First meiosis errors in immature oocytes generated by stimulated cycles

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Objective: To investigate chromosomal errors detected by first polar body (PB) biopsy in relation to the nuclear maturity of the oocytes.
Design: Retrospective study.
Setting: Reproductive medicine unit.
Patient(s): Eighty-seven cycles were examined by PB biopsy for aneuploidy. Indications were maternal age ≥38 years (49 cycles), repeated IVF failures (22 cycles), and others (16 cycles).
Intervention(s): First polar bodies were analyzed for the chromosomes 13, 16, 18, 21, and 22 in both in vivo and in vitro matured oocytes. Euploid oocytes were inseminated by intracytoplasmic sperm injection.
Main Outcome Measure(s): Chromosomal status of the analyzed oocytes, development after intracytoplasmic sperm injection, pregnancy, and implantation rates.
Result(s): In in vitro matured oocytes, the proportion of chromosomal abnormalities was higher than in in vivo matured oocytes (70% vs. 54%, $P<.005$), with complex abnormalities being the prevailing defect (62% vs. 40%, $P<.001$). Conversely, the presence of an extra chromatid or the lack of a chromatid was more frequent in in vivo than in in vitro matured oocytes (55% vs. 34%, $P<.001$).
Conclusion(s): The low viability of in vitro matured oocytes from stimulated cycles could be related to a significantly higher proportion of chromosomal abnormalities compared with in vivo matured oocytes. Complex abnormalities, involving two or more chromosomes, gave the strongest contribution to the detected increase. (Fertil Steril® 2006;86:629–35. ©2006 by American Society for Reproductive Medicine.)

Key Words: Aneuploidy, chromosomal abnormalities, fluorescence in situ hybridization, immature oocytes, polar body

Dosage imbalance of the chromosomal complement is usually associated with inviability. For this reason, it is not surprising that the frequency of meiotic errors is quite uncommon in most organisms, with the notable exception of the human species. It is estimated that proportions ranging between 10% and 30% of fertilized human oocytes are aneuploid (1). The clinical consequences are severe; approximately a third of miscarriages are aneuploid.

As the majority of chromosomal abnormalities in embryos are maternal in origin, the study of aneuploidy in oocytes could represent a valuable tool for preselection of viable oocytes. This approach is especially advantageous in the case of patients with a poor prognosis for pregnancy who are at risk of generating a high proportion of aneuploid oocytes. According to the data obtained from clinical pregnancies, the importance of meiosis I versus meiosis II errors varies among chromosomes, although maternal meiosis I errors predominate among the majority of trisomies (2, 3). This could be due to the peculiar timing and modality of female meiosis, in which the first meiotic division involves homologous chromosome segregation rather than sister chromatids in a process that begins prenatally and reaches completion only after ovulation.

Clinically relevant information on oocytes has derived from the testing of polar bodies (PBs) by fluorescence in situ hybridization on IVF patients (4, 5). According to the results from several thousand oocytes, the reported incidence of aneuploidy in women aged ≥34 years varies between 32% and 52% and reflects the effect of maternal age on meiotic errors. These studies, based on the analysis of five chromosomes, have confirmed the prevalence of errors in meiosis I with 71% of abnormalities in the first polar body (PB1). Overall, 42% of oocytes had meiotic abnormalities in PB1 whereas the second PB was normal, and 29% displayed abnormalities in both meiotic divisions (6, 7).

In light of these findings, the removal and testing of PB1 can be performed to evaluate the outcome of meiosis I and

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predict the chromosomal status of the resulting oocyte. If the procedure is performed soon after oocyte collection and follows a short hybridization protocol, oocyte insemination could be performed according to the results of the chromosomal analysis. This approach could be especially valuable in those countries in which restrictions are imposed on the number of oocytes to be inseminated (8, 9).

In human assisted reproduction techniques, controlled ovarian hyperstimulation promotes the development of multiple follicles and oocytes. Approximately 10% of these oocytes are immature at the time of collection and require further maturation in vitro. Some of them succeed completing maturation spontaneously after 4–24 h in culture, but others degenerate. The performance of these oocytes is reported to be lower, possibly owing to a defective cytoplasmic maturation (10) resulting in a reduced implantation potential (11).

In the present study, the frequency and type of chromosomal errors detected by PB1 are reported in relation to the nuclear maturity of the oocytes at the time of PB biopsy. This was done to ascertain whether a correlation could exist between delayed oocyte maturation and chromosomal status as defined by PB1 testing.

MATERIALS AND METHODS

Patients

Between March 2004 and March 2005, 221 infertile couples underwent 248 assisted conception cycles combined with chromosomal analysis of PB1. This study included 87 cycles in which PB biopsy was performed both on oocytes that were at the metaphase II (MII) stage at the time of hyaluronidase treatment and on in vitro matured oocytes. Inclusion criteria to the PB biopsy program were maternal age ≥38 years (49 cycles, mean age 41.0 ± 2.4 years, mean number of previous cycles 2.3 ± 3.0) or repeated IVF failures (22 cycles, mean female age 33.9 ± 1.5 years, mean number of previous cycles 3.2 ± 1.8). Other poor prognosis indications were present in the remaining 16 cycles (mean female age 33.5 ± 2.4 years, mean number of previous cycles 0.5 ± 0.5), such as recurrent abortions and poor response to hormonal stimulation.

Induction of multiple follicular growth was accomplished by administering exogenous gonadotropins after a long desensitization protocol with long-acting GnRH analog (12). Oocytes were collected transvaginally via ultrasound guidance at 33–34 h after hCG administration and cultured in HTF medium (Lifeglobal; Guilford, CT), in a 5% CO_2 humidified gas atmosphere at 37°C.

The study was discussed and approved by our Institutional Review Board.

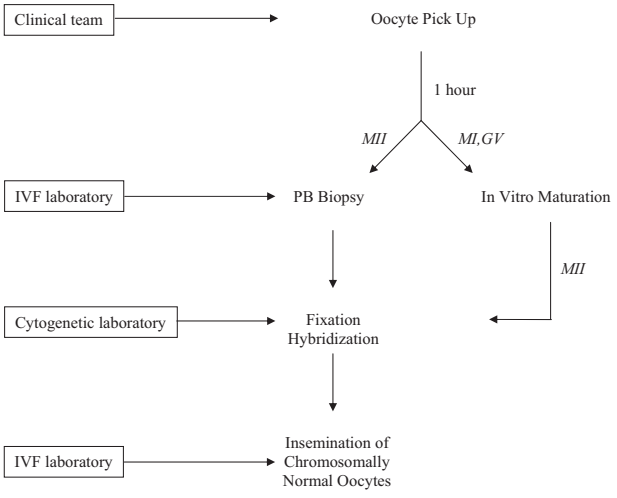
Polar Body Biopsy and Analysis by Fluorescence in Situ Hybridization

As described in Figure 1, at approximately 1 hour after collection oocytes were denuded by hyaluronidase treatment (40 IU/mL; Medicult, Jyllinge, Denmark) and scored for morphology and nuclear maturation. Polar body biopsy on MII oocytes started immediately, whereas immature oocytes, either in metaphase I (MI) or germinal vesicle (GV) stage, according to the results of a previous study (10), were cultured in the presence of FSH and LH (1 IU/mL rFSH + 10 IU/mL rLH) and scored again 4–6 hours later and at the time of fertilization control. At that time, in vitro matured oocytes were biopsied for PB analysis.

For PB biopsy, each oocyte was manipulated individually in HEPES-buffered medium supplemented with 10% human serum albumin in 0.1 mol/L sucrose, overlaid with preequilibrated mineral oil. A slit of 20–25 μm was opened mechanically in the zona pellucida by passing a glass microneedle through the perivitelline space tangentially to the oocytes, as previously described (13). The cut was completed by repeat-

FIGURE 1

Schematic representation of oocyte biopsy and insemination. At approximately 1 hour after retrieval, oocytes were evaluated for nuclear maturity. Polar body biopsy and fixation started immediately on MII oocytes, and insemination was performed as soon as the results of the chromosomal analysis were available (approximately 6 hours after retrieval). Immature oocytes were cultured and scored again 4–6 hours later and at the time of fertilization control. Concomitantly, in vitro matured oocytes were biopsied, and those diagnosed as normal for the tested chromosomes were inseminated. In all (fixation, hybridization, washings, and reading) the time for PB analysis was approximately 4–5 hours. Intracytoplasmic sperm injection was performed after the same time interval following PB biopsy in both in vivo and in vitro matured oocytes.



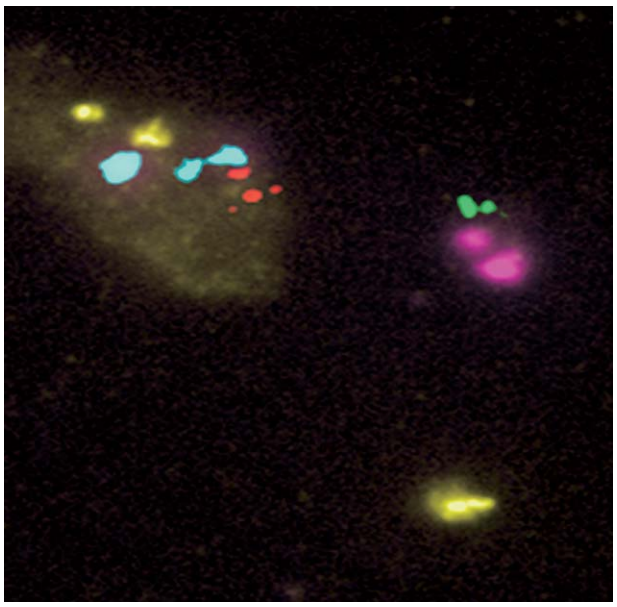
Magli. Chromosomal status of immature oocytes. Fertil Steril 2006.

edly rubbing the microneedle against the holding pipette, and the PB was aspirated with a polished glass pipette (12 μm inner diameter). After biopsy, the oocyte was thoroughly rinsed and incubated until the time of insemination, and the PB1 was processed in the cytogenetic laboratory for chromosomal analysis.

By using a pulled glass pipette of approximately 40–50 μm inner diameter, the PB1 was transfered to water, fixed with methanol and acetic acid (proportion 3:1) on a glass slide, and dehydrated in ethanol. For the chromosomal analysis, five probes specific for the chromosomes 13, 16, 18, 21, and 22 (Multivision PB Panel; Vysis, Downers Grove, IL) were hybridized to the fixed PB1s for 2 hours. Because PB1 is the mirror image of the oocyte, the detection of double-dotted signals (one dot per chromatid) indicates that no errors occurred at meiosis I. Alternatively, the presence or lack of two additional dots implies that the oocyte is nullisomic or disomic, respectively, possibly giving rise to an aneuploid embryo. Another source of meiotic error is due to predivision of chromatids (14), which causes the presence or absence of a chromatid (a dot signal) with consequent possible aneuploidy of the corresponding oocytes (Fig. 2). The presence of anomalies involving two or more chromosomes was defined as complex abnormality.

FIGURE 2

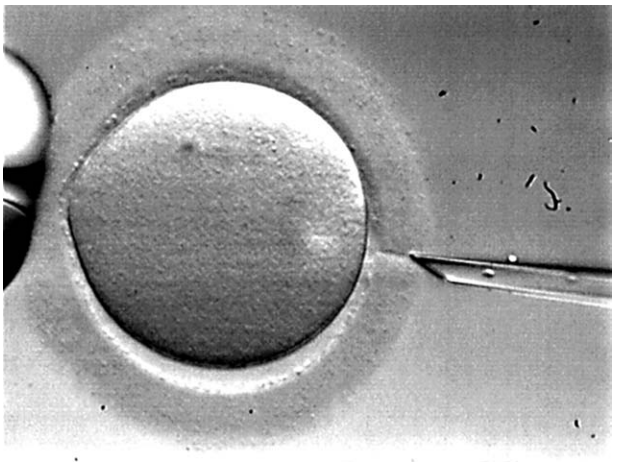
First PB analyzed for the chromosomes 13 (red), 16 (aqua), 18 (pink), 21 (green), and 22 (yellow). All the signals are double-dotted, with the exception of that corresponding to chromosome 22. The PB was diagnosed as abnormal and the corresponding oocyte was not selected for insemination.



Magli. Chromosomal status of immature oocytes. Fertil Steril 2006.

FIGURE 3

Insemination of chromosomally normal oocytes by ICSI. The injection needle was introduced through the same breach that was previously opened in the zona to remove the PB.



Magli. Chromosomal status of immature oocytes. Fertil Steril 2006.

Oocyte Insemination, Control of Oocyte Fertilization and Embryo Development

After completion of the chromosomal analysis, insemination of the normal oocytes was performed by intracytoplasmic sperm injection (ICSI) with the injection needle introduced through the breach already opened in the zona (Fig. 3). Intracytoplasmic sperm injection was used to avoid the high incidence of polyspermy that could result from the opening made in the zona pellucida. According to the current IVF regulation in Italy stating that no more than three embryos can be generated, a maximum of three oocytes were inseminated per cycle, and the remaining chromosomally normal oocytes were cryopreserved. Priority was given to in vivo matured oocytes, but in vitro matured oocytes with a normal chromosomal complement were also inseminated in such a number that did not exceed the formation of 3 embryos.

Regularly fertilized oocytes were cultured individually in fresh medium (Global Blastocyst Medium; Lifeglobal) and scored daily at regular time intervals. Number and morphology of nuclei and blastomeres and the percentage of fragmentation were recorded. Grade I embryos represented those with regular morphology and development according to the time of observation.

Embryo Transfer and Pregnancy Outcome

Embryo culture was extended to the blastocyst stage when there were three fertilized oocytes growing regularly. In this case, embryos were transfered to blastocyst growing medium and scored at 88 and 112 hours after insemination with the aim of favoring the natural selection in culture. This was

TABLE 1

Overall results in patients entering the study.

No. cycles	87
No. patients	75
Age, y (mean ± SD)	37.8 ± 4.3
No. previous cycles (mean ± SD)	2.2 ± 2.6
No. oocytes	698
No. biopsied oocytes (%)	564 (81)
No. diagnosed oocytes (%)	527 (93)
No. oocytes normal for the chromosomes 13, 16, 18, 21, 22	218 (41)
No. inseminated oocytes	207
No. fertilized oocytes (%)	136 (66)
No. generated embryos (%)	129 (95)
No. transferred cycles (%)	66 (76)
No. clinical pregnancies (%)	14 (21)
No. spontaneous abortions	1
No. ectopic pregnancies	1
Implantation rate (%)	14.6

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done with the purpose of avoiding the transfer of three embryos because, according to the current IVF regulation in Italy, embryo cryopreservation is forbidden and all the generated viable embryos must be transfered.

Clinical pregnancies were defined by the presence at ultrasound scanning of a gestational sac with fetal heartbeat. The implantation rate was calculated by the ratio between the number of gestational sacs with fetal heartbeat and the total number of embryos transfered.

Statistical Analysis

Data were analyzed by χ^2 analysis applying the Yates correction, with 2 × 2 contingency tables.

RESULTS

The clinical outcome of the 87 cycles included in the study is described in Table 1. A total of 564 oocytes were biopsied and a diagnosis was obtained in 527 (93%), with 218 (41%) normal for the analyzed chromosomes. After ICSI, regular fertilization occurred in 66% of the 207 inseminated oocytes, which originated 129 embryos. Embryo transfer was performed in 66 cycles, yielding 14 clinical pregnancies, with an implantation rate of 14.6%.

