Analysis of unfertilized oocytes subjected to intracytoplasmic sperm injection using two rounds of fluorescence in-situ hybridization and probes to five chromosomes

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Chromosomal aberrations are the major cause of pre- and post-implantation embryo wastage and some studies suggest that half of all human concepti have a chromosomal abnormality. Analysis of gametes provides information on the origin of these chromosomal aberrations. The purpose of this study was to develop a reliable multi-probe fluorescence in-situ hybridization (FISH) procedure that would enable us to investigate aneuploidy in unfertilized oocytes subjected to intracytoplasmic sperm injection (ICSI). Oocytes were spread with HCl and Tween 20 solution, and then two rounds of triple-probe FISH were performed on each oocyte using directly-labelled centromeric probes: chromosomes 1, 7, 15 (overnight hybridization); chromosomes 1, X, Y (2 h hybridization). After the first round, the slides were counterstained and evaluated, and the positions of FISH signals were recorded. For the second round, the counterstain was removed and the second probe cocktail was applied. The chromosome 1 probe was an internal control for the two hybridization procedures, while the Y chromosome probe was used to detect sperm DNA. To evaluate the method, a total of 79 oocytes from 27 patients were studied. Of these, 67 (84.8%) were successfully spread and 97% of these oocytes exhibited discernible FISH signals. Upon lysis, oocytes exhibited one or more DNA fragments (mean 1.9, range 1–3). Of the 65 analysable oocytes, 17 (26.2%) displayed a normal haploid chromosome constitution with paired spots for the two chromatids. A further 23 oocytes (35.4%) showed an ambiguous chromosome complement due to an abnormal number of DNA fragments which may have resulted from loss of DNA during spreading or to an abnormal oocyte, while 25 oocytes (38.4%) displayed aneuploidy for one or more of the chromosomes studied. In conclusion, this new approach is a quick and efficient method with which numerical chromosomal abnormalities in human oocytes can be studied; interpretation of the patterns of DNA fragments and FISH signals requires further clarification.

Key words: aneuploidy/chromosome/FISH/ICSI/oocyte

Introduction

Aneuploidy has been observed for almost every chromosome in human spontaneous abortions (Hassold et al., 1980), whereas in human live born infants, aneuploidy is generally only seen for chromosomes 13, 18 and 21 and the sex chromosomes (De Grouchy and Turleau, 1984). Some estimates suggest that up to 50% of all human concepti have a chromosomal abnormality (Boue et al., 1975), and chromosomal aberrations which arise during gametogenesis and early embryonic development play a significant role in fetal loss (Chard, 1991). The introduction of in-vitro fertilization (IVF) has made it possible to study meiotic and mitotic errors during oogenesis, spermatogenesis, fertilization and early embryogenesis and assess the contribution of these errors to chromosomal abnormalities in embryos and fetuses.

Many chromosome studies on human gametes have been performed by karyotyping, a technique which is considered by many to be the gold standard. Chromosomal abnormalities in human oocytes have been studied by karyotyping (Martin et al., 1991; Edirisinghe et al., 1992; Angell et al., 1993; Angell, 1994; Roberts and O’Neill, 1995) and it has been suggested that a high proportion of human oocytes (25–30%) are chromosomally abnormal (Plachot et al., 1988; Benkhalifa et al., 1990; Pellestor, 1991b). The two main difficulties encountered while studying chromosomes in human oocytes are first, the scarcity of oocytes, and second, the unusual, condensed nature of the chromatids on the metaphase II spindle (Pellestor, 1991b). The condition of the chromatids hampered reliable analysis by karyotyping and necessitated the introduction of a modified protocol based on Tarkowski (1966) to spread the chromatids. This method uses methanol:acetic acid (3:1) fixation and has major drawbacks such as loss of cells and/or loss of morphology, which reduces the efficiency, reliability and reproducibility. This is a great disadvantage in the study of aneuploidy, where the results must be reliable and accurate.

