Embryo morphology and development are dependent on the chromosomal complement

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Objective: To analyze embryo morphology in relation to the corresponding chromosomal status, in order to evaluate the effects of aneuploidy over fragmentation, delayed cleavage, and arrested cleavage.
Design: Retrospective study.
Setting: Reproductive Medicine Unit, Società Italiana di Studi di Medicina della Riproduzione, Bologna, Italy.
Patient(s): A total of 662 patients with a poor prognosis for pregnancy underwent 916 cycles of preimplantation genetic diagnosis for aneuploidy.
Intervention(s): The chromosomes XY, 13, 15, 16, 18, 21, and 22 were analyzed in blastomeres biopsied from day 3 embryos.
Main Outcome Measure(s): Embryo morphology, chromosomal status of the analyzed blastomeres, and spreading and reanalysis of nontransferred embryos.
Result(s): The incidence of chromosomal abnormalities was significantly higher in arrested or slow-cleaving embryos, and in embryos cleaving too fast, compared to embryos with eight cells at 62 hours after insemination. The presence of an uneven number of blastomeres or fragments scattered in the perivitelline space was associated with an increased incidence of chromosomal abnormalities.
Conclusion(s): A correlation between embryo development and chromosomal complement makes the incidence of chromosomal abnormalities significantly higher in embryos dividing according to a time frame and a symmetry plan which are different from what are expected. The type of fragmentation is also related to chromosomal status, which explains why the extrusion of fragments might severely affect embryo viability. (Fertil Steril® 2007;87: 534–41. ©2007 by American Society for Reproductive Medicine.)

Key Words: Aneuploidy, blastomere biopsy, cleavage rate, chromosomal abnormalities, embryo fragmentation, embryo morphology, fluorescence in situ hybridization, preimplantation genetic diagnosis

The development of IVF technology has enabled the study of the initial phases of embryonic growth, and has provided valuable information on gametes and embryo physiology. This permits the creation of novel techniques and culture conditions aimed at supporting the development of viable embryos. However, variable rates of cleavage, associated with different morphologies and degrees of fragmentation, remain characteristic of human preimplantation embryos. It is not known whether this is because of in vitro culture systems after follicular stimulation with high doses of gonadotropins, or if it is a characteristic of human species (1–3).

Throughout preimplantation development, embryos must undergo a series of critical events that impose a strong selection over their survival potential, i.e., the fertilization process, the first mitotic divisions, the activation of the embryonic genome, and blastomere compaction and cavitation, ending with the formation of an expanded blastocyst. Every step is combined with a substantial reduction in the number of embryos which, following the current criteria of morphological evaluation, can progress in their development toward implantation. In this context, there is a continuous search for scoring criteria which might enable embryo selection to be reliably performed as early as possible during the culture period.

The assessment of the chromosomal constitution of in vitro-generated embryos has become part of this framework, and the selection of embryos to be transferred can be based on both the genetic diagnosis and embryonic morphology. The results obtained have demonstrated that a great number of in vitro-generated embryos carry chromosomal disorders, with some of these arising from meiotic nondisjunction, and others during fertilization and the first mitotic divisions (4–6). A correlation was found between embryonic morphology and chromosomal defects such as chaotic mosaicism, polyploidy, and multinucleation (7–9).

The aim of this study was to analyze the correlation between embryonic morphology and the corresponding chromosomal status, in order to evaluate the effects of aneuploidy over fragmentation, delayed cleavage, and arrested cleavage. It is unknown whether these features contribute substantially to the low viability that characterizes the human embryo compared to other animal species. It is clear that some embryos classified as chromosomally normal fail

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to reach the blastocyst stage and implantation, whereas others, carrying chromosomal disorders, can regularly develop to the blastocyst stage (10, 11).

MATERIALS AND METHODS

Patients

A total of 662 patients with a poor prognosis for pregnancy (maternal age ≥36 years, ≥3 IVF failures, microsurgical epididymal sperm aspiration (MESA)-testicular sperm extraction (TESE) patients with at least 1 IVF failure, and ≥2 previous miscarriages) (12) attended the Reproductive Medicine Unit, Società Italiana di Studi di Medicina della Riproduzione, Bologna, Italy, to undergo IVF treatment cycles for infertility in combination with aneuploidy testing. Between September 1996–February 2004, they performed 916 preimplantation genetic diagnosis (PGD) cycles after induction of multiple follicular growth that was performed by administering exogenous gonadotropins after a long desensitization protocol with long-acting GnRH analogues (13). Oocytes were collected transvaginally via ultrasound guidance 34 hours after HCG administration, and cultured in human tubal fluid supplemented with 5% human serum albumin (HSA), in a 5.3% CO_2 humidified gas atmosphere at 37.3°C. Insemination was performed with conventional IVF or intracytoplasmic sperm injection (ICSI), depending on sperm indices and the couple's reproductive history. The study was discussed and approved by our institutional review board.

Fertilization Assessment, Biopsy of Embryo, and Fluorescence in Situ Hybridization

Oocytes were checked for the presence of pronuclei and polar bodies 16 hours after insemination (14). Regularly fertilized oocytes were cultured individually, and scored at 40, 62, 88, and 112 hours after insemination. The number and morphology of nuclei and blastomeres, and the percentage and types of fragmentation (concentrated in one area of the perivitelline space or scattered among blastomeres) (1), were recorded.

For the biopsy procedure, embryos with at least four cells (developing from two cells on day 2) and no more than 40% fragmentation, were manipulated individually in HEPES-buffered medium overlaid with preequilibrated mineral oil. One blastomere was gently aspirated with the use of a polished glass needle, which was introduced into the perivitelline space after opening a breach of approximately 20 μm in the zona pellucida with acidic Tyrode's solution. After blastomere biopsy, embryos were thoroughly rinsed and transferred to blastocyst growing medium. In the case of embryos with >5 cells, the blastomere to be biopsied was chosen among those with a size corresponding to the results of the third cleavage division.

The biopsied blastomere was transferred to hypotonic solution, the nucleus was fixed in methanol:acetic acid on a glass slide, and dehydrated in increasing ethanol series (15). Multicolor fluorescence in situ hybridization (FISH) was used in a two-step protocol for the simultaneous detection of chromosomes XY, 13, 14, 15, 16, 18, 21, and 22 (15, 16). Beginning in December 1998, the probe specific for chromosome 14 was removed from the panel and substituted with the probe for chromosome 17 in 25 consecutive cycles. From March 1999, the probe specific for chromosome 1 replaced the one specific for chromosome 17. In July 2001, the probe for chromosome 17 was replaced by a telomeric probe specific for chromosome 21 that was thus screened twice, both in the first and second panels (17). These modifications were made in an effort to identify other chromosomes beyond XY, 13, 15, 16, 18, 21, and 22 which could determine aneuploidy at very early stages, whereas the double check for chromosome 21 was decided on the basis of clinical implications related to this chromosome aneuploidy (18).

The scoring criteria for FISH signals were previously described (17, 19). Briefly, euploidy, haploidy, and polyploidy were defined by the presence of two sets, one set, and ≥3 sets, respectively, for the tested chromosomes. Monosomy and trisomy were defined by the presence of an abnormal number of copies for one or two chromosomes, whereas the presence of ≥3 chromosomes with an abnormal number of copies defined the embryo as complex abnormal.

Embryo Fixation and Analysis

A total of 1,415 embryos had all their blastomeres fixed and analyzed. Of these, 562 had not been selected for embryo biopsy, because they were arrested in development from day 2 to day 3 in at least one blastomere, while the remaining 853 embryos were diagnosed as nontransferable after PGD, and were reanalyzed to estimate the overall rate of mosaicism and to verify the efficiency of the technique (18). For the reanalysis, nuclei were fixed and hybridized with the same probes used for PGD, following the same protocol described above.

Statistical Analysis

Data were analyzed by chi-square analysis, applying Yates' correction, with 2 × 2 contingency tables.

