997; Scott, 2003). It has been reported indeed that the position of the second PB marks the polar axis that predicates the plane of the first mitotic division (Gardner, 2001). This step is so important

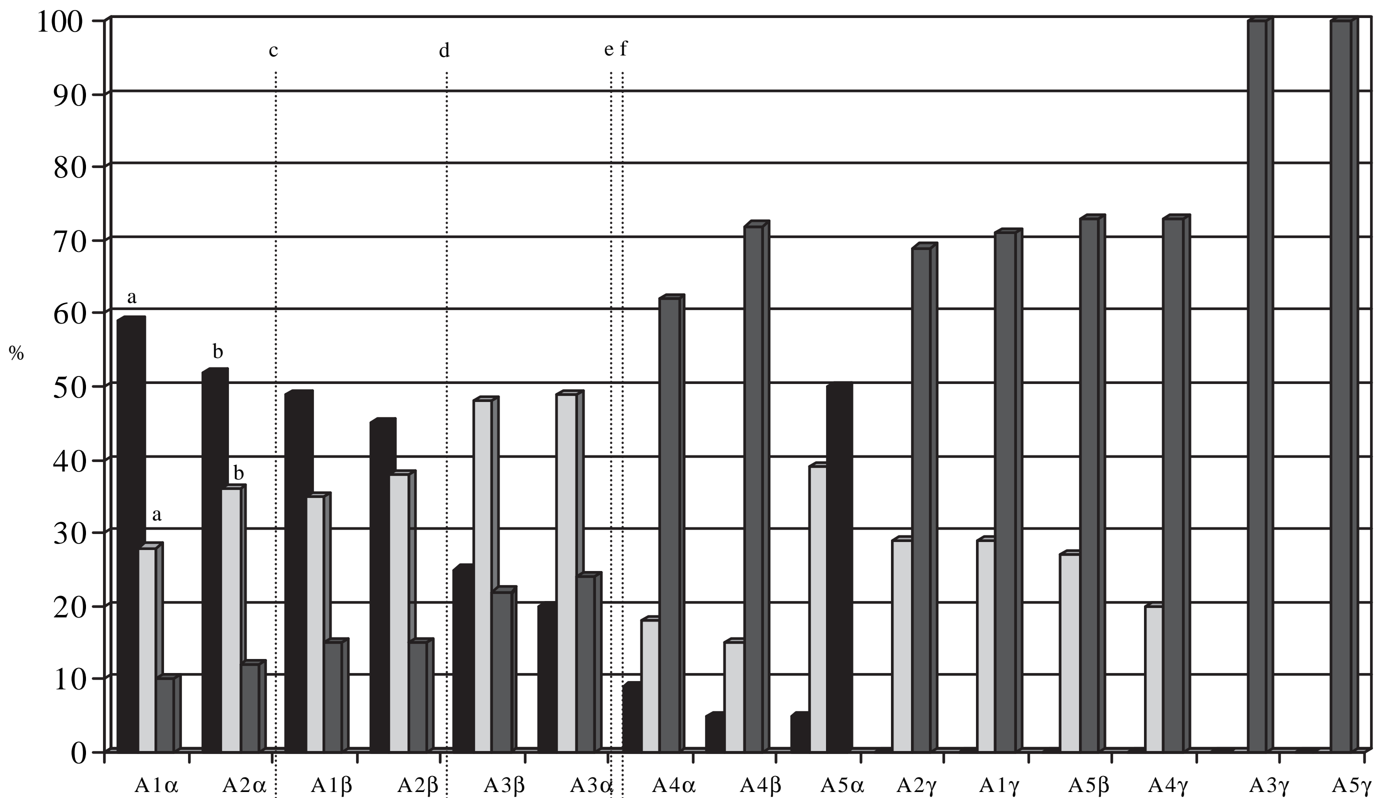

Figure 2. Percentage of embryos with a normal chromosomal complement (black bars), monosomies or trisomies (light-grey bars) and complex abnormalities (dark-grey bars) in relation to the configurations of pronuclear zygote morphology. Values with same letter are statistically different. [a]$P < 0.001$ and [b]$P < 0.01$. [c]$P < 0.025$ represents the percentage of chromosomally normal embryos in A1α, A2α versus A1β and A2β; [d]$P < 0.001$ represents the percentage of chromosomally normal embryos in A1β and A2β versus A3β and A3α; [e]$P < 0.001$ represents the percentage of chromosomally normal embryos in A3β and A3α versus A4α, A4β, A5α, A2γ, A1γ, A5β, A4γ, A3γ, and A5γ; [f]$P < 0.001$ represents the percentage of embryos with complex abnormalities in A1α, A2α, A1β, A2β, A3β and A3α versus all the other configurations.