In recent years, fluorescence in-situ hybridization (FISH) has also been used to study chromosomes in human gametes. FISH has been used on biopsied blastomeres and polar bodies for preimplantation genetic diagnosis (PGD) of X-linked diseases and aneuploidy (Griffin et al., 1992; Harper et al., 1994; Veiga et al., 1994; Munné et al., 1995a, b), as well as to study early embryogenesis (Benkhalifa et al., 1993; Munné et al., 1994; Muggleton-Harris et al., 1995; Sultan et al., 1995). FISH has also been used to investigate aneuploidy in spermatozoa from healthy donors and infertility patients (reviewed by Downie et al., 1997) and in the study of oocytes (Benkhalifa et al., 1996; Dyban et al., 1996; Wall et al., 1996).
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Coonen et al. (1994) presented an alternative method for preparing and isolating interphase nuclei from preimplantation embryos using HCl and Tween 20 which offers high reproducibility, good cellular morphology and a high FISH efficiency (Coonen et al., 1994; Harper et al., 1994; Muggleton-Harris et al., 1995).

Since its introduction in 1992 (Palermo et al., 1992), intracytoplasmic sperm injection (ICSI) has revolutionized the treatment of male factor infertility. However, the overall fertilization rate is only 60–70% and so a considerable number of oocytes are potentially wasted. Flaherty et al. (1995) documented that 82% of unfertilized ICSI oocytes were still at metaphase II and the majority (71%) of these contained a swollen sperm head, indicating that fertilization failure was due to defective oocyte activation and not to the injection technique. Similar results were obtained by Dozortsev et al. (1994), Sousa and Tesarik (1994), and Wall et al. (1996).

Furthermore, it has been shown that these injected but unactivated oocytes can be activated using calcium ionophore (Tesarik and Sousa, 1995) or sperm extracts (Palermo et al., 1997) and in many cases this resulted in development of pronuclei. The safety of this approach has not been proven and it would be imprudent to use this procedure clinically until the safety of the method has been properly assessed.

The aim of this study was to develop and assess a reliable FISH method for studying chromosomal anomalies in unfertilized, metaphase II ICSI oocytes. We used the spreading technique of Coonen et al. (1994) and a novel FISH procedure involving two triple-probe hybridizations to enable a larger number of chromosomes to be studied. Directly-labelled centromeric probes for chromosomes 1, 7, 15, X and Y were used. The chromosome 1 probe was included in both rounds as an internal control for the specificity, sensitivity and efficiency of the FISH procedure.

**Materials and methods**

**Source of oocytes**

Unfertilized oocytes were obtained from 27 women undergoing ICSI in the Reproductive Medicine Units at The Queen Elizabeth Hospital and Wakefield Clinic. The study was approved by the Ethics of Human Research Committee at The Queen Elizabeth Hospital. Our protocols for patient selection, ovarian stimulation, collection and preparation of spermatozoa and oocytes, and ICSI have been described by Payne et al. (1991, 1994) and Payne and Matthews (1995). Of the 343 oocytes recovered from the 27 patients (mean age: 31.3; range: 21.3–40.8), 263 were inseminated and 147 (56%) fertilized normally. The oocytes used in this study (n = 79) had all been injected with a spermatozoon, but failed to show any evidence of fertilization when examined 17 h post-injection using differential interference contrast (DIC) optics. They all possessed a first polar body (PB) or a fragmented PB.

**Spreading of oocytes**

Oocytes were spread about 1–2 h after the fertilization check (18–20 h post-injection) using the method developed by Coonen et al. (1994). Using a fine glass pipette, oocytes were transferred in a minimal volume of culture medium to microdrops (1–2 µl) of 0.01 N HCl and 0.1% Tween 20 in bi-distilled water on Super Starfrost Plus® slides (Menzel Glaeser, Braunschweig, Germany). This spreading solution caused the zona pellucida to dissolve, the oolemma to lyse immediately, and the cytoplasm to dissolve, leaving intact the metaphase II DNA of the oocyte and, if present, the DNA of the first PB. Once all the cytoplasm had been removed by gently adding fresh spreading solution, the individual DNA fragments remained attached to the slide. During the whole procedure, oocytes were watched constantly using an inverted microscope. Photographs were taken using Fujichrome® 400 ASA Sensia film. Slides were subsequently air dried, washed with phosphate buffered saline (PBS, pH 7.0) for 3 min and dehydrated through an ascending ethanol series. Slides were stored at room temperature for up to 1 week or processed immediately for FISH.