RESULTS

In total, 5,227 embryos generated by patients with a normal karyotype were analyzed by means of FISH. Based on observations performed 62 hours after insemination, 4,665 embryos were selected for chromosomal analysis after single cell biopsy. In addition, 562 embryos had all their blastomeres analyzed, as from day 2 to day 3 they were arrested in development (two cells, three cells, or four cells that did not cleave from day 2, or that had only one divided blastomere). All blastomeres were also analyzed in 853 embryos which were diagnosed as nontransferable after PGD, accounting for a total of 1,415 embryos which were spread and completely analyzed, as shown in Tables 1 and 2.

TABLE 1

Fluorescence in situ hybridization results from the reanalysis of 853 biopsied embryos in relation to the diagnosis as assessed by PGD.

PGD	No. of embryos (%)	Reanalysis		
		Confirmed (%)	Normal (%)	Other abnormalities (%)
Normal	178	174 (98)		4 (2)
Monosomic	233	202 (87)	10 (4)	21 (9)
Trisomic	255	225 (88)	18 (7)	12 (5)
Haploid	19	17 (89)		2 (11)
Polyploid	34	29 (85)	2 (6)	3 (9)
Complex abnormalities	134	132 (99)	2 (1)	
Total	853	779 (91.4)	32 (3.7)	42 (4.9)

Magli. Embryo morphology and aneuploidy. Fertil Steril 2007.

The results of the reanalysis of 853 biopsied embryos are given in Table 1. Confirmation of PGD results was obtained in 779 embryos (91.4%), while the rest were either normal (32 embryos, 3.7%) or carriers of other abnormalities (42 embryos, 4.9%). In agreement with previous studies, these results confirmed that the FISH diagnosis of one cell has a clinically relevant inaccuracy, corresponding to 3.7% (18). For the purpose of the present study, this error rate was considered negligible, and all data deriving either from one-cell analysis or from the study of all blastomeres were pooled and evaluated in their totality, regarding the diagnosis of their chromosomal complement.

Table 2 shows the results of the chromosomal analysis performed on 562 embryos which were arrested in development in at least one blastomere on day 3 compared to day 2 (141 with two cells, 299 with three cells, and 122 with four cells). The proportion of chromosomally abnormal embryos was significantly higher in completely arrested embryos versus those which were arrested in one blastomere (97% versus 91%, respectively; $P<.05$). In all cases, the greatest majority of defects was due to mosaicism and/or the presence of multinucleated blastomeres, with a significantly higher incidence in completely arrested embryos versus those which were arrested in one blastomere (97% versus 85%; $P<.001$).

As shown in Figure 1, these data, combined with data derived from the PGD program, permitted us to evaluate the chromosomal status of all cleavage stages observed on day 3. The incidence of chromosomal abnormalities was significantly lower at the 7–8-cell stage (50%) compared to all other stages, and was highest in embryos with two or three cells or four cells on the morning of day 3 that were arrested in at least one blastomere (94% for two- or three-cell embryos, and 85% for four-cell embryos, versus 50% at the 7–8-cell stage; $P<.001$). Slow-cleaving embryos (four cells

TABLE 2

Fluorescence in situ hybridization results on 562 day 3 embryos which were arrested in development in at least one blastomere, in relation to the stage on day 2.

No. of cells	Completely arrested		Arrested in one blastomere	
No. of cells on day 3	2	4	3	4
No. of cells on day 2	2	4	2	3
No. of embryos diagnosed by FISH	141	36	299	86
No. abnormal after FISH (%)[c]	138 (98)[a]	33 (92)	275 (92)	75 (87)[a]
No. mosaics/MNB (%)[d]	135 (96)[b]	31 (86)	260 (87)	68 (79)[b]

Note: MNB = multinucleated blastomeres. Values with the same superscript are significantly different.
[a] $P<.005$.
[b] $P<.05$.
[c] Completely arrested (97%) versus arrested in one blastomere (91%), $P<.05$.
[d] Completely arrested (97%) versus arrested in one blastomere (85%), $P<.001$.