Table 2 summarizes the results derived from PB1 analysis in the oocytes that were at the MII stage at the time of hyaluronidase compared with those that matured in vitro. The nuclear maturation was complete in 384 oocytes that underwent immediately PB biopsy, whereas the procedure was performed later on 180 in vitro matured oocytes, with 146 from MI oocytes and 34 from GV oocytes. Failure of diagnosis owing to polar body fragmentation or degeneration was more frequent in in vitro matured oocytes compared with in vivo matured oocytes (11% vs. 5%, $P<.025$). The proportion of abnormal oocytes for the tested chromosomes was higher in in vitro matured oocytes (70% vs. 54%, $P<.005$). The presence of an extra chromatid (23 univalents + 1 chromatid) or the lack of a chromatid (22 univalents + 1 chromatid) was significantly higher in MII oocytes (55% vs. 34% in in vitro matured oocytes, $P<.001$), whereas complex abnormalities demonstrated the opposite trend (40% in MII vs. 62% in in vitro matured oocytes, $P<.001$).

The frequency of aberrations involving different chromosomes varied. Chromosome 21 was the most prone to aneu-

TABLE 2

Results of PB1 analysis according to the maturation stage at the time of hyaluronidase treatment: MII vs. MI and GV which subsequently matured to MII.

		MI + GV in vitro matured		
Variable	**MII**	**Total**	**MI**	**GV**
No. biopsied oocytes	384	180	146	34
No. diagnosed oocytes (%)	366 (95)[a]	161 (89)[a]	133 (91)	28 (82)
No. FISH normal (%)	169 (46)[b]	49 (30)[b]	34 (26)[f]	15 (54)[f]
No. FISH abnormal (%)	197 (54)[c]	112 (70)[c]	99 (74)[g]	13 (46)[g]
No. missing/extra chromatid (%)	108 (55)[d]	38 (34)[d]	35 (39)	3 (23)
No. missing/extra chromosome (%)	9 (5)	5 (4)	4 (4)	1 (8)
No. complex abnormalities (%)	80 (40)[e]	69 (62)[e]	60 (61)	9 (69)

Note: FISH = fluorescent in situ hybridization; GV = germinal vesicle stage; MI = meiosis I stage; MII = meiosis II stage; PB1 = first polar body. Values with same superscript are significantly different: [a] $P<.025$; [bc] $P<.005$; [de] $P<.001$; [fg] $P<.01$.

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TABLE 3

Fertilization and embryo development in relation to the maturation stage at the time of hyaluronidase treatment.

Variable	MII	MI + GV in vitro matured
No. inseminated oocytes	160	47
No. fertilized oocytes (%)	107 (67)	29 (62)
No. generated embryos (%)	102 (95)	27 (93)
No. grade I embryos (%)	60 (59)	10 (37)
No. transfered embryos (%)	81	22

Note: Abbreviations as in Table 2.

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ploidy in in vivo matured oocytes (4.3%). In the group of in vitro matured oocytes, the highest level of variation was related to chromosome 13, whose frequency corresponded to 6.7%, followed by chromosomes 21 (6.3%) and 22 (5.7%). Although chromosomes 16 and 18 presented similar frequency of variation irrespective of their maturation status (3.3% and 2.5%, respectively, in in vivo matured oocytes vs. 3.9% and 3.6% in in vitro matured oocytes), the other three analyzed chromosomes, 13, 21 and 22, showed significantly higher incidence of aneuploidy in in vitro (6.7%, 6.3% and 5.7% respectively) compared with in in vivo matured oocytes (2.5%, 4.3% and 3.4%, respectively).

As reported in Table 3, the fertilization rate between the two groups of oocytes was similar as was the rate of embryo development. The proportion of grade I embryos was slightly lower in the group of in vitro matured oocytes, although the difference was not statistically significant.

A total of 22 embryos derived from in vitro matured oocytes were transfered in 19 cycles; in 8 cycles (mean maternal age 39.4 ± 4.9 years) they were the only embryos transfered (n = 11) and did not generate any implantation. In the remaining 11 cycles (mean maternal age 38.2 ± 3.2 years), 11 in vitro matured embryos were transfered along with 11 embryos developed from in vivo matured oocytes, yielding one pregnancy with a single implantation. Only embryos derived from in vivo matured oocytes were replaced in 47 cycles (mean maternal age 37.2 ± 4.2 years), in whom pregnancy and implantation rates were 28% and 19.2% respectively.

The rate of maturation was 73% for MI oocytes (146 of 200) and 42% for GVs (34 of 81, $P<.001$). The analysis of PBs from in vitro matured oocytes demonstrated that the incidence of chromosomal abnormalities was higher in matured M1s (74%) compared with matured GVs (46%, $P<.01$) (Table 2).

DISCUSSION

The achievement of complete maturation in the oocyte is crucial for the developmental competence of the resulting embryo. Although nuclear maturation can be easily accomplished in vitro, a concomitant maturation of the cytoplasm seems not to occur properly, as demonstrated by the absence of specific proteins in the cytoplasm of in vitro matured oocytes (10). Accordingly, the implantation rate of in vitro matured oocytes is significantly lower compared with that of in vivo matured oocytes, implying that the complexity of the whole process is dramatically affected by the maturation conditions (11). Nevertheless, during the last years the efforts devoted to study and improve the in vitro maturation of human oocytes have led to several pregnancies, which are promising for a wider clinical application (15–18).

Superovulation in assisted conception cycles entails suppression of the endogenous secretion of gonadotropins and the induction of multiple follicular growth by exogenous FSH followed by the trigger of ovulation by hCG injection. Even in such a controlled condition, the proportion of oocytes reaching complete maturation can differ irrespective of similar follicular sizes, and a small proportion of collected oocytes are at the MI or GV stage. This delayed maturation generally results in a lower developmental competence and could be attributed to the inability of the follicles to respond to hCG administration (17). The primary cause could be represented by an insufficient blood supply or by a reduced number of LH receptors, as substantiated by the frequent nonexpansion of the corresponding cumuli. The in vitro maturation of these oocytes is generally achieved by culture in medium supplemented with FSH and LH, although pregnancies have also been reported in the absence of gonadotropins or steroids (19–21). In any case, pregnancy and implantation rates are lower compared with mature oocytes (9.5% and 4.5%, respectively (22).

These results suggest that the clinical application of in vitro matured oocytes in stimulated cycles are of limited relevance, especially when considering that approximately 85%–90% of the retrieved oocytes are at the MII stage. This proportion might vary in poor-prognosis patients, namely, those of advanced reproductive age and poor responders. In these cases, in vitro maturation is worth attempting to increase the number of oocytes available for insemination.

The standard stimulation conditions also play a role, and possibly the combination of mild stimulation protocols and the performance of oocyte pick-up at 33–34 h after hCG could have contributed to raising the proportion of immature oocytes in the present study. The decision to make patients undergo mild hormonal stimulation protocols was consequent to the current Italian legislation on IVF that limits the number of oocytes to be inseminated to three (8). The poor results obtained so far with oocyte cryopreservation (23) made a milder hormonal stimulation a preferable option.

This approach determined a significant increase in the proportion of immature oocytes in comparison with the data

derived before the introduction of the law. Performing the oocyte collection 2–3 hours earlier than the conventional 36 hours after hCG, and the early denuding of the recovered oocytes, possibly added another factor contributing to reducing the percentage of MII oocytes. This figure was 55% (384 of 698) at the observation performed 1 hour after the retrieval. However, considering that 64% of the immature oocytes reached the MII stage and that the great majority of them extruded the first PB at approximately 4 hours after the hyaluronidase treatment, the percentage of immature oocytes resulting from a conventional ICSI timing would have been 26%.

