GENETICS

FISH Analysis of Six Chromosomes in Unfertilized Human Oocytes After Polar Body Removal

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Purpose: To develop an improved technique for estimating chromosomal abnormalities in human oocytes by fluorescence in situ hybridization (FISH) and to correlate the position of single chromatids with the chromosomal status of the oocytes.

Methods: Oocytes that were at metaphase II about 17–20 hr after insemination or intracytoplasmic sperm injection (ICSI) were treated with pronase to remove the zona pellucida and polar body (PB) and then spread on slides using HCl and Tween 20. Two rounds of FISH were performed using direct-labeled probes: chromosomes 1, 13, 21 (round 1); chromosomes X, 7, 18 (round 2).

Results: Of the 63 oocytes from 18 patients (mean age, 32 years), 48 (76%) had one DNA complement as expected, 9 (14%) had 2 DNA complements, 3 (5%) gave incomplete FISH signals, and 3 (5%) were not analyzable. Of the 48 oocytes with one set of DNA, 48% were haploid, 44% were aneuploid for one or more chromosomes, and 8% were polyploid. We also found an increased frequency of predivision of chromatid bivalents in aneuploid oocytes, especially for chromosome 21.

Conclusions: This technique enables simultaneous assessment of six chromosomes in human oocytes, and therefore can be useful for accurately determining the incidence and causes of genetic imbalances in human oocytes and apparently low fertilization rates.

KEY WORDS: aneuploidy; FISH; oocyte; polar body.

INTRODUCTION

The introduction of assisted reproductive technologies (ART) such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) has made it possible to study early events in human reproduction and assess the contribution of spermatogenesis, oogenesis, fertilization, and early embryogenesis to the generation of chromosomal abnormalities. Since there appears to be no correlation between the chromosomal status and fertilizing ability of human gametes (1), it is important to consider the separate contributions of the oocyte and spermatozoon to the high aneuploidy rate in human embryos (2). Oocyte meiosis is sensitive to endogenous and exogenous factors, which can lead to chromosomally abnormal oocytes and hence abnormal zygotes (3). However, the signs of chromosomal imbalance in oocytes are less obvious than in spermatozoa, in which reduced concentration, motility, and morphology are readily apparent; therefore sensitive and reliable techniques are required to study chromosomal anomalies in otherwise morphologically normal oocytes.

The incidence of chromosomal aberrations in unispermated human oocytes has been reported as 8–50% and the rate of chromosomally abnormal oocytes appears to be high in cases of fertilization failure after IVF (27%) (4–8). In addition, studies on metaphase II human oocytes revealed an unexpected class of chromosome abnormalities, which manifest as single chromatids, rather than whole chromosomes comprising a dyad of chromatids. These studies also revealed a close correlation, with respect to frequency, distribution, and maternal age, between oocyte data and trisomies in aborted fetuses and live births (9–11). Aberrations

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arising de novo from meiotic nondisjunction (NDJ), especially paternal NDJ, contribute to chromosomal abnormalities in newborns after ICSI (8,12).

There are two main impediments to the study of chromosomes in human oocytes. First, there is a scarcity of oocytes, especially uninseminated oocytes, and unfertilized oocytes from ART cycles are usually the only readily available source of oocytes for chromosomal analysis. Second, the unusual and condensed nature of the chromatids on the metaphase II spindle (6,7) hampers reliable analysis by karyotyping. This necessitated the introduction of a modified protocol based on Tarkowski (13) to spread the chromosomes, but even this method has major drawbacks such as loss of cells and/or loss of morphology, which reduces the number of analysable oocytes and compromises the efficiency and accuracy of the analysis [reviewed in Warburton (3)].

