Aneuploidies of chromosomes 1, 4, and 6 are not compatible with human embryos' implantation

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Objective: To verify whether chromosomes 1, 4, and 6 have a role in determining oocyte viability.
Design: Retrospective study.
Setting: Reproductive Medicine Unit, Società Italiana Studi Medicina della Riproduzione, Bologna, Italy.
Patient(s): Eighty-five patients with a normal karyotype who had undergone an assisted conception cycle with chromosomal analysis of first polar bodies for chromosomes 13, 15, 16, 18, 21, and 22 (first panel). A clinical pregnancy was obtained in 43 patients, whereas 42 patients were not pregnant.
Intervention(s): After conclusion of clinical pregnancies to delivery or abortion, first polar bodies from 85 patients were reanalyzed for chromosomes 1, 4, and 6 (second panel).
Main Outcome Measure(s): Aneuploidy frequency, clinical pregnancy outcome.
Result(s): The aneuploidy rate contributed by chromosome 1, 4, and 6 to the oocytes that were normal for the first panel was significantly higher in the nonpregnant patients (28%) versus the pregnant patients (11%), whereas no difference resulted between term pregnancies (11%) and abortions (10%). This trend was also observed when studying the first polar bodies from the oocytes that originated the transferred embryos. The frequency of aneuploidy for chromosomes 1 and 4 was comparable with that of chromosomes 15, 16, 21, and 22.
Conclusion(s): Aneuploidy of chromosomes 1, 4, and 6 seems to be related to failed implantation and not to spontaneous abortions. (Fertil Steril® 2010;■:■–■. ©2010 by American Society for Reproductive Medicine.)

Key Words: Aneuploidy, FISH, clinical pregnancy, delivery rate, implantation, abortion

During oogenesis, the sequential processes of DNA division are not devoid of errors that, during meiosis I, are basically due to the coexisting mechanisms of [1] bivalent nondisjunction or [2] predivision of sister chromatids (1–3). The analysis of the first polar body by fluorescence in situ hybridization (FISH) and by comparative genomic hybridization (CGH) largely has confirmed this theory on the origin of aneuploidy (4–7).

These techniques, FISH especially, have been used extensively in IVF for the preconception diagnosis of aneuploidy and have revealed that a sizeable part of aneuploidy, one third approximately, is observed in meiosis II (8, 9). However, most trisomic syndromes in clinical pregnancies derive from meiosis I (3), and it is believed that one third of meiotic I errors are resolved in meiosis II. This could be due either to a higher recombination rate that impairs the separation of bivalents (10) or to premature chromatid separation that would produce chromatids to be distributed at random, with some being distributed correctly at meiosis I but incorrectly at meiosis II.

These considerations imply that the chromosomal status of the resulting zygote can be predicted only if both polar bodies are studied. Nevertheless, the biologic and clinical relevance of meiosis I errors makes the testing of the first polar body of significance to the point that this approach has been used in assisted conception cycles as a tool for the selection of the oocytes to be inseminated (7, 11).

Received September 6, 2009; revised and accepted December 1, 2009.
M.C.M. has nothing to disclose. L.G. has nothing to disclose. A.C. has nothing to disclose. S.M. has nothing to disclose. F.R. has nothing to disclose. A.P.F. has nothing to disclose.
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The great majority of data reported from polar body testing by FISH regards chromosomes 13, 16, 18, 21, and 22 (7–9, 11, 12). Recent data coming from CGH experiments on polar bodies have demonstrated that other chromosomes give a substantial contribution to aneuploidy, suggesting that many errors are missed if only these five chromosomes are tested (13).

The aim of this study was to verify whether the retrospective analysis of the first polar body with additional chromosomes could contribute any correlation with the corresponding embryo viability in a group of pregnant and nonpregnant patients, who had completed an intracytoplasmic sperm injection cycle in combination with the testing for six chromosomes (chromosomes 13, 15, 16, 18, 21, and 22). These patients had been treated in the same period, and only pregnancies with a known outcome (either term pregnancies or abortions) were included. Their first polar bodies were rehybridized with the probes specific for the chromosomes 1, 4, and 6, and the information derived from the testing of the nine chromosomes was correlated with the clinical outcome.

MATERIALS AND METHODS

Patients

Between February 2005 and March 2008, 413 infertile couples with a normal karyotype underwent 564 assisted conception cycles in which chromosomal analysis of the first polar body was performed. Among them, 43 pregnant patients were selected retrospectively to enter this study and matched with 42 patients treated in the same period who did not become pregnant. Each pregnant patient was matched to the nonpregnant patient having similar characteristics of age, reason of infertility, and indication for chromosomal analysis.

Patients' indications for aneuploidy testing were maternal age $\geq$38 years (n = 52, mean age 40.1 $\pm$ 1.9 years), repeated IVF failures (n = 27, mean female age 34.2 $\pm$ 2.5 years), and recurrent abortions (n = 6, mean female

age 34 ± 3.4 years). After controlled ovarian stimulation (14, 15) oocytes were collected and cultured in HTF medium (Sage, Cooper Surgical Inc., Pasadena, CA) supplemented with human serum albumin (Sage), in a 5% CO_2 humidified gas atmosphere at 37°C.

Polar Body Biopsy and FISH

One hour after retrieval, the first polar bodies were biopsied mechanically from metaphase II oocytes and processed for the analysis by FISH of chromosomes 13, 15, 16, 18, 21, and 22, here denominated as first panel (11). When the FISH analysis was completed, the corresponding slides were stored at −20°C.

Oocyte Insemination, Control of Fertilization, and Embryo Transfer

On the basis of FISH results, insemination was performed by intracytoplasmic sperm injection at approximately 5 hours after oocyte retrieval on euploid oocytes up to a maximum of three per patient as established by the national law on IVF (16). Regularly fertilized oocytes were cultured individually in fresh cleavage medium (Sage) supplemented with 10% human serum albumin and scored daily every 24 hours (17, 18).

Embryo transfer was performed on day 3, and clinical pregnancies were defined by the presence of a gestational sac with fetal heartbeat. The implantation rate expressed the ratio between number of gestational sacs with fetal heartbeat and total number of embryos transferred.

Fluorescence in Situ Hybridization Reanalysis for Chromosomes 1, 4, 6

The slides with the first polar bodies belonging to the oocytes generated by the 85 patients included in the study were reanalyzed for chromosomes 1, 4, and 6 (here defined as second panel; Fig. 1). Hybridization with the probes for chromosomes 1 (CEP 1, Spectrum Orange, Abbott Molecular, Abbott Park, IL), 4 (CEP 4, Spectrum Green, Abbott Molecular), and 6 (CEP 6, Spectrum Aqua, Abbott Molecular) followed the same conditions described above.

Before the study was started, the efficiency of first polar body reanalysis was tested in 10 oocytes donated for research in which the first polar bodies were hybridized with use of the first panel probes and then rehybridized with the second panel. The corresponding oocytes were hybridized directly with the second panel (and not with the first panel) to estimate any possible error due to the process of rehybridization. After rehybridization, a clear result was obtained in nine first polar bodies, whereas in the remaining first polar body the signals were highly fragmented and could not be interpreted. The results from the nine first polar bodies were compared with the corresponding oocytes and yielded a full match considering the polar body as the mirror image of the oocyte.

