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The chromosomal analysis of human oocytes. An overview of established procedures

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The cytogenetic survey of mature human oocytes has been and remains a subject of great interest because of the prevalence of aneuploidy of maternal origin in abnormal human conceptuses, and the lack of understanding about the non-disjunction processes in human meiosis. The first attempts to analyse the chromosomal content of human female gametes were made in the early 1970s, and led to limited data because of the paucity of materials and the inadequacy of the procedure used. The years to follow brought a resurgence of interest in this field, because of the development of human IVF techniques which made oocytes unfertilized *in vitro* available for cytogenetic analysis. Numerous studies have since been performed. However, the difficulties in obtaining good chromosome preparations and of performing accurate chromosome identification have reduced the viability of these studies, resulting in large variations in the reported incidences of chromosomal abnormalities. The further introduction of new procedures for oocyte fixation and the screening of large oocyte samples have allowed more reliable data to be obtained and to identify premature chromatid separation as a major mechanism in aneuploidy occurrence. The last decade has been privileged to witness the adaptation of molecular cytogenetic techniques to human oocytes, and thus various powerful procedures have been tried not only on female gametes, but also on polar bodies, involving sequential and multicolour fluorescent *in situ* hybridization (FISH) labelling, comparative genomic hybridization (CGH), spectral karyotyping and alternative methods such as primed *in situ* labelling (PRINS) and peptide nucleic acid (PNA) techniques. A large body of data has been obtained, but these studies also display a great variability in the frequency of abnormalities, which may be essentially attributable to the technical limitations of these *in situ* methods when applied to human oocytes. However, molecular cytogenetic approaches have also evidenced the co-existence of both whole chromosome non-disjunction and chromatid separation in maternal aneuploidy. In addition, the extension of these techniques to oocyte polar body materials has provided additional data on the mechanism of meiotic malsegregation. Improvements of some of these techniques have already been reported. The further development of new approaches for the *in situ* analysis of human meiosis will increase the impact of cytogenetic investigation of human oocytes in the understanding of aneuploidy processes in humans.

Key words: aneuploidy/FISH/karyotype/oocyte/polar body

Introduction

Data on the incidence of chromosomal defects in humans are available from various sources. A number of surveys established the frequencies of chromosomal aberrations in livebirths, stillbirths, spontaneous abortions (Hassold *et al.*, 1980; Hassold and Chiu, 1985; Jacobs *et al.*, 1987; Jacobs, 1992) and more recently in preimplantation embryos (Munné *et al.*, 1994; Braude *et al.*, 2002). All these studies indicate that chromosome aneuploidy is the major cause of pregnancy wastage. Molecular studies using DNA polymorphic markers have demonstrated that the large majority of autosomal aneuploidies originate from maternal meiosis, with a marked prevalence of meiosis I segregation errors (Nicolaidis and Petersen, 1998; Hassold and Hunt, 2001). Consequently, the chromosomal constitution of human oocytes has been a subject of great interest for many years. Various procedures were developed or adapted for performing

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the chromosomal investigation of human oocytes. To date, many studies have been reported with results that can look divergent, even contradictory in some respects.

In this review, we summarize the data resulting from the different methods applied to human oocytes, and discuss the strengths and the limitations of these approches, as well as their potential impact on the identification of chromosomal abnormalities and the interpretation of data.

Karyotyping studies of human oocytes

The preliminary assays

The cytogenetic study of human oocytes started only after the perfecting of *in vitro* maturation techniques of female gametes (Edwards, 1965), and the development of adapted fixation techniques (Tarkowski, 1966). However, the initial attempts to explore the chromosomal content of human oocytes were severely limited by the lack of materials and the inadequacy of the technology. The first recorded description of human oocyte II chromosomes was by Edwards (1965) on a small sample of 15 *in vitro*-matured oocytes. The quality of chromosomes obtained did not allow cytogenetic analysis, but Edwards already stressed that the technique provided an excellent method for studying meiotic chromosome abnormalities, especially with reference to maternal ageing. Subsequently, Yuncken (1968) and Jagiello *et al.* (1968) reported results of chromosomal analysis on human oocytes. Jagiello *et al.* (1968) presented the cytogenetic examination of both metaphase I and metaphase II oocytes, after ovulation induction of 12 patients considered for

Table I. Results of karyotyping studies on human oocytes, using Tarkowski fixation technique

Reference	Age of women (years)	Origin	No. metaphases II analysed	% aneuploidy	% polyploidy	% structural abