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Uses and limitations of two molecular cytogenetic techniques for the study of arrested embryos obtained through assisted reproduction technology

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ABSTRACT. We studied chromosomal abnormalities in arrested embryos produced by assisted reproductive technology with fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH) in order to determine the best technique for evaluating chromosomal aneusomies to be implemented in different situations. We examined individual blastomeres from arrested embryos by FISH and arrested whole embryos by CGH. All of the 10 FISH-analyzed embryos gave results, while only 7 of the 30 embryos analyzed by CGH were usable. Fifteen of the 17 embryos were chromosomally abnormal. CGH provided more accurate data for arrested embryos; however, FISH is the technique of choice for screening in preimplantation genetic diagnosis, because the results can be obtained within a day, while the embryos are still in culture.

Key words: Embryos, Comparative genomic hybridization, Fluorescence *in situ* hybridization

INTRODUCTION

The morphological variability of *in vitro*-generated human embryos has been the subject of numerous studies. Several morphological scoring systems to assess embryo viability have been proposed for the successive stages of human embryo development (Racowsky et al., 2003).

Despite enormous effort towards the improvement of pregnancy rates, only 15 to 30% of *in vitro*-developed embryos are able to achieve a biochemical/clinical pregnancy (Wright et al., 2003; Pandian et al., 2003). It is known that unbalanced chromosomal complement frequently does not affect embryo ability to reach the blastocyst stage *in vitro* (Evsikov et al., 2000). Recent studies have shown that the contribution of chromosomal abnormality to first trimester pregnancy loss is nearly 70% (Fritz et al., 2001). Using a panel of nine chromosome probes, Bielanska et al. (2002) showed that arrested mosaic embryos had a higher incidence of chaotic abnormalities than a non-arrested group.

Prior to the development of molecular cytogenetic techniques, it was very difficult to study the chromosomal complement of cells that were not in division. The advent of fluorescent *in situ* hybridization (FISH) allowed a more efficient assessment of chromosomal imbalance in all or most cells (Verlinsky and Evsikov, 1999). The incidence of abnormalities observed in human cleavage stage embryos using this technique is quite variable, ranging from 15 to 85%. Also, the inclusion of this technique for preimplantation genetic diagnosis resulted in an approximately 5% increase in implantation success rate and 14% fewer abortions after implantation (Munne et al., 1999).

In these studies, the incidence of haploidy, aneuploidy and polyploidy was much higher in the preimplantation embryos than in clinical pregnancies; up to 50% of embryos were mosaic, containing two or more chromosomally different cell lines. Various types of aneuploidy were identified, including consistently abnormal embryos (e.g., all blastomeres aneuploid or polyploid), abnormal mosaic embryos (most blastomeres abnormal alongside some normal blastomeres) and chaotic mosaics (blastomeres with randomly different chromosome complements).

Comparative genomic hybridization (CGH) is a technique that allows us to obtain a complete karyotype (Kallioniemi et al., 1992). It does not require a complete set of chromosome probes, and it can be used to obtain information from as little as a single cell (Vollaire et al., 1999).

We compared FISH with CGH on arrested embryos to evaluate their advantages and disadvantages in the assessment of chromosomal aberrations.

MATERIAL AND METHODS

Sample collection

This research project was approved by the Halitus Ethics, Teaching and Research Committee. Patients signed a consent form, permitting us to include their arrested embryos in the study.

After fertilization, embryos were cultured according to published protocols for up to three days (El-Toukhy et al., 2003). The embryos were graded according to cell number, degree of fragmentation and presence of equal-sized cells, and were classified based on the following

criteria: Grade A: good quality embryos; Grade D: bad quality embryos; Grades B and C were intermediate. Forty arrested embryos were used for this research. Arrested embryos are those that stop development, showing more than 50% fragmentation and having dark, contracted blastomeres. Ten of them were fixed on a slide and the individual blastomeres were used for FISH (Xu et al., 1998). The other 30 were placed in PCR tubes with 5 µl distilled water in order to amplify the total DNA with a degenerate oligonucleotide primer (DOP) PCR reaction (Vollaire et al., 1999).