Statistical Analysis

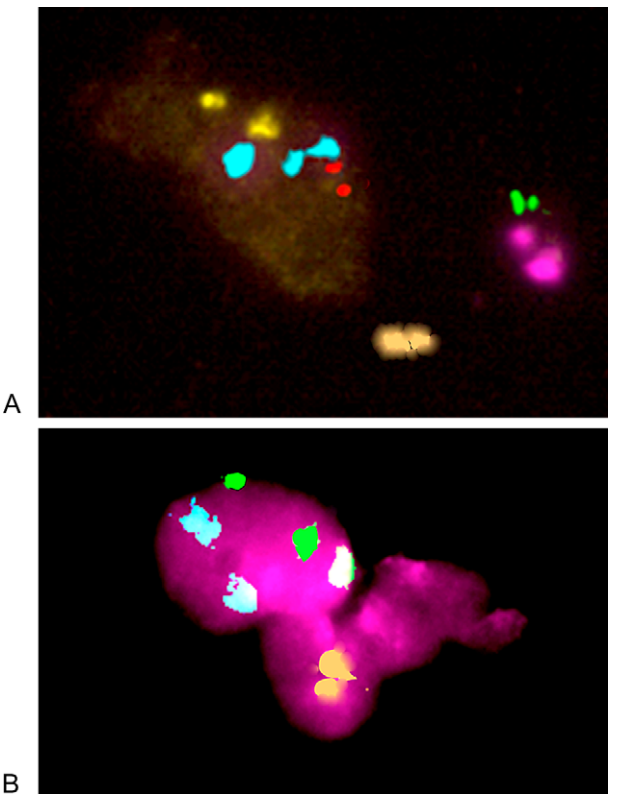
Data were analyzed by Fisher's exact test and χ^2 analysis applying the Yates correction, 2 × 2 contingency tables.

RESULTS

A total of 538 oocytes from the 85 cycles included in the study underwent first polar body biopsy for the chromosomes 13, 15, 16, 18, 21, and 22 (Table 1). A result was obtained in 490 first polar bodies (91%), 289 were classified as euploid for the tested chromosomes, and 234 were inseminated. Failure to obtain FISH results in 48 first polar bodies was mostly due to poor quality of the chromatin (36 cases) or to first polar body fragmentation (8 cases), whereas technical problems occurred in only 4 cases. No differences were found in the proportion or mean numbers of euploid oocytes between pregnant and nonpregnant patients (57% vs. 61%), nor in the rates of fertilization (83% vs. 77%) or cleavage (93% vs. 96%). The transfer of 162 embryos (1.9 ± 0.8 per patient) yielded

FIGURE 1

Chromosomal analysis of first polar body by multicolor FISH. (**A**) During the treatment cycle, the first polar body was tested for chromosomes 13 (red), 15 (orange), 16 (aqua), 18 (pink), 21 (green), and 22 (yellow). Three signals for chromosome 16 were present indicating that the oocyte was hypohaploid for chromosome 16. (**B**) The same polar body was rehybridized with the probes specific for the chromosomes 1 (orange), 4 (green), and 6 (aqua). The presence of three aqua signals indicated that the oocyte was hypohaploid also for chromosome 6.



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43 clinical pregnancies, of which 32 went regularly to term with 44 babies born and 11 ended in spontaneous abortion.

To further investigate the chromosomal status of the 490 first polar bodies diagnosed for the first panel probes, hybridization with the second panel probes was performed. After FISH reanalysis for chromosomes 1, 4, and 6, a result was obtained in 395 first polar bodies (81%), of which 256 were euploid after the first panel and 139 were aneuploid.

As represented in Table 2, 208 first polar bodies were euploid for the nine tested chromosomes after analysis with both panels (208/395, 53%), 123 were aneuploid for at least one chromosome in one panel (75 aneuploid only for the first panel + 48 aneuploid only for the second panel = 123/395, 31%), and 64 showed abnormalities for at least one chromosome in both panels (64/395, 16%). Regarding the distribution of abnormalities, chromatid errors (77%) were more frequent than chromosome errors. This was especially true in the first panel chromosomes where chromatid errors occurred in 81% of abnormalities versus 70% in the second panel chromosomes ($P<.025$). For both panels, there was a preponderance of missing

TABLE 1

Overall outcome of 85 patients undergoing chromosomal analysis of first polar body for the chromosomes 13, 15, 16, 18, 21, and 22 (first panel).