**DNA probes**

Five centromeric probes were used: (1) pUCC 1.77 (satellite III, insert size 1.77 kb), specific for the centromeric region of human chromosome 1 (Cooke and Hindley, 1979); (2) p71t (alphoid, insert size 0.68 kb), recognizes a tandem repeat in the centromeric region of human chromosome 7 (Waye et al., 1987); (3) pBam X (alphoid, insert size 2.0 kb), specific for the centromeric region of human chromosome X (Willard et al., 1983); (4) DYZ1 (satellite II, insert size 2.1 kb), specific for the long arm of human Y chromosome (Cooke et al., 1982); and (5) CEP15, a human chromosome 15 probe labelled with Spectrum Orange (Vysis, Downers Grove, IL, USA). Probes other than CEP15 were labelled by nick translation with either FITC-12-dUTP (Boehringer Mannheim, Mannheim, Germany) or TRITC-4-dUTP (Amersham International plc, Buckinghamshire, UK). Probes were dissolved in hybridization mixture [60% formamide (FA), 2× SSC buffer] and used at a final concentration of 1 ng/µl in hybridization solution (60% FA, 2× SSC, 10% dextran sulphate, tRNA, ssDNA).

**FISH protocol**

Two rounds of FISH were applied to each oocyte. The first round used a probe cocktail for chromosomes 1, 7 and 15. After screening, evaluation and recording of the slides, the second round was performed with a probe cocktail for chromosomes 1, X and Y. The human chromosome 1 probe was used as an internal control for FISH efficiency, sensitivity and specificity, while the V-chromosome probe was used as a control for sperm DNA.

Slides were pretreated using a protocol adapted from Harper et al. (1994). Briefly, they were treated with pepsin (Sigma Chemical Co., St Louis, MO, USA; 100 µg/ml) in 0.01 N HCl for 15 min at 37°C to remove any remnants of cytoplasm and make the nuclei accessible to the probes. The slides were rinsed twice in bi-distilled water, followed by phosphate buffered saline (PBS), then fixed in 1% paraformaldehyde in PBS for 5 min at 4°C. After fixation, the slides were rinsed twice in PBS followed by one rinse in bi-distilled water, and dehydrated through an ethanol series (70–96–96–100–100%). Ten µl of hybridization mixture containing the first three probes (1-FITC and 1-TRITC, 7-FITC and 15-Spectrum Orange©) was 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, the slides were washed twice in 2× SSC, 0.05% Tween 20 for 5 min at 42°C, then twice in 0.1× SSC for 5 min at 60°C, twice in 4× SSC, 0.05% Tween 20 for 5 min at room temperature, then dehydrated through an ethanol series and mounted in Vectashield antifade (Vector laboratories, Burlingame, CA, USA) containing 0.01 µg/ml 4,6-diamidino-2-phenylindole (DAPI) to counterstain the DNA.

Slides were examined using an Olympus Vanox-AHBT3 fluorescence microscope equipped with a triple band-pass filter. After
analysis and recording of results from the first FISH round, and on
the same day, DAPI was removed with two washes in 4× SSC, 0.05% Tween 20 for 5 min at room temperature. The slides were
dehidrated, and the probes for the second FISH round were applied
(1-FITC and 1-TRITC, X-FITC and Y-TRITC). A coverslip was
sealed on the slide, and the denaturation and hybridization steps were
performed as described for the first round, except that hybridization
proceeded for only 2 h at 37°C. The slides were washed as described
above but with only one 5 min wash at each step, then they were
counterstained with DAPI and analysed. Photographs were taken
using Fuji® 400 ASA film.

Evaluation and scoring criteria
The specificity of each FISH probe was tested concurrently on
interphase nuclei obtained from ethanol-fixed suspensions of male
leukocytes (5×10⁶/ml). Using the conditions described above, the
hybridization efficiency was optimal and there was only limited non-
specific binding.