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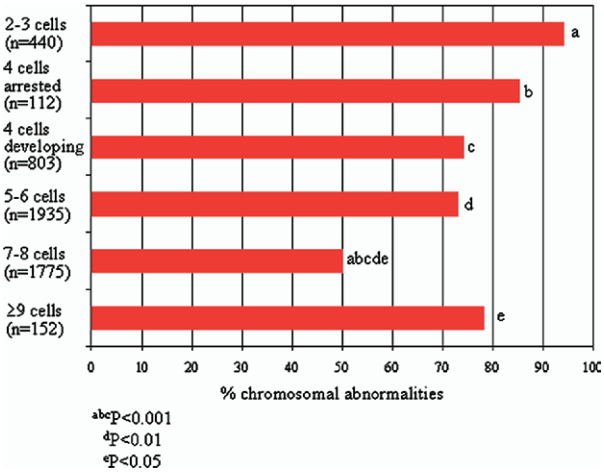
FIGURE 1

Chromosomal abnormalities and cellular stage, 62 hours after insemination.



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which had been two cells on day 2), moderately slow-cleaving embryos (five or six cells), and very fast-cleaving embryos (≥9 cells) had similar proportions of chromosomal abnormalities (74%, 73%, and 78%, respectively).

Figure 2 shows the results of 4,665 embryos which underwent embryo biopsy. When the PGD data were analyzed for each cell stage in detail, the lowest incidence of chromosomal abnormalities was found in embryos with eight cells (48%). In addition, significant differences were detected when comparing embryos with an uneven number of blastomeres to those with an even number of cells that were closest to their stage. Hence, embryos with five cells had more chromosomal abnormalities then those with six cells (79% versus 71%, respectively; $P<.001$), and embryos with seven cells were more abnormal than those with eight cells (56% versus 48%, respectively; $P<.001$). This trend was not maintained in embryos with a higher number of blastomeres: those with nine cells had fewer abnormalities than those with 10 cells (64% versus 83%, respectively; $P<.025$), while no differences were reported in even faster-cleaving embryos.

To investigate the significance of a higher proportion of abnormalities in embryos with an uneven number of blastomeres, the data from 316 embryos with five and seven cells were extrapolated from those reported in Table 1. According to the results of PGD, 48 embryos were euploid and 268 were chromosomally abnormal (Table 3). The spreading and FISH analysis of all blastomeres revealed that the great majority of the dominant blastomeres were highly abnormal (100% in embryos diagnosed as euploid after PGD, and 98% in embryos carrying chromosomal abnormalities at PGD). In both cases, multinucleation and complex abnormalities were the prevailing defects, followed by polyploidy. Most of the small-sized blastomeres in the embryos diagnosed as abnormal after PGD were chromosomally abnormal (95% in five-cell embryos, and 87% in seven-cell embryos), but chromosomal abnormalities were detected even in a proportion of small-sized blastomeres from the embryos diagnosed as euploid after PGD (25% in five-cell embryos, and 13% in seven-cell embryos).

Finally, the numerical analysis of chromosomes was related to the percentage and type of fragmentation in relation to the cellular stage of biopsied embryos. As shown in Figure 3, the incidence of chromosomal abnormalities in embryos with four, five, or six cells was similar, irrespective of the grade and type of fragmentation. In the 754 embryos with seven cells, the total incidence of chromosomal abnormalities was 53% in the absence of fragments, 55% with 1%–10% fragmentation, 58% with 11%–20% fragmentation, 67% with 21%–30% fragmentation, and 66% with 31%–40% fragmentation. When the results were analyzed for type of fragmentation (Fig. 3), there was a tendency toward higher proportions of chromosomal abnormalities in embryos with a scattered distribution of fragments, compared to those with fragments concentrated in one area. This difference was statistically significant in the presence of 21%–40% fragmentation (80% with scattered fragments versus 57% with concentrated fragments; $P<.03$). In the 1,021 eight-cell embryos studied, the incidence of chromosomal abnormalities was 47% with no or 1%–10% fragmentation, and 49%, 56%, and 60% with 11%–20%, 21%–30%, and 31%–40% fragmentation, respectively. Similar to seven-cell embryos, the proportion of chromosomal abnormalities was significantly higher in embryos with 21%–40% fragmentation that had a scattered distribution of fragments versus those where fragments were concentrated (70% vs. 50%, respectively; $P<.03$). Both in seven-cell and eight-cell em-

FIGURE 2

Chromosomal abnormalities and cellular stage in embryos having ≥5 cells at the observation performed 62 hours after insemination.