To overcome these limitations, researchers have recently turned to fluorescence in situ hybridization (FISH), which enables the detection of whole chromosomes, centromeres, and unique sequences, including interstitial regions (14), and therefore is better suited to the study of oocyte chromosomes. We recently developed a method for studying chromosomal anomalies in metaphase II oocytes using the spreading technique of Coonen *et al.* (15) and a novel FISH procedure involving two triple-probe hybridizations to enable up to six chromosomes to be studied simultaneously (16). This novel approach enabled us to analyze 85% of the oocytes, a marked improvement on the low efficiencies (30–50%) obtained by karyotyping [Nakaoka *et al.*, (17); reviewed by Plachot (18)]. However, it was not always possible to differentiate between DNA from the first PB and the metaphase II chromosomes, which hindered interpretation of the results. The aim of the present study was to bypass this problem by removal of the polar body (PB) prior to spreading the oocytes so that only oocyte chromosomes were analyzed. Two rounds of FISH and direct-labeled probes for chromosomes 1, 7, 13, 18, 21, and X were used, enabling simultaneous detection of six different chromosomes in each oocyte.

MATERIALS AND METHODS

Source of Oocytes

Unfertilized oocytes were obtained from 18 couples undergoing ICSI ($n = 8$) or routine IVF ($n = 10$) in the Reproductive Medicine Unit in Adelaide. Of the

344 oocytes recovered, 200 (58%) fertilized normally and 111 of the unfertilized oocytes were used in this study. At 17–20 hr postinsemination, these oocytes all possessed a first PB, either normal or fragmented, and showed no signs of fertilization. The mean female age at oocyte collection was 32 years (range, 26–39). Protocols for patient selection and treatment have been described by Payne *et al.* (18,19) and Payne and Matthews (20). The study was approved by the Ethics of Human Research Committee at The Queen Elizabeth Hospital.

Zona Pellucida Removal

A 0.5% (wt/vol) solution of pronase (from *Streptomyces griseus*, specific activity 7000 U/g; Boehringer-Mannheim, Castle Hill, NSW, Australia) was prepared in HEPES-buffered human tubal fluid (HTF) culture medium (21) and frozen in aliquots at -20°C . On the day of use, aliquots were thawed, vortexed, and then clarified by centrifugation for 20 sec at 14,000g. Oocytes were processed individually, 20–24 hr postinsemination. Zona removal was performed in 50- μl microdrops, and oocytes were transferred between drops using fine-drawn glass pipettes (inner diameter slightly larger than the oocyte). Each oocyte was transferred into a drop of HEPES-HTF containing 0.1% human serum albumin (HSA), then into a drop of pronase solution. It was drawn in and out of the pipette four to five times, incubated for 30–60 sec, then aspirated in and out of the pipette again until the zona was partially removed. It then was transferred to a fresh drop of HEPES-HTF containing 0.1% HSA, after which final removal of the zona and PB was achieved by further aspiration. The oocyte was then rinsed in a fresh drop of HEPES-HTF containing HSA and spread on a slide.

Spreading of Oocytes

Oocytes were spread on slides using a modification of the method reported by Coonen *et al.* (15), as detailed in Martini *et al.* (16). Briefly, oocytes were transferred using a fine glass pipette to Super Starfrost Plus slides (Maenzel Glaeser, Germany). Microdrops of 0.01 *N* HCl and 0.1% Tween 20 in bidistilled water were added. This spreading solution caused the zona pellucida, the oolemma, and the cytoplasm to lyse first and then to dissolve, leaving the metaphase II DNA of the oocyte intact on the slide. Slides were subsequently air dried, washed, and dehydrated through an ascending ethanol series. They were stored at -20°C

in Adelaide before being shipped to Maastricht. The slides were then kept at room temperature for up to 1 month before being processed for FISH.

DNA Probes

Six probes were used: (a) pUCC 1.77 (satellite III, insert 1.77 kb), specific for the centromere of human chromosome 1 (22); (b) p7t1 (alphoid, insert 0.68 kb), which recognizes a tandem repeat in the centromere of human chromosome 7 (23); (c) LSI13, specific for chromosome 13q14 and labeled with Spectrum Green (Vysis, Downers Grove, IL); (d) L1.84 (alphoid, insert 0.68 kb), specific for the centromere of human chromosome 18 (24); (e) LSI21, specific for 21q22.13–21q22.2 labeled with Spectrum Orange (Vysis); (f) and pBam X5 (alphoid, insert 2.0 kb), specific for the centromere of human chromosome X (25).