Analysis of the FISH signals was performed according to the
criteria set by Hopman et al. (1988) and adapted by Munne et al.
(1995a, b). Minor hybridization spots that had much lower fluores-
cence intensity were not scored, and spots found in close proximity
to one another, interconnected or in paired arrangements, were counted
as one signal. Minor hybridization spots were considered to be cross-
hybridization to non-target chromosomes, while paired arrangements
were interpreted as sister chromatids or split signals. Due to the
compact nature of the DNA, we considered the occurrence of two
single spots for a chromosome to be disomy only if a single signal
was present for the other chromosomes. The opposite situation, in
which all the chromosomes examined showed two signals except
one, was interpreted as monosomy for the latter chromosome.

Results

Efficiency of the protocol
A total of 79 of the unfertilized oocytes were used for this
study. Twelve were lost during the spreading procedure and
67 were successfully spread and fixed onto clean slides, giving
a spreading efficiency of 84.8%. After the first FISH round,
two of the 67 cells were unanalysable due to the presence of
cytoplasmic remnants covering the DNA, but the remaining
65 oocytes were analysed successfully in both FISH rounds.
The overall FISH efficiency was 97.0%. The signals for
chromosome 1 were identical in both FISH rounds for every
oocyte.

FISH results
Oocytes exhibited one or more DNA fragments and were
categorized into three groups based on the pattern of DNA
and the number and arrangement of hybridization signals
(Figure 1).

Group I consisted of 17 oocytes (26.2%) which displayed
two DNA fragments and a normal haploid chromosomal
constitution with paired spots for the two chromatids of each
univalent chromosome, except the Y chromosome if present
(Munne et al., 1995a, b; Wall et al., 1996) (Figure 1). A
normal metaphase II oocyte has a single univalent chromosome
from each pair, and all the chromosomes should appear as two
close spots representing the two chromatids; the same pattern
should occur in the first PB, both in interphase and metaphase
(Figure 1, patterns i–ii; Figure 2).

Group II consisted of 23 oocytes (35.4%) which displayed
an ambiguous chromosome complement due to an abnormal
number of DNA fragments (Figure 1, patterns i–vii). These
Figure 2. (a–c) Spreading of the oocyte. (a) Oocyte with first polar body and no pronuclei. At the beginning of spreading, the oolemma is still present. (b) Lysis of the polar body; the DNA is visible. (c) Isolated metaphase DNA. (d–i) Fluorescence in-situ hybridization (FISH) results. Directly labelled probes for chromosome 1 (yellow), 7 (green), 15 (red) in the first FISH round (d, f, h), and chromosome 1 (yellow) and X (green) in the second FISH round (e, g, i), hybridized to DNA (blue) of unfertilized intracytoplasmic sperm injection (ICSI) oocytes. (d, e) First and second FISH rounds respectively on the same DNA. In (d) signals for the chromatids are close to each other, representing dyads. In (e), the X-chromosome is clearly distinguishable from the remnants of the first hybridization. The signals for chromosome 1 are at the same location as in the first round. (f, g) First and second FISH rounds respectively on the same DNA. Example of pre-division of the dyads. The chromatids are clearly separated from each other in (f) and are almost at opposite poles. In (g), the X-chromatids are at the same pole. (h, i) First and second FISH rounds respectively on the same DNA. Example of aneuploidy for all the chromosomes analysed. In (h) three chromatids of chromosome 1 are visible, three for chromosome 7 and four for chromosome 15. In (i), three chromatids for chromosome 1 are evident and four copies of chromosome X.
Table I. Number of oocytes with single chromatid hyperhaploidy and hypohaploidy

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Hyperhaploidy</th>
<th>Hypohaploidy</th>
<th>Total chromatids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>~</td>
<td>221</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>3</td>
<td>209</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>3</td>
<td>193</td>
</tr>
<tr>
<td>X</td>
<td>1</td>
<td>16</td>
<td>192</td>
</tr>
</tbody>
</table>

...omies might be explained by two hypotheses, the first that abnormalities are triggered by exogenous hormone induced ovulation, and the second that in-vitro ageing of oocytes induces spindle instability and a subsequent loss or scattering of chromosomes. To date, no evidence has been obtained for the first hypothesis (Pellestor, 1991b; Edirisinghe et al., 1992), and the second one has also been questioned (Pellestor, 1991a; Roberts and O’Neill, 1995; Macas et al., 1996). Nevertheless, it should be kept in mind that anomalies in metaphase II oocytes contribute to aneuploidy at later cleavage stages.