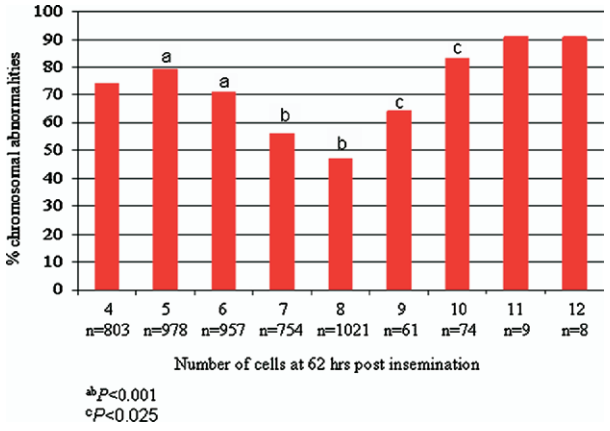

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TABLE 3

Spreading and FISH analysis of 316 embryos with five or seven cells, 62 hours after insemination.

	Chromosomally normal at PGD	Chromosomally abnormal at PGD
No. of embryos	48	268
No. of abnormal dominant blastomeres determined by FISH (%)	48 (100)	263 (98)
Type of abnormality		
Polyploidy (%)	3 (7)	26 (10)
Complex abnormalities (%)	18 (37)	84 (32)
Multinucleated blastomeres (%)	27 (56)	153 (58)
No. of abnormal small-sized blastomeres determined by FISH (%)		
Five-cell embryos (%)	48/190 (25)	999/1,052 (95)
Seven-cell embryos (%)	37/283 (13)	1,386/1,593 (87)

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bryos, the incidence of chromosomal abnormalities did not vary according to the percentage of fragmentation in the case of embryos that had fragments concentrated in one area of the perivitelline space.