Probes other than LSI13 and LSI21 were labeled by nick translation. The chromosome 18 probe was labeled with FITC-12-dUTP (Boehringer-Mannheim), the chromosome 7 probe was labeled with TRITC-4-dUTP (Amersham, Buckinghamshire, UK), and aliquots of the chromosome 1 and X probes were labeled with FITC-12-dUTP and TRITC-4-dUTP. Probes were dissolved in 60% formamide [FA], 2x SSC, and used at a final concentration of 1 ng/ μ l in hybridization solution (60% FA, 2x SSC, 10% dextran sulfate, tRNA, ssDNA).

The specificity of each probe was tested concurrently on interphase nuclei obtained from ethanol-fixed, single-cell suspensions of human leukocytes. Hybridization efficiencies were optimized using the above conditions and there was limited nonspecific binding of probes.

FISH Protocol

Two rounds of FISH were performed on each oocyte (16). The first round used probes for chromosomes 1, 13, and 21. After evaluation and scoring, the second round was performed using probes for chromosomes 7, 18, and X. Briefly, slides were treated with pepsin (100 μ g/ml) for 15 min at 37°C to remove any remnants of cytoplasm and make the nuclei accessible for hybridization to the probes, then they were fixed in paraformaldehyde for 5 min at 4°C, rinsed in PBS, then bidistilled water, and dehydrated through an ethanol series. Ten microliters of hybridization mixture containing the first three probes (1-FITC and 1-TRITC, 13-Spectrum Green and 21-Spectrum Orange) were added to the slide and sealed under a coverslip. The

nuclear and probe DNA were denatured simultaneously for 3 min at 72°C, then incubated overnight in a moist chamber at 37°C. After hybridization, slides were washed, dehydrated, and mounted in Vectashield antifade (Vector Laboratories, Burlingame, CA) containing 0.01 μ g/ml of 4,6-diamidino-2-phenylindole (DAPI) to counterstain the DNA. After analysis and recording of results from the first FISH round, and on the same day, DAPI was removed with two washes, the slides were dehydrated, and the probes for the second round were applied (X-FITC and X-TRITC, 18-FITC and 7-TRITC). A coverslip was sealed on the slide, and denaturation and hybridization were performed as for the first round, except that hybridization proceeded for only 2 hr at 37°C. The slides were washed, counterstained with DAPI, and reanalyzed.

Evaluation and Scoring of Slides

Slides were examined using a DMRBE fluorescence microscope (Leica, Wetzlar, Germany) equipped with a triple band pass filter block containing FITC, TRITC, and DAPI filters. The stringent scoring criteria used have been described by Martini *et al.* (16). Paired signals reflect the unique nature of oocyte chromosomes at metaphase II, highly contracted, with sister chromatids widely separated and loosely adjoined in the centromere (26). The positions of sister chromatids were scored as dyads, when the signals were close to each other (normal situation), or as predivision, when the signals were separated. Images were recorded using a CCD camera, green, red, and blue filters, and the Image Pro System (Metasystem, Sandhausen, Germany). Sharpness was enhanced in Photoshop 3.0 (Adobe Systems Inc., San Jose, CA) at 120 pixels per inch (see Fig. 1).

RESULTS

A total of 111 oocytes were prepared for FISH analysis. After the first FISH round, the DNA of 63 oocytes was visualized using DAPI, giving a spreading efficiency of 57%. Of these 63 oocytes, 57 were analyzed in both FISH rounds, yielding a FISH efficiency of 91%.