	Pregnant	Nonpregnant	Total
No. biopsied oocytes	303	235	538
No. FISH-diagnosed oocytes (%)	277 (91)	213 (91)	490 (91)
No. euploid oocytes (%) (mean ± SD)	158 (57) (3.6 ± 1.4)	131 (61) (3.1 ± 1.8)	289 (59)
No. inseminated oocytes	125	109	234
No. fertilized oocytes (%)	104 (83)	84 (77)	188 (80)
No. embryos (%)	97 (93)	81 (96)	178 (95)
No. transferred embryos (mean ± SD)	93 (2.1 ± 0.8)	69 (1.6 ± 0.8)	162 (1.9 ± 0.8)
No. clinical pregnancies	43	0	43
No. abortions	11	0	11

Note: Values are number (percentage) unless otherwise noted.

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signals in first polar body (56% of abnormalities in the first panel and 51% in the second panel). The total proportion of euploid first polar bodies in the pregnant patients (127/229, 55.5%) was not different from that detected in the nonpregnant patients (81/166, 49%). However, the aneuploidy rate contributed by the second panel to the oocytes that were found to be normal for the first panel was superior in the nonpregnant patients (28%) versus the pregnant patients (11%, $P<.001$). This difference was not detected in the group of oocytes that were diagnosed as aneuploid after the first panel, and the proportion of aneuploidy for the second panel was not significantly different in pregnant (41%) versus nonpregnant patients (55%).

The group of pregnant patients was analyzed according to the pregnancy outcome and compared with the nonpregnant patients, accounting for a total of 256 first polar bodies, which had been diagnosed as euploid for the first panel. The proportion of oocytes that were aneuploid for the second panel chromosomes did not differ in relation to the pregnancy outcome, being 11% for term pregnancies (12/93) versus 10% for spontaneous abortions (4/34). When compared with the nonpregnant patients, both figures were significantly lower (28%, 32/81; $P<.001$ and $P=.039$, respectively).

The analysis of the data then was concentrated on the chromosomal status of the 162 oocytes from which the transferred embryos were generated (they were all euploid for the first panel chromosomes) (Table 3). A result was obtained for 151 oocytes (93%) and demonstrated a significantly lower percentage of aneuploid oocytes for the chromosomes 1, 4, and 6 in the group of pregnant patients versus the nonpregnant patients (8% vs. 32%, respectively, $P<.001$). For the pregnant patients, there was no significant difference in the aneuploidy rate for chromosomes 1, 4, and 6 when comparing term pregnancies and spontaneous abortions.

The analysis was restricted further to 17 pregnant patients with 100% implantation rate, with 16 delivering 25 babies and 1 ending in spontaneous abortion. In the subgroup of term pregnancies, all the diagnosed oocytes were normal for chromosomes 1, 4, and 6, whereas in the subgroup of abortions the only oocyte that was analyzed was aneuploid for the second panel. Unfortunately, the abortive material was not karyotyped.

Besides this case, no pregnancies resulted after the transfer of embryos developed from oocytes that were abnormal for the second panel chromosomes, as demonstrated by the outcome in pure transfers (15 single ETs and two transfers with two embryos, which both derived from oocytes aneuploid for chromosomes 1, 4, or 6). Finally, the frequency of aneuploidy for single chromosomes was evaluated (Fig. 2). Apparently, chromosomes 1 and 4 had similar rates of

TABLE 2

Results obtained from the reanalysis of 395 first polar bodies with probes specific for the chromosomes 1, 4, and 6 (second panel).

	Pregnant	Nonpregnant	Total
No. oocytes euploid for first panel	143	113	256
No. oocytes euploid for second panel (%)	127 (89)	81 (72)[a]	208 (81)
No. oocytes aneuploid for second panel (%)	16 (11)	32 (28)[b]	48 (19)
No. oocytes aneuploid for first panel	86	53	139
No. oocytes euploid for second panel (%)	51 (59)	24 (45)	75 (54)
No. oocytes aneuploid for second panel	35 (41)	29 (55)	64 (46)

Note: Values are number (percentage) unless otherwise noted. First panel: chromosomes 13, 15, 16, 18, 21, 22. Second panel: chromosomes 1, 4, 6.