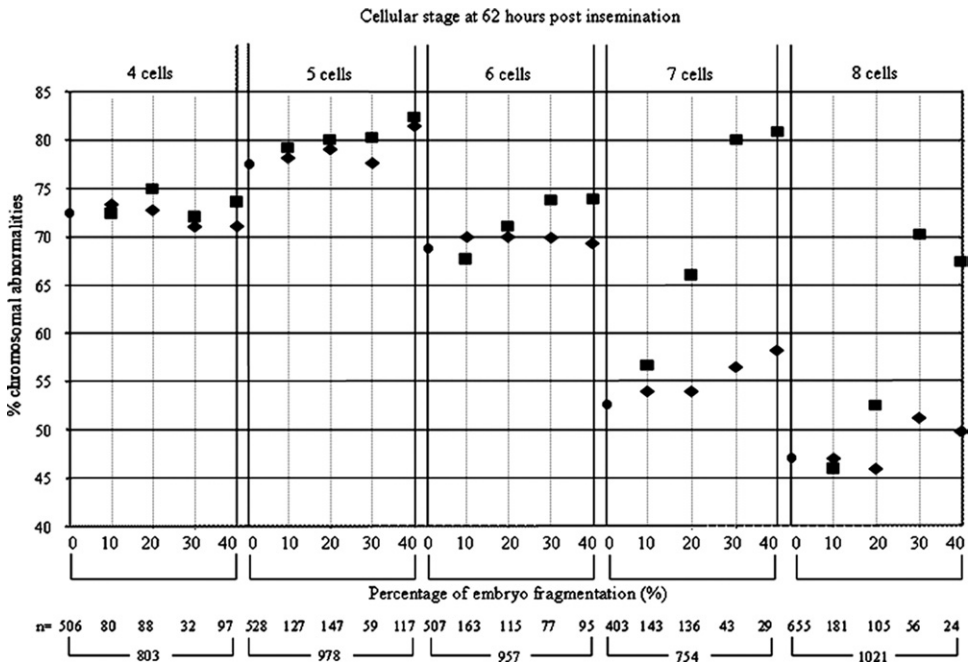
DISCUSSION

After fertilization, embryonic development initiates by the syngamy of the male and female pronucleus. The probability of further development is partially determined at these early stages by virtue of the genetic and epigenetic contributions received from both gametes. Subsequent errors in the execution of this developmental program, or deviations from the time frame that is scheduled for a correct synchrony and sequence of events, can cause embryonic mortality.

The meiotic pathway is a highly conserved mechanism through which haploid gametes are generated. The frequency

FIGURE 3

Percentage of chromosomal abnormalities in day 3 embryos in relation to cellular stage and percentage of fragmentation. The proportion of chromosomally abnormal embryos was evaluated in embryos with no fragments (●), in embryos whose fragments were concentrated in one area of the perivitelline space (♦), and in embryos having fragments scattered among blastomeres (■).



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of meiotic errors is quite uncommon in most organisms, with the notable exception of the human species, in which 10%–30% of oocytes carry chromosomal abnormalities (20). This condition has grave implications, because approximately one-third of spontaneous abortions are aneuploid. The presence of chromosomal abnormalities in embryos is rarely compatible with viability.

Accordingly, the FISH analysis of day 3 preimplantation embryos demonstrated that an abnormal chromosomal complement is more frequent in embryos dividing in a time frame that is different from what is expected; the highest incidence occurs in slow-cleaving embryos (7–9). As shown in the present study, there is a tight correlation between chromosomal status and embryonic development, implying that the timing of division is affected by the normality of the nuclear condition (Figs. 1, 2). The highest proportion of abnormalities was detected in arrested and slow-dividing embryos. These aberrations are mainly represented by multinucleation and mosaicism, which are related to negative effects that result in delayed timing of cleavage and increased fragmentation (4, 7–9). The main causes of this irregular development could reside in environmental factors or culture systems (21, 22), but also in low oxygen tension in follicular fluid (23). The consequences could be the occurrence of nuclear replication without cytokinesis, the fragmentation of nuclei, or defective chromosome movements at anaphase, giving rise to complex abnormalities with mosaicism and multinucleation. Another relevant factor could involve sperm centrosome dysfunction or an altered number of centrioles in the fertilizing spermatozoon. This could lead to aberrant spindle assembly, with a consequent abnormal pairing and disjunction of chromosomes at mitosis, and the generation of mosaic embryos (24, 25).

Interestingly, an accelerated cleavage rate is associated with chromosomal patterns that are similar to those described for slow-cleaving embryos. Embryos presenting with $\geq$9 blastomeres at 62 hours after insemination have the same chances of being chromosomally abnormal as slow-cleaving embryos. These observations suggest that timing is a crucial factor in determining embryonic development. In agreement with previous observations, any deviation from the expected cleavage stage has dramatic effects on implantation (2, 26, 27), as demonstrated by the fact that embryos which have already completed the third division at 62 hours after insemination have the highest chances of being chromosomally normal (Fig. 2).

Closely related to the effect of timing is the concept of a synchrony of events occurring in early embryogenesis. Oocyte viability is strictly dependent on a synchronized nuclear and cytoplasmic maturation that must occur properly to have the oocyte ready for meiosis resumption, fertilization, and cleavage. In humans, the first morphological evidence of synchronized development and polarity occurs at fertilization. At this stage, the distribution and number of nucleoli within the two pronuclei, and the position of the second polar body with respect to the first, seem to be a direct consequence of the chromosomal status of the pronuclear zygote (14, 28, 29). It was proposed that after fertilization, cleavage occurs according to axes of symmetry whose orientation is fundamental in the determination of the developing embryo (29). The planes of successive cell divisions control the distribution of maternal transcripts and proteins to the originating blastomeres, whose subsequent differentiation depends on the presence and concentration of these substances (30–33). There are some data demonstrating that a clear polarity of four-cell embryos exists, in which only one cell is destined to develop the clone that produces hCG in the developing blastocyst (34). It is thought that this cell is the first one to start the second division, and probably is farthest away from the second polar body. Although the functional significance of polarity is still largely unknown, a correct sequence of events seems to be crucial for the determination of lineages in the blastocyst, the inner cell mass, and the trophectoderm (35, 36).