Oocytes were categorized into three groups based on the number of DNA fragments and the number and arrangement of hybridization signals. After spreading, only one DNA fragment (oocyte metaphase II) should be seen on the slide (group I). However, sometimes the DNA split and two fragments were detected (group

Fig. 1. FISH results on human oocytes. Images from the first round of FISH are shown in a, c, e, and g, while b, d, f, and h are from the second round. In the first round the signals are chromosomes 1 (yellow), 13 (green) and 21 (red), while in the second round the signals are chromosomes X (yellow), 18 (green) and 7 (red). DNA is counterstained blue. (a,b) Haploid oocyte, with paired signals in the first round and hypohaploidy for chromosome 18 in the second round. (c,d) Haploid oocyte with paired signals in the first round for chromosomes 1 and 13 and hyperhaploidy and predivision for chromosome 21. The second round shows hyperhaploidy for chromosome 7 and predivision of chromatid bivalents. (e,f) Triploid oocyte with paired signals for all chromosomes in both rounds. (g,h) Haploid oocyte showing predivision of chromatid bivalents for all six chromosomes and hyperhaploidy for chromosome 13 in the first round.

II). Finally, in some cases there were too many DNA fragments, perhaps due to segregation of the chromosomes, and the results were not analyzable (group III).

Group I ($n = 48$, 76.2%) had one DNA complement and a haploid ($n = 23$, 36.5% of total, 47.9% of group I), aneuploid ($n = 21$, 33.3% of total, 43.8% of group I), or polyploid ($n = 4$, 6.3% of total, 8.3% of group I) chromosomal constitution. A normal metaphase II oocyte has a single univalent chromosome from each pair and all the chromosomes should appear as two close dots representing the two chromatids (dyads). However, some signals may be at considerable distance from each other, indicating that predivision of the chromatids has begun (9).

The term "aneuploidy" indicates variation of the correct number of chromosomes (euploidy). In Table I we report the findings related to a loss (hypohaploidy) or gain (hyperhaploidy) of the single chromatids. Some oocytes presented an abnormal number of chromosomes for only one of the six chromosomes analyzed, while others had a complex situation (i.e., loss of one chromosome or chromatid but gain of another, loss of two chromosomes). In some, the chromatids were adjoined, while in others a predivision of dyads had occurred. The occurrence of hyperhaploidy and hypohaploidy is shown in Table I.

Group II ($n = 9$, 14.3%) exhibited two DNA complements: a haploid chromosome constitution in both DNA complements with only one chromatid signal in each complement ($n = 3$) (perhaps originating from a metaphase II DNA that had split); a haploid chromosome constitution in both complements with dyads and single chromatids ($n = 1$) (from a metaphase II DNA and perhaps a precondensed spermatozoa); one haploid complement and one aneuploid complement ($n = 3$); and aneuploidy in both DNA complements ($n = 2$). Group III ($n = 6$, 9.5%) members were unanalyzable ($n = 3$) or yielded incomplete results due to failure of one of the hybridization rounds ($n = 3$).

The results for haploid and aneuploid oocytes from group I (one DNA fragment) were further analyzed to determine whether there was any correlation between

aneuploidy and predivision of chromatid bivalents (27). The findings are presented in Table II. Haploid oocytes usually displayed dyads using centromeric probes for chromosomes 1, X, 7, and 18 (83–91% dyads) as well as using LSI probes for chromosomes 13 and 21 (74–78% dyads). Aneuploid oocytes, however, showed an increased rate of predivision of chromatid bivalents for all the chromosomes studied, especially chromosome 21 (66%).

DISCUSSION

We recently reported a two-round, multiprobe FISH method for simultaneously assessing up to six chromosomes in human oocytes. This was a significant improvement on karyotyping of oocytes in terms of efficiency and an improvement on other FISH techniques in terms of the number of chromosomes studied (16). A similar approach recently was described by Liu *et al.* (28) for studying six different chromosomes in blastomeres from human embryos. However, our first study encountered a restriction in differentiating between oocyte and PB DNA. After spreading oocytes, the chromosomes are clustered together in DNA "fragments." DAPI counterstaining allows the visualization of the DNA, but still it is difficult to differentiate DNA from the PB and DNA from the oocyte, especially if the PB was fragmented at the time of fixation.