[a] $P<.001$, compared with pregnant.

[b] $P<.001$, compared with pregnant.

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TABLE 3

Results obtained from the reanalysis of 162 first polar bodies belonging to oocytes that were inseminated and the corresponding embryos of which were transferred.

	Pregnant			
	Term pregnancies	Spontaneous abortions	Total	Nonpregnant
No. oocytes euploid for first panel	69	24	93	69
No. oocytes diagnosed for second panel	63	20	83	68
No. oocytes euploid for second panel (%)	59 (94)	17 (85)	76 (92)	46 (68)[a]
No. oocytes aneuploid for second panel (%)	4 (6)	3 (15)	7 (8)	22 (32)[b]

Note: These oocytes were all euploid for the first panel chromosomes. Values are number (percentage) unless otherwise noted.
[a] $P<.001$, compared with pregnant total.
[b] $P<.001$, compared with pregnant total.

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variation (10.1% and 11.6%), whereas chromosome 6 showed an aneuploidy rate of 4.5%. When compared with the chromosomes that were tested in the first panel, chromosomes 1 and 4 showed levels of aneuploidy that were similar to those characterized in chromosomes 15, 16, 21, and 22.

DISCUSSION

Recent data from CGH on polar bodies confirm that chromosomes 13, 15, 21, and 22 are exposed to the highest variation frequency, followed by chromosomes 16 and X (19, 20). Accordingly, the probes specific for these chromosomes are included in the majority of panels used for preimplantation genetic diagnosis of oocytes and embryos (21). Chromosome 18 is usually tested too, because of viability of its trisomic form that is responsible for the Edwards syndrome.

Results from CGH also have demonstrated that segregation errors occur at a notable frequency even for those chromosomes that normally are not involved in aneuploid pregnancies (5, 6, 13, 22). These studies estimated that when FISH is performed with a limited number of probes ranging from 5 to 9, between 47% and 38% of aneuploidies would have been missed with the consequent misdiagnosis of 34% and 25% oocytes, respectively. However, a recent study on blastocysts demonstrated that this estimate should be reviewed in light of the occurrence of complex abnormalities (23). According to the authors, if it is true that a nine-probe panel only detects a limited amount of abnormalities (56%) compared with CGH, most undetected abnormalities occur simultaneously with those detected, for which the nine-probe panel identifies at the end 86% of abnormal embryos (23).

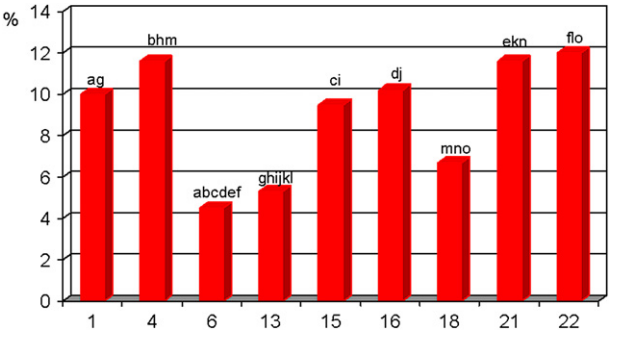
Combining the information provided by the data from clinical pregnancies with that derived from polar body and embryo analysis, it is clear that some aneuploidies are scarcely viable or not viable at all (13). The results from the present study are in agreement with these considerations at least for what regards chromosomes 1, 4, and 6, the aneuploidy of which seemed to be associated with failed implantation and not with spontaneous abortions (Table 3).