Fluorescence *in situ* hybridization

A FISH protocol was used on cells from arrested embryos. Six of them were dissected to study some of their cells, while others were completely disaggregated, and every cell was used.

Cells were prepared *in situ*, on a poly-L-lysine-coated slide, by treating them in a hypotonic solution (1% sodium citrate in distilled water) containing 6% human serum albumin (HSA) (6 mg HSA/ml) and fixing them *in situ* with 3:1 methanol-acetic acid. The area to be hybridized was delineated by a glass pen in order to facilitate cell localization after FISH. Three sets of centromeric probes (18,X and 21-13) and a Y satellite III probe were developed and used in a FISH procedure. The centromere of chromosome 18 was labeled with Spectrum red. Centromeres of chromosomes 21/13 were labeled with Spectrum green. The centromere of chromosome X was labeled with biotin, and the Yq satellite III labeled with digoxigenin. Probes were produced by a combination of micro-dissection technique (Mühlmann-Díaz et al., 1995) and amplifying fragments with universal primers (Telenius et al., 1992), followed by amplification and labeling with specific primers (Hou and Wang, 1997).

FISH procedure

The probe set was obtained by co-precipitating all probes, which were previously checked for specificity in normal human lymphocyte spreads. Preoptimized amounts of probe were used in the hybridization mix, which was heated for 5 min at 85°C, placed on the hybridization area under a cover slip (about 5 µl for every embryo) and sealed with rubber cement. A co-denaturation procedure was then applied for 8 min at 68°C, and slides were allowed to hybridize overnight.

Six sequential washes were done in 50% formamide in 2x SSC (two times), 2x SSC (two times) and 2x SSC with 0.1% Tween (two times). Slides were then mounted in DAPI antifade and signals were observed under an epifluorescence microscope (Olympus BX51). At this point, only signals from the centromeres 18 (red) and 21/13 (green) were detected because X and Y were not yet developed (Figure 1A). Images of each cell and their signals were registered with an Optronics camera and saved as a computer file. Coverslips were then removed and a new round of the last four rinses was applied. Indirect labeled signals were then developed with a combination of FITC-antidigoxigenin antibody and streptavidin Texas red, which bind the digoxigenin and the biotin-labeled probe, respectively. Slides were incubated for 20 min at 37°C and then rinsed four times (as above) before remounting them again in DAPI antifade. Slides were placed under a fluorescent microscope and re-evaluated (Figure 1B). In general, the first signals of direct probes were now faded. New signals were compared with the

old ones in the saved files in order to distinguish 18 and 21/13, from X and Y, thereby obtaining the full set of information.

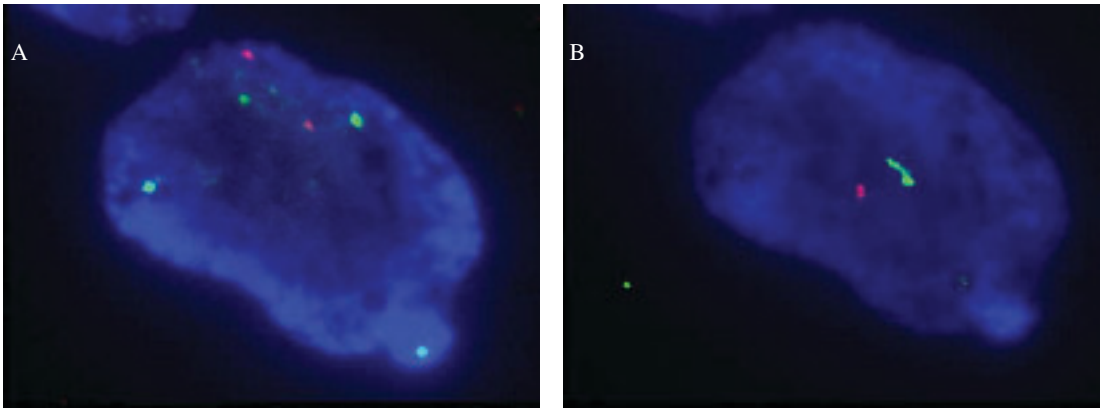

Figure 1. Cell from an arrested human embryo studied by FISH with normal outcome for the chromosomes. **A.** Cell with four green signals corresponding to chromosomes 21 and 13 and two red signals from chromosome 18. **B.** Same cell developed for indirect probes: Y digoxigenin (green) and X biotin (red).