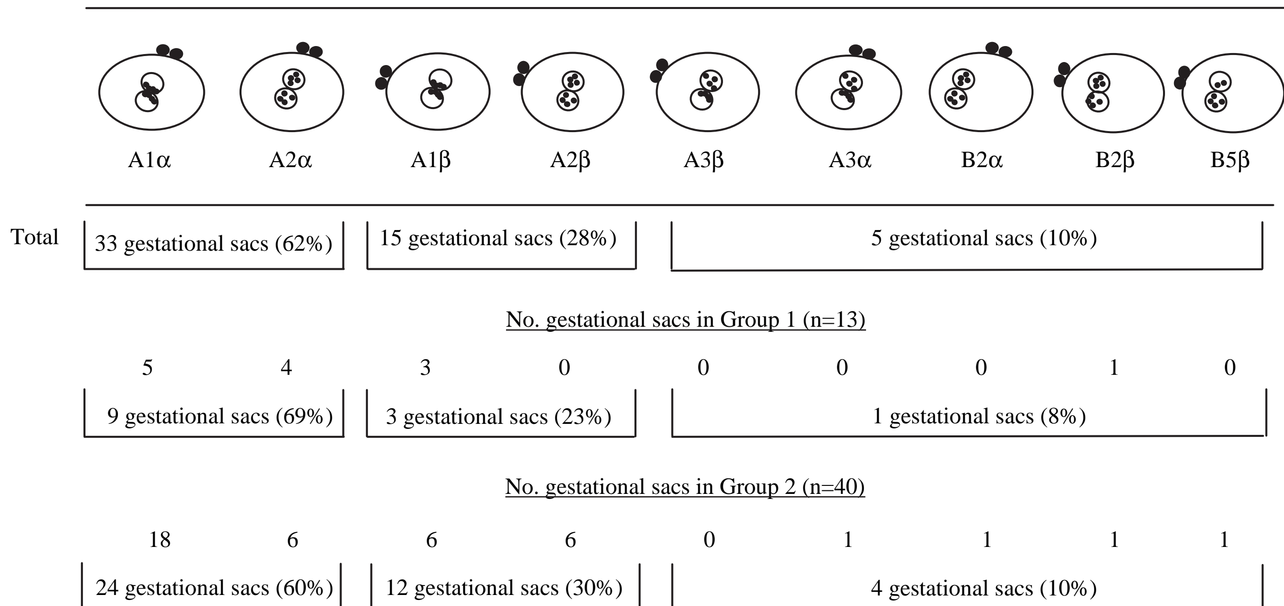

Figure 3. Diagram representing the pronuclear zygote configurations of 53 implanted embryos for which a direct correlation with pronuclear zygote morphology could be established. Thirty-three of them (62%) belonged to the configurations A1α and A2α, deriving 9 from group 1 (69% of 13 implanted embryos of known pronuclear morphology in this group) and 24 from group 2 (60% of 40 implanted embryos of known pronuclear morphology in this group); 15 (28%) belonged to the configurations A1β and A2β, deriving 3 from Group 1 (23%) and 12 from Group 2 (30%). The 5 remaining sacs (10%) derived from the configurations A3α, B2α, B2β (2 sacs) and B5β.

that in murine 4-cell embryos, the cell located furthest away from the second PB is the first starting the second division and giving rise to the clone producing HCG in the developing blastocyst (Gardner, 2002; Hansis *et al*., 2002; Gardner, 2003).

In light of these considerations, it is not surprising that a correlation exists between pronuclear zygote morphology and embryo viability. The consequences of incorrect positioning of pronuclei could cause several effects such as the unequal distribution of mitochondria to blastomeres, incorrect positioning of centrosomes or not proper alignment of nuclei on the polar axis (Garello *et al*., 1999; Van Blerkom *et al*., 2000; Tesarik *et al*., 2002). In all cases, abnormal development will follow. These findings are confirmed by the present results in Group 2 patients, in which irregular morphology of pronuclei (patterns C, D, E and pattern γ) is associated with poor embryo development and with a very low proportion of euploidy (Tables III and V). Concomitantly, complex abnormalities involving more than three of the tested chromosomes were by far the most frequent type of chromosomal abnormality. More indications came from the observation of nucleolar morphology for which the development to good-quality embryos was significantly higher in the configurations having large-size nucleoli in both pronuclei (patterns 1 and 2). Accordingly, these were the patterns with the highest proportions of chromosomally normal embryos, whereas small-size scattered nucleoli (pattern 4) and pronuclei containing nucleoli of different number and size (pattern 5) resulted in embryos which were abnormal in their majority, especially due to complex abnormalities (Table IV).

These morphological features could be related to the activity of DNA transcription and protein synthesis. These processes are very active in immature oocytes, in which nucleoli actively synthesize RNA and proteins, but decrease with maturation and nucleoli disappear at the MII stage. In pronuclear zygotes, NPBs appear while full nucleoli are formed over the first mitotic cycles leading to entirely competent nucleoli when the embryonic genome activates (Flechon and Kopecny, 1998). Cytoplasmic factors developed during oocyte maturation have a key role in regulating these events (Tesarik and Kopecny, 1989, 1990).