Therefore, in the present study we modified the technique of Martini *et al.* (16) by removing the zona pellucida and PB before spreading the oocyte, thus ensuring that only oocyte DNA was assessed. We recognize that correlative information from the PB was lost by this approach, but at the time of the study this was considered to be less important than clear assessment of the chromosomal complement of the oocyte itself. Furthermore, PB results also can be unreliable due to difficulties in visualizing PB chromosomes (29) and poor correlation between results in the PB and embryo (30). Durban *et al.* (14) described a

Table I. Incidence (Number) of Hyperhaploidy and Hypohaploidy

Chromosome	Hyperhaploidy	Hypohaploidy	Chromatids
1	10	—	52
7	17	1	58
13	2	12	32
18	15	5	52
21	3	16	29
X	6	8	40

Table II. Incidence (%) of Chromatid Dyads in Haploid and Aneuploid Oocytes

Chromosome	Haploid ($n = 23$)	Aneuploid ($n = 21$)
1	91.3	60.3
7	82.6	51.7
13	73.9	50.0
18	87.0	51.7
21	78.3	34.2
X	91.3	73.3

procedure to obtain first PB chromosome complements with defined chromosome morphology, so we may see clinically relevant improvements in this regard in the near future.

Removal of the PB prior to spreading reduced the interpretative problems encountered in our earlier study; however, the efficiency with which oocytes were spread and able to be analyzed (i.e., DNA with signals on the slide) was lower (57% vs. 85%) (16). There are several explanations. First, a single cell is analyzed and techniques such as spreading and fixation of the cells, as well as FISH, always encompass a risk of losing the cells, for example, by dissolution of the oolemma in the pipette during spreading or by damaging the DNA during denaturation steps. Second, pronase treatment could have reduced the adhesiveness of the oolemma via proteolysis of membrane proteins. Finally, the storage and shipping conditions may have resulted in the loss of some oocyte DNA from slides. We would expect a higher efficiency if such a study were undertaken in one laboratory without extended transport and storage of slides.

Incidence of Aneuploidy in Human Oocytes

We found a high rate of aneuploidy in unfertilized oocytes in this study. These data are preliminary given the small sample size, but similar findings were reported in our previous study (16) and in other studies on human oocytes (8). Dyban *et al.* (26) recorded aneuploidy rates as high as 37% for chromosomes X and 18 after FISH analysis, which is in agreement with earlier karyotyping reports on metaphase II human oocytes. The results of the present study revealed an overall aneuploidy rate of 44% (48% in oocytes with one DNA fragment, group I). There were different frequencies and patterns for each of the chromosomes studied, which is not surprising given that meiotic NDJ is nonrandom and exhibits chromosome-specific patterns (8,30). We found an excess of hyperhaploidy for chromosomes 1, 7, and 18, an excess of hypohaploidy for chromosomes 13 and 21, and an equal distribution of hyperhaploidy and hypohaploidy for chromosome X. Locus-specific probes such as those used for chromosomes 13 and 21 have different spatial arrangements than centromeric probes and the hypohaploidy found might be caused by artificial loss of chromatids. However, if this were to occur, it should be equal for all the chromosomes analyzed, and since we found an increase in the number of signals for chromosomes 1, 7, and 18, it also might reflect the true situation. Karyotyping studies on oocytes often

report a higher incidence of hypohaploidy than hyperhaploidy and this is attributed to loss of chromosomes (31). The incidence of aneuploidy, therefore, is often calculated by doubling the hyperhaploidy rate, resulting in so-called "conservative estimates" of aneuploidy. This was not required in our study because hypohaploidy and hyperhaploidy were represented equally.