The culture of immature oocytes was performed in the present study, where a program of chromosomal analysis on PB1 was proposed to patients in whom the need of oocyte selection was especially strong. This approach combined the presence of a poor prognosis indication with the possibility of having available an additional criterion for oocyte selection.

In this context, patients with an age factor or repeated IVF failures, which were recommended to undergo preimplantation genetic diagnosis (PGD) for aneuploidy on embryos before the banning of PGD due to the current Italian IVF regulation (8, 24–27), were now proposed to have PB1 biopsy done on their oocytes. According to the results obtained, errors at meiosis I were significantly higher in in vitro matured oocytes at MII, whereas the rates of fertilization and cleavage, as well as the proportion of grade I embryos, did not differ between the two groups (Tables 2 and 3). As expected, the viability of the resulting embryos was higher for mature oocytes, confirming that a maturation process entirely accomplished in vivo provides the best developmental competence. It should be kept in mind that the PB2s were not investigated in this study, implying that the incidence of abnormalities in the fertilized oocytes could be even higher. Because approximately 30% of meiotic errors are originated at the second meiotic division, the total proportion of chromosomally abnormal oocytes could further increase, to close to 80%, for in vitro matured oocytes. This might explain why these oocytes are unlikely to contribute to conceptions.

In agreement with other reports (6, 7), in the groups of in vivo matured oocytes the most common error found in PB1 was related to chromatid nondisjunction (55% vs. 34% in in vitro matured oocytes, $P<.001$) (Table 2). Conversely, the prevailing defect in in vitro matured oocytes was represented by complex abnormalities (62% vs. 40% in in vivo matured oocytes, $P<.001$) (Table 2).

Following DNA duplication during the S phase, sister chromatids remain physically connected until their separation at anaphase. This connection is essential for the migration of sister chromatids to occur correctly. Molecular mechanisms have been recently demonstrated to trigger nondisjunction at meiosis which leads to an increase in the incidence of aneuploidy with a clear correlation with female age (28). Because the quality of the oocyte strictly depends upon the maturity of the ooplasm, a higher incidence of meiotic errors may be the consequence of a defective cytoplasmic maturation or loss of synchrony in nuclear and cytoplasmic maturation.

Accordingly, high proportions of chromosomal abnormalities have been detected in oocytes with cytoplasmic defects, suggesting that these anomalies could be related to degenerative cytoplasmic alterations (29). This could happen in association with, or represent the cause of, maturation arrest or delay. Some factors could negatively affect the proportion of oocytes confronting these conditions, such as advanced female age and unsuitable microenvironmental conditions such as low intracellular pH or accumulation of medications used for anesthesia (i.e., diprivan). Presence or absence of cumulus cells could also be critical. The consequences are possibly represented by spindle defects and chromosomal malsegregation or, similarly to the mouse, damage to the chromatin (29–31). As expected, acrocentric chromosomes are especially susceptible to malsegregation, and this tendency could be exacerbated in a condition of defective maturation (24).

According to the results of this study, a significant difference was observed depending on the source of in vitro matured oocytes, with chromosomal abnormalities significantly higher in those matured from MI (74%) than in those derived from GV oocytes (46%, $P<.001$) (Table 2). These data suggest that, although more MI can reach the MII stage compared with GV oocytes, a delayed maturation due to a retarded germinal vesicle breakdown seems to be less prone to cause meiotic errors. Alternatively, performing the pickup at 33 hours after hCG, and the early denuding of the recovered oocytes, could damage the spindle of those oocytes which are late in MI and still possess an MII spindle.

In conclusion, the low viability of in vitro matured oocytes from stimulated cycles could be related to a significantly higher proportion of chromosomal abnormalities compared with oocytes that were at the MII stage at the time of hyaluronidase treatment. Complex abnormalities, defects related to two or more chromosomes, represent the strongest contribution to the detected increase. These findings may suggest that a delay in oocyte maturation in response to hormonal stimulation could be related to an abnormal cytoplasmic maturation having severe consequences on chromosome segregation at meiotic divisions.

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