...mercial studies on human oocytes (Benkhalifa et al., 1996; Dyban et al., 1996; Wall et al., 1996), but only single probe FISH was applied to a small number of oocytes in these studies.

In this study, we applied a multi-probe FISH procedure to 65 oocytes which had been spread using the improved method of Coonen et al. (1994). A combination of five different chromosome probes gave a good insight into the genetic constitution of these oocytes. We found that the paired signals for a chromosome were not always tightly adjoined and, in many cases, one signal was quite distant from the other (Figure 2). We scored a pair of similar signals as one chromosome complement and estimated the number of chromatids in accordance with the number of individual signals. Paired chromosome signals reflect the unique nature of the oocyte’s chromosomes at this stage of the second meiotic division: highly contracted, with sister chromatids widely separated and loosely adjoined in the centromeric region (Dyban et al., 1996).

Two biological events might influence the interpretation of FISH signals in oocytes. First, it has been suggested that predivision of dyads into two single chromatids is a frequent phenomenon prior to the first meiotic division (Angell, 1991; Angell et al., 1994; Munné et al., 1995a, b; Dyban et al., 1996). In this case, two discrete signals for a chromosome would be observed instead of two conjoined signals (Figure 2). However, this interpretation might be ambiguous due to pre-division of the chromatids before metaphase I or overlap of the signals for the two chromatids (Munné et al., 1995a, b). Second, chromatid overlap would produce a dyad with only a single hybridization signal instead of a pair. Munné et al. (1995a, b) presented preliminary results which showed that when the DNA in the first PB was very condensed, all the chromosome-specific signals appeared as single signals instead of as paired signals. In this study, we applied the same scoring criteria as Munné et al. (1995a, b) and scored a DNA fragment with single signals as normal, because the probability of having a monosomy for all five chromosomes due to pre-division of all the chromatids is remote (Munné et al., 1995a, b).
The results for Group II oocytes (35.4%) were difficult to interpret because the chromosome constitution appeared to be haploid, and either dyads or single chromatids were present; however, the number of DNA fragments was inconsistent with the expected number that should derive from the first PB and the metaphase II DNA. We found a subgroup containing only one DNA fragment which may have been due to artefactual loss of DNA during spreading. However, another subgroup contained three DNA fragments, which might indicate an unrecognized decondensed sperm head, fragmented PB DNA or a potentially polyploid oogonium.

The inclusion of a Y chromosome probe to detect sperm DNA was of less use than expected. Sperm heads were recognized in 10 oocytes; three displayed a signal for the Y chromosome, but the sperm head was undecondensed in the other seven oocytes which would have hindered efficient hybridization of the Y probe (Martini et al., 1995). Moreover, decondensed sperm heads which yielded hybridization signals (single chromatids), but were X-bearing, might not have been recognized as such and consequently those oocytes could have been misclassified as Group II oocytes. Schmiady et al. (1996) recently reported a variable degree of chromosome condensation during premature chromosome condensation (PCC) of the sperm nucleus in unfertilized ICSI oocytes, and the occurrence and variation in sperm PCC might influence the rate of misclassification of sperm nuclei as oocyte DNA.