There is still debate as to whether or not these high aneuploidy frequencies are overestimates, but they might be explained in several ways. First, some of the oocytes, especially the ICSI oocytes, would have contained partially decondensed sperm nuclei (32), and extra signals from the sperm DNA might have led to overestimation of hyperhaploidy in haploid oocytes (16,31). This is unlikely, however, because the sperm nuclei usually would be insufficiently decondensed to yield signals, and it seems more probable that this would have elevated the polyploidy rate rather than the hyperhaploidy rate. An additional explanation for the high aneuploidy rate is a distinct identification of chromosome-specific aneuploidies, whereas karyotyping mostly classify aneuploidy using the broad Denver classification. Moreover, the 20 to 24 hr period between insemination and fixation, and/or the pronase treatment, might have adversely affected the meiotic spindle in some of the oocytes, leading to de novo aberrations during culture. There also might be a collateral patient-to-patient variability, which influences the total aneuploidy rate (3). Finally, the cells analyzed belong to a cohort of oocytes forced to mature by exogenous hormonal stimulation and subjected to more aggressive technical handling than they would in a normal in vivo situation.

Predivision of Chromatid Bivalents

Angell (33) karyotyped human oocytes and reported that hyperhaploidy was not due to extra whole chromosomes (bivalents) but rather to an extra single chromatid or two single chromatids. This suggests that precocious division (predivision) of chromatid bivalents during the first meiotic division, leading to single chromatids, is the most likely mechanism for trisomy formation, rather than NDJ of whole bivalents as previously thought. Paired signals corresponding to paired chromatid bivalents (dyads) were reported in several FISH studies on human oocytes (26,34,35); however, the detection sensitivity in those studies was low because only single-probe FISH was applied to a small number of oocytes. Marquez *et al.* (36) recently reported the identification of chromosomes in human

oocytes and PB using spectral karyotyping and FISH. They also reported data on NDJ of whole bivalents and predivision of bivalents at meiosis I. This new technique may be a promising alternative to karyotyping and warrants further study. Nakaoka *et al.*, (17) found that in 388 karyotyped oocytes, of the 33 aneuploidies, 48.5% showed loss or gain of dyads, while 51.5% showed loss or gain of single chromatids (predivision).

We reported in our previous study (16) that the centromeres of chromatids were sometimes far apart from each other and unpaired, but we did not relate this to the chromosomal constitution of the oocyte. In the present study, we found an association between aneuploidy and the relative positions of the chromatids. Haploid oocytes generally presented dyads (frequency 74–91%), with the lowest percentages being associated with the acrocentric chromosomes (chromosomes 13, 21) and the use of locus-specific probes that do not bind to the centromeric region. Aneuploid oocytes displayed an increased frequency of predivision for all six chromosomes, especially the acrocentric chromosomes (chromosomes 13, 21). These results support the hypothesis that trisomy formation in humans can occur as a result of a predivision of bivalents at meiosis I (11, 27). Since chiasma frequency declines with increasing maternal age, the reduction in chiasma frequency may be accompanied by the production of univalents, probably at the origin of NDJ (33). Alterations in cell cycle progression might in turn be the principal cause for maternal age-related predisposition to NDJ (37). Moreover, Angell (11) studied oocytes that had been in culture for ~70 hr prior to fixation. This period is longer than the total culture time for the oocytes used in our study. Munne *et al.*, (38) and Dailey *et al.* (39) also suggested that some type of abnormalities found by Angell (40) and Angell (9) might be artifacts specifically related to time in culture. Unfortunately, the patient group in the present study was too small to search for correlations between aneuploidy, predivision of bivalents, and maternal age. This warrants further investigation.

CONCLUSIONS

The aim of this study was to improve existing techniques for detecting aneuploidy in human oocytes. This was achieved by removing the zona pellucida and PB with pronase before spreading oocytes, followed by two rounds of multiprobe FISH enabling assessment of six different chromosomes. This provided good insight into the chromosomal constitution of each oocyte and

yielded more data than standard multiprobe FISH approaches. The preliminary data, thus, obtained suggested a high rate of aneuploidy and an association between predivision of chromatid bivalents and aneuploidy, supporting the hypothesis of Angell (11). This method should be useful for determining the contribution of oocytes to chromosomal abnormalities in human embryos.

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