Comparative genomic hybridization

A modified protocol for single cell amplification was used (Wells and Delhanty, 2000). Each arrested embryo was placed in a PCR tube with 5 µl endonuclease-free water and covered by a drop of PCR oil. The tube was heated for 5 min at 95°C to break cells and relax DNA, then 15 µl of PCR reaction mixture was added. The mixture consisted of 1.5 µM DOP primers, 200 µM dNTPs and 2 U thermosequenase (Thermo-sequenase cycle sequencing kit, USB) in the manufacturer's buffer. A PCR reaction followed: 12 ramp cycles (annealing at 37°C and then raising the temperature slowly for 4 min up to 72°C) and 30 regular PCR cycles (56°C annealing temperature 72°C extension) (Kuukasjarvi et al., 1997).

We checked whether this first reaction had a positive outcome by running the DNA in a minigel alongside a ladder standard. When the fragments were between 100 and 600 bp, we labeled the product with a regular PCR reaction in the presence of a fluorochrome. Standard DNA was obtained from about 10 male cells, following the same procedure as for embryos; that DNA was labeled with another fluorochrome.

We used Spectrum red-dUTP (Vysis Inc.) for the test (embryo) DNA and Spectrum green-dUTP for the control (normal male) DNA. One microgram of each DNA along with 30 µg COT (Human DNA enriched in repetitive sequences) were co-precipitated and resuspended in 20 µl of hybridization mixture. This was used to hybridize a slide spread with normal human male cells (46,XY). Co-denaturation was done at 73°C for 8 min. After overnight hybridization, rapid washes were done by rinsing for 2 min with 0.4x SSC 0.3% NP40 at 70-72°C, followed by 2x SSC 0.1% NP40 at room temperature.

We mounted the slides with DAPI. Image analysis was done with an epifluorescence Olympus BX51 microscope, and a Cool-Snap camera was used to capture the images. Metaphase analysis was done semi-automatically using various programs: Image-Pro, Excel and Cario-CGH (which was under development at the time of our experiments and kindly loaned by Bioanalitica S.A., Argentina).

RESULTS

Among the 40 embryos studied, we obtained full results for 17 by one of the two techniques. Fifteen of these 17 were chromosomally abnormal.

FISH technique allows us to study differences among cells of the same embryo (Table 1). We dissected few blastomeres from six of the embryos, and not all the cells were available for analysis. Among the set of five chromosomes studied by FISH, only those involved in any aneusomy for each embryo were included (Table 1). Most of the embryos were mosaic, which meant that not every cell had the same alteration. FISH technique does not provide information about the chromosomes that are not specifically studied.

Table 1. Embryos partially analyzed by FISH.

Embryo	No. of blastomeres*	No. of normal blastomeres	Aneusomic chromosomes	Embryo
A	3	0	X/0	Abnormal
B	2	0	X/0	Abnormal
C	2	0	21-13; 18	Chaotic mosaic
D	2	1	21-13; 18	Abnormal mosaic
E	2	0	X; 13-21; 18	Chaotic mosaic
F	2	0	X; 18	Chaotic mosaic
G	21	2	X/Y; 21-13; 18	Abnormal mosaic
H	7	2	X/Y; 21-13; 18	Abnormal mosaic
I	14	7	21-13; 18	Abnormal mosaic
J	18	1	21-13; 18	Chaotic mosaic

*Embryos were analyzed after cell by cell biopsy of two or three cells per embryo, or by disaggregating them on a slide (at least seven cells per embryo). They were classified according to their FISH results in each of the blastomeres. They were considered: Abnormal: when all cells had the same chromosome complement (one cell line). Abnormal mosaic: when there were two cell lines in the embryo; one normal and the other abnormal. Chaotic mosaic: when there were more than two different cell lines within the same embryo, or no normal cells were found among the abnormal cells.

An example of an analyzed blastomere is shown in Figure 1, illustrating the same cell hybridized with a mixture of direct and indirect probes. This example is a normal blastomere for the chromosome set that we studied.

CGH technique did not differentiate chaotic or mosaic embryos in our experimental design, since we used the whole arrested embryo. We analyzed at least four chromosomes of each pair for each case-embryo. Therefore, the resulting data are an average of at least four separate chromosomes for each chromosome number.

Normal ploidy red/green ratio was found to fluctuate from 0.8 to 1.2. Ploidy standardization was done with normal cells, using 48,XXXX cell DNA against normal female metaphases (Piper et al., 1995; Yu et al., 1997). We included the Y chromosome in this analysis because of its importance for embryo development and its involvement in some genetic disorders. Results were interpreted as follows: centromeric regions were not considered for the analysis. Red/green ratio from 0.8 to 1.2 for a given fragment or complete chromosome was classified as a normal chromosome in the embryo; ratios from 1.2 to 1.5 = trisomy; ratios from 1.5 to 1.8 = tetrasomy; ratios from 0.8 to 0.5 = monosomy, and a ratio below 0.5 was nullisomy. We did not have any result above 1.8 or below 0.5 (Table 2). Since we studied embryos as a

whole, not each cell by its self, the abnormality must be common to every cell or at least to most of them.

Table 2. Karyotype results from comparative genomic hybridization studies from seven arrested embryos.

Case embryo	Results*
2	Normal, 46,XY
7	47,X,+2,+4.
21	44,XY,dup(10)(p10),del(11)(p10),+16,-18,-18
22	46,Y,+20
25	Normal 46,XX
26	48,XY,+6,-9,+13,+16
28	50,XXYYY,-4,-5,del(10)(p10),+13,+18,+21

*International System for Human Cytogenetic Nomenclature.

Figure 2 shows an example of the results for the metaphases for one case.

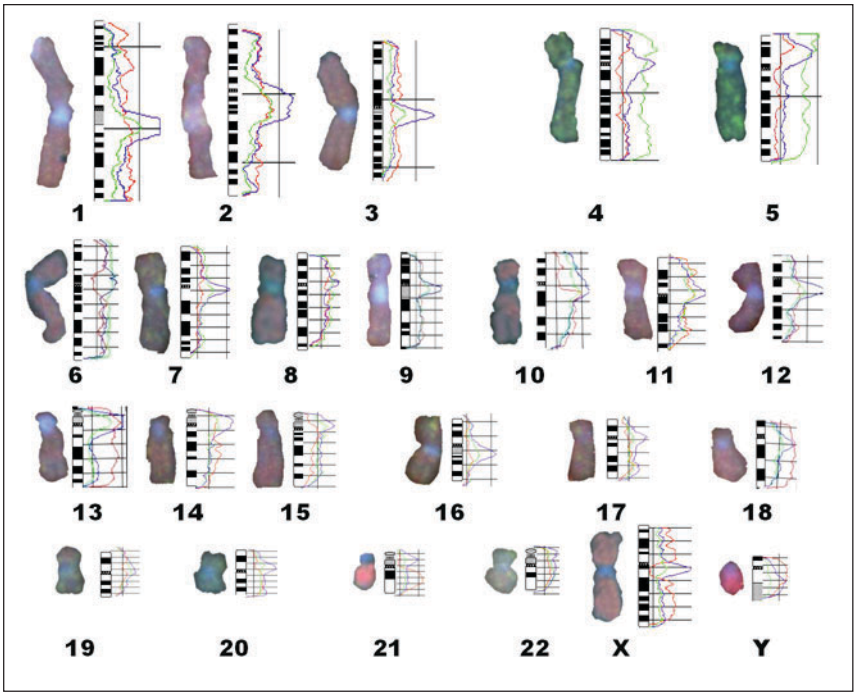

Figure 2. Comparative genomic hybridization for embryo 28. Each chromosome is aligned with the corresponding ideogram and a three color, line profile. The profile is the average of at least four chromosomes and shows the relative proportion of each color. Blue line = DAPI (COT); red line = Spectrum red (embryo DNA); green line = Spectrum green (normal competitive DNA).