Considering that fertilization failure after ICSI is typically associated with a complete failure of oocyte activation, rather than with incomplete activation or premature initiation of the block to polyspermy (Dorzortsev et al., 1994; Sousa and Tesarik, 1994; Flaherty et al., 1995), there might be a genetic imbalance in some oocytes which predisposes them to this maturation arrest. DNA repair systems are less effective in dealing with chromosome imbalances in the resting stage of meiotic oocytes (Ashwood-Smith and Edwards, 1996). The most common anomaly in human oocytes is disomy or nullisomy for various chromosomes, and this probably arises during the first meiotic division (Angell et al., 1994; Griffin, 1996). The presence of a univalent chromosome can affect the alignment and segregation of other chromosomes in the complement (Hunt et al., 1995), presenting an increased risk of NDJ, aneuploidy and trisomy in an embryo (Hassold and Jacobs, 1984; Soewarto et al., 1995). Indeed, we found oocytes in which some of the chromatids were a considerable distance apart and this might represent the beginning of anaphase lag or pre-division of dyads that could lead to an abnormal separation of the chromatids and consequently to aneuploidy (Angell et al., 1994).

The high level of chromosome X hypohaploidy found in this study (Table I) can be explained by one or more of the following theories. First, the use of FISH enables one to identify chromosome-specific aneuploidy, whereas karyotyping mostly classify aneuploidy using the broad Denver classification which does not allow classification of chromosome-specific aneuploidy. For example, the high rate of chromosome X hypohaploidy we report might correspond to the hypohaploidy rate reported for group C chromosomes by karyotyping (Martin et al., 1991; Pellestor, 1991b; Roberts and O’Neill, 1995; Macas et al., 1996). Second, our sample population might not have been representative of the general gamete population. Third, pre-division of chromatids and subsequent loss of DNA material increases with time in culture and has been proposed as a major mechanism of aneuploidy by Dalley et al. (1996). Finally, we cannot exclude the possibility that chromatids were inadvertently lost, although if this were to occur it should be equal for all the chromosomes analysed and not specific for the X chromosome. On the other hand, the high frequency of hyperhaploidy for chromosome 1 is surprising, since this is the only chromosome that has not been observed as a trisomy in spontaneous abortions. However, trisomy 1 has been observed in an 8-cell human pre-embryo (Watt et al., 1987) and it is therefore possible that trisomy 1 is common in human concepti but that they are lost at an early preimplantation stage.

The fact that we found a higher percentage of aneuploid cells (38.4%) compared to karyotype studies (25–30%) (Plachot et al., 1988; Benkhalifa et al., 1990; Pellestor, 1991b) might be due to the small number of oocytes we analysed, but it might also more accurately reflect the true situation. The spreading technique we used enabled us to analyse a very high percentage (85%) of the oocytes, and FISH on interphase and metaphase cells is more sensitive and more efficient than karyotyping because the DNA probes used for FISH are chromosome-specific. In contrast, only about half of the oocytes karyotyped prior to 1991 yielded results, and in those, very few chromosome-specific aneuploidies were identified (Pellestor, 1991a). An additional limitation of karyotyping is that the arrangement and morphology of the chromosomes is critical for obtaining a diagnosis, and many oocytes do not meet these criteria and therefore remain unanalysed even though they were not lost during spreading. The protocol used in this study gave high spreading and FISH efficiencies and enabled us simultaneously to investigate up to five different chromosomes in each oocyte.

Like all techniques, however, there are limitations to the approach used in this study. For instance, spreading and FISH always encompass a risk of losing the oocyte, although this is the same with any technique. The identification of two ‘spots’ in an oocyte is not as reassuring as the clear identification of two chromosomes by cytogenetic analysis, nor can FISH results be easily verified. Perhaps a combination of the two techniques, FISH on previously karyotyped oocytes (Wall et al., 1996) would provide more accurate verification of results in individual oocytes. It would also be helpful to be able to identify, with certainty, whether a particular DNA fragment originated from the metaphase II chromosomes of the oocyte, the first PB, or a decondensed sperm head. This would clarify the dilemma we encountered with Group II oocytes. Further studies will help to clarify the stringent scoring criteria needed to achieve this and to correctly distinguish between paired and single chromatids. It is unrealistic to think that this FISH procedure will ever provide error-free estimation of aneuploidy in oocytes; however, it is much quicker and easier to perform than karyotyping, and it enables rapid and reliable accumulation of information about the chromosome constitution of large numbers of human oocytes.
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