These observations suggest that disturbances in the scheme of cell division according to a precise symmetry might be strictly related to embryo development. To shed light on the possible causes of altered cleavage, embryos with an uneven number of blastomeres were investigated in this study. The analysis by FISH of all their blastomeres revealed that chromosomal abnormalities were especially frequent in embryos with abnormal morphology. As shown in Table 3, the great majority of dominant blastomeres analyzed by FISH in five-cell and seven-cell embryos were abnormal, and had the same types of abnormalities detected in arrested embryos (multinucleation, complex abnormalities, and polyploidy). This is another point in favor of the close association between chromosomal complement and cell division, although it must be clear that these findings refer to observations performed 62 hours after insemination. In other words, it is not meant to say that all embryos with an uneven number of blastomeres are not viable, but to report that in a comparative study based on precise timing of observations, a pattern of cleavage different from the eight-cell stage has less probability of further development. In addition, because diploid cells characterize most mosaic human embryos (6, 10, 11), the fate of their development is strictly dependent on which cell line will prevail, although it is plausible that the ratio between euploid and aneuploid cells may need to be below a critical ratio. Conversely, in very fast-cleaving embryos, the presence of an uneven number of blastomeres does not seem to be associated to chromosome abnormalities. In this case, the tendency to an accelerated cleavage could be the most relevant factor related to the chromosomal condition.

Finally, interest has focused on the types and percentage of fragmentation in the perivitelline space in relation to the cell number on day 3 (1). As blastomere fragmentation is so frequent in human embryos, the question arises as to whether it is preferable to transfer a slow-cleaving embryo, e.g., five or six cells on day 3, with no fragmentation, or a regularly

cleaving embryo, e.g., still seven or eight cells on day 3, with variable degrees and types of fragmentation. According to recent reports, fragments can be positively related to mosaicism, because they could contain lagging or broken chromosomes originating through spindle errors (8, 37). Moreover, the loss of polarized proteins into fragments could heavily compromise embryonic development (31). It can be postulated that these effects are more severe if fragments are scattered in the perivitelline space, as they are originated from several or all blastomeres (1).

According to the data in the present study, when considering embryos with no fragments or with a concentrated pattern of fragments, the incidence of chromosomal abnormalities seems to vary according to the cellular stage, irrespective of the percentage of fragmentation. Conversely, in the presence of scattered fragmentation, the occurrence of chromosomal abnormalities is significantly higher in seven-cell or eight-cell embryos compared to when fragments are concentrated in one area (Fig. 3). On the other hand, for slow-cleaving embryos (4–6 cells on day 3; Fig. 3), there is apparently no correlation between fragmentation and total number of chromosomal abnormalities; possibly the delayed stage of cleavage is the prominent factor related to chromosomal disorders. Altogether, these observations suggest that eight-cell embryos on day 3 are always preferable for transfer even in the presence of fragmentation, especially if fragments are concentrated in one area of the perivitelline space.

It is remarkable indeed that even in embryos with good morphology (eight cells with no fragmentation), almost 50% of them are still abnormal, suggesting that PGD for aneuploidy could be considered a valid option, at least for patients with a poor prognosis (12, 38).

In conclusion, the study of the chromosomal complement in preimplantation embryos suggests that timing coordination of the processes occurring from the early stages of oogenesis are essential to regulate many aspects of embryo growth and morphogenetic movements. This happens according to physiological clocks that are active in the oocyte, in the fertilized zygote, and in the individual blastomeres forming the embryo. Their spatial and sequential integration represents the crucial step toward embryonic death or survival.

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