DISCUSSION

We compared applications and constraints of two cytogenetic methods, FISH and CGH,

using arrested embryos obtained by assisted reproductive technology. Both techniques are extremely useful and provide important information on interphase cells (Muhlmann, 2002). Using both methodologies, we confirm that chromosomal aneusomies play an important role in the arrest of cleavage stage human embryos obtained by assisted reproductive technology procedures.

The five selected chromosomes studied by FISH are those that comprise the most frequent viable aneusomies. Some blastomeres were normal by FISH, but all of the embryos were chromosomally abnormal. We found 80% chaotic embryos; these cannot survive more than a few divisions. Even though some of the cells were normal for the five chromosomes, we cannot be certain of the ploidy of the unstudied chromosomes.

Using CGH, we found about 71% aneusomic embryos. The way in which we used CGH does not allow us to determine a chaotic or mosaic feature. We might have cases in which complementary aneusomies are compensated within an embryo, rendering a normal result in CGH. The characteristic of a CGH study allows us to obtain an aneusomic result that "dominates" the embryo; that means that the aneusomy is present in all or most of the cells. Meanwhile it can "dilute" an aneusomy present in one cell only. Therefore, when an abnormality is found, it must be present in all or almost all cells of the embryo. CGH technique in arrested embryos can inform any chromosome or segmental aneusomy, allowing us to identify chromosomal anomalies not commonly found in spontaneous early abortions.

In our investigation, the efficiency of FISH procedure was better than CGH, since all of the 10 cells analyzed by FISH rendered some results, while for CGH we obtained results in only 7 of 30 embryos. CGH has a higher sensitivity; since it allows us to study the whole set of chromosomes, and even to detect unbalanced translocations. On the other hand, FISH, as a screening method, uses only centromeric probes, therefore translocations cannot be evaluated. Neither the CGH nor the FISH method resolve balanced translocation.

For preimplantation genetic diagnosis, FISH can be resolved in few hours (Wilton et al., 2003); so, after biopsy, the embryo can be kept in culture until the ploidy for those chromosomes is reported and transfer can be accomplished. CGH takes much more time, so biopsied embryos need to be frozen (interfering with embryo viability).

A drawback for both technologies, as they are commonly used, is that they do not detect balanced translocations, which are sometimes found originating strong phenotypic abnormalities. For these reasons, only when the possible translocation is suspected, can a combination of telomeric centromeric sequences be useful using FISH technique (Van Assche et al., 1999; Coco et al., 2000).

CONCLUSION

The applicability of either technique depends on the study to be performed. CGH technique allows us to perform a complete karyotype from interphase cells, so it gives us more information in arrested embryos. By studying arrested embryos from the same patient, a regular pattern could eventually be found, which would allow us to design a better FISH protocol for future preimplantation genetic diagnosis. For us, the biggest problem was the low amplification efficiency, which surely can be improved. CGH does not detect abnormal ploidy. Haploidy, triploidy and tetraploidy have been shown by other techniques to occur in early embryos and also in clinical pregnancies (Perandones et al., 1998).

We obtained results by FISH from 100% of the analyzed embryos; these were ready within one day. Based on our practical experience, we believe FISH to be faster and more efficient for routine screening of preimplantation genetic diagnosis. Viable trisomies can be checked accurately and quickly, in order to proceed to embryo transfer. CGH is more time consuming, and embryo freezing, with its consequences, is required while performing the studies. For early and hard to culture abortion products, CGH would be the method of choice, especially because enough DNA can be extracted to make it work with higher efficiency than for blastomeres, and a complete karyotype can be studied.

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