The reasons for which these chromosomes were selected to retrospectively reanalyze first polar bodies already having results for the chromosomes 13, 15, 16, 18, 21, and 22 were based on the findings coming from CGH studies indicating chromosomes 1 and 4 as being highly variable (5, 6), whereas chromosome 6 was chosen to investigate its stability because of discrepant results from previous studies (5, 6).

As reported in Table 2, the majority of oocytes that were normal for the first panel were also euploid for the second panel (81%). This was especially true in the pregnant patients where this proportion rose to 89% versus 72% for the nonpregnant patients ($P<.001$), suggesting that aneuploidy of chromosomes other than 13, 15, 16, 18, 21, and 22 could have been the cause for failed implantation, although the lack of information on the second meiotic division might have disregarded aneuploidies having an effect on implantation. On the other hand, when considering the transferred embryos, the incidence of aneuploidy for chromosomes 1, 4, and 6 in the corresponding oocytes (that were all normal for the first-panel chromosomes) was similar between term pregnancies and spontaneous abortions but was significantly higher in the nonpregnant patients (32%) compared with the pregnant patients (8%, $P<.001$). These findings suggest that chromosomes 1, 4, and 6 possibly have no involvement in miscarriages but more likely in implantation (Table 3). Accordingly, from the reanalysis of 26 first polar bodies belonging to oocytes that were inseminated and the corresponding embryos of which were transferred and all implanted, no anomalies for chromosomes 1, 4, and 6 were found in term pregnancies. Only one

FIGURE 2

Aneuploidy events in first polar body for single chromosomes. Chromosomes 6, 13, and 18 showed the lowest rate of variation. The *P* values are for comparisons between values with the same superscript letter. [aefhkl] $P<0.001$; [bd] $P<0.005$; [cjo] $P<0.01$; [gimn] $P<0.025$.



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oocyte from the group of spontaneous abortions that was transferred and implanted could be reanalyzed and found to be abnormal for the second panel chromosomes, but this finding was obviously meaningless. Besides this case, no other implantation resulted from the pure transfer of embryos originated from oocytes that were aneuploid for the second panel chromosomes.

The observations on the frequency of single chromosome variation were in agreement with previous findings in which the 23 chromosomes were studied in oocytes (5, 6, 13, 24, 25). Although generally based on a small number of cases, all these studies came to the common conclusion that aneuploidy occurs at high frequency also in chromosomes that were not predicted to be variable by data from clinical pregnancies. The screening for all chromosomes possibly could have a positive effect on implantation, and the clinical application of CGH to polar body testing is regarded as the most promising approach (13). Forthcoming data on larger numbers are expected to shed light on the frequency of aneuploidy for each chromosome in human oocytes.

When working with single cells, as typical for preimplantation genetic diagnosis and preimplantation genetic screening, the risk of misdiagnosis due to technical artefacts is of major concern. This could have been especially likely in the present study, where a second cycle of hybridization on first polar bodies was performed. Preliminary experiments actually were performed to verify the accuracy and reliability of this approach. The results were reassuring as demonstrated by the high concordance between first polar bodies and corresponding oocytes, but the high proportion of FISH failure after the reanalysis compared with the first analysis (19.4% vs. 8.9%) suggests that the DNA quality could have been affected by the repeated procedure. Nonetheless, it is reassuring that similar conclusions on the chromosomal status of first polar bodies have been achieved by different methods, namely those testing a limited number of chromosomes such as conventional FISH (9, 26), as well as other techniques detecting all 23 chromosomes such as CenM-FISH (multiple FISH using centromere-specific probes with different combinations of five fluorochromes) (24) and CGH (5, 6, 13).

In conclusion, the study of the first polar body by means of several distinct approaches has contributed information of both clinical and scientific value on the incidence of aneuploidy after the first meiotic division. With this knowledge in mind, further treatment and counseling of infertile couples could be modified drastically with the aim of offering the highest chances of success to infertile patients.

Acknowledgment: This work is dedicated to the memory of Yury Verlinsky, Ph.D., pioneer of polar body biopsy, who introduced the authors to this technique.

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