The dynamic features of this process are manifest in pronuclear zygotes, whose morphology varies with time in relation to the coalescence of NPB to originate nucleoli (Payne *et al*., 1997). Several configurations can be observed indeed that could represent a transition phase towards NPB coalescence into nucleoli and subsequent juxtaposition. For this reason, in this study, the observations were made at 16-h post-insemination to make comparisons of development with respect to time. It has been reported that delayed or abnormal coalescence of NPB could derive from a decreased amount of ATP available to the oocyte, consequent to insufficient vascularization or oxygenation to the follicle. Consequently, a switch from oxidative phosphorylation to glycolysis for ATP production will occur (Kaelin, 2002), and oocytes that are metabolically depressed might not have enough energy to properly organize the achievement of full competence and all the events related to pronuclear formation. In addition, the tight association between the energy availability and the correct alignment of chromatin onto the mitotic plate could represent the cause of errors occurring at meiosis and mitosis with the generation of chromosomal abnormalities resulting in aneuploid or mosaic embryos (Wilding *et al*., 2003).

The results obtained from the insemination of oocytes diagnosed by PB1 analysis (Group 1 of this study, Table II) emphasize

how important is the role of the oocyte in determining the following steps of development. Most pronuclear zygotes derived from euploid oocytes had centralized, juxtaposed pronuclei (pattern A, 86%) and polar bodies in the longitudinal or perpendicular axis (patterns α and β, altogether 98% of zygotes). This is in agreement with the results in Group 2, where according to the chromosomal analysis of preimplantation embryos, these patterns were those with the highest proportion of euploidy. Similarly, 73% of zygotes in Group 1 showed the configurations A1α, A2α, A1β and A2β which, in Group 2, were those with the greatest proportion of chromosomally normal embryos (Figures 1 and 2). From a more detailed analysis, the configurations A1α and A2α appeared to be the most frequently related to a normal chromosomal complement as suggested by the fact that 43% of pronuclear zygotes developed from euploid oocytes in Group 1 belonged to the above configurations. Accordingly, in preimplantation embryos from Group 2, while in most configurations the proportion of euploidy was comparable to that of monosomy and trisomy, A1α and A2α gave rise to embryos in which the incidence of euploidy was superior to all chromosomal defects. According to these results, these two configurations seem to be the best candidates for embryo transfer due to the highest chances of normal implantation. The common denominator between A1α and A2α versus A1β and A2β is represented by pattern α that designates the location of pronuclei as parallel to the axis. Apparently, this identifies pronuclei that have already rotated onto the axis, whereas pronuclei in pattern β still have to undergo rotation (Scott, 2003). These findings suggest that such a slight delay could be more frequent in the presence of aneuploidy. The other combinations involving pattern α behave differently, and this is probably due to the developmental condition that in the oocyte is associated with the nucleolar morphology of patterns 3, 4 and 5.

Unfortunately, no results were available on the morphology and performance of aneuploid oocytes. Nevertheless, the correspondence of the results yielded in the two study groups by means of two independent analyses suggests that the morphology of the fertilized oocyte is highly predictive of its chromosomal condition and subsequent cleavage outcome.

Further confirmation to the proposed correlation between chromosomal condition, pronuclear zygote morphology and embryo development was given by the data from implantation. As shown in Figure 3, in cases of correspondence between transferred embryos and gestational sacs, the great majority of embryos that developed to fetal heart beat derived form the two configurations A1α and A2α, followed by A1β and A2β. This was also confirmed by the implantation rate that was calculated by adding the data derived from the non-pregnant patients. According to the results in this study, the configurations A1α and A2α, A1β and A2β have been identified as those more frequently related to a normal chromosomal condition, and this was true for both study groups, confirming that the fate of an embryo has profound roots in the oocyte. These findings also imply that the chromosomal analysis of PB1 permits to refine the criteria of oocyte selection, for which a higher proportion of good-quality embryos is obtained. Although this strategy cannot estimate the chromosomal abnormalities arising from the second meiotic division and those generated at fertilization and during the first mitoses, the insemination of FISH-selected oocytes seems to result in the formation of pronuclear zygotes with the best scores. In other words, the advantage derived from the selection of euploid oocytes for insemination could, at least partially, compensate those derived from the chromosomal analysis of preimplantation embryos, as suggested by the similar clinical pregnancy rate per oocyte retrieval obtained in both study groups. Therefore, even under the restrictive Italian law on IVF, selecting oocytes on the basis of their chromosomal status has positive implications on the clinical outcome (Ferraretti *et al.*, 2006). This conclusion could possibly be valid mainly for those couples in which the female gamete is especially prone to aneuploidy. Conversely, a severe male factor condition could give especial relevance to sperm-derived aneuploidy and to post-meiotic abnormalities. In this case, establishing a possible correlation between embryo aneuploidy and pronuclear zygote morphology would be especially interesting.

In conclusion, the pronuclear zygote morphology could represent the earliest point at which the quality of the fertilized oocyte can be evaluated. A selection based on its observation could assist in designating not only the embryos in which the chances of implantation are negligible but also those with the highest chances of normal implantation. In this way, criteria for a priority in selecting or deselecting embryos for transfer are available from the earliest phases of oocyte development. This could be of great value for all laboratories performing clinical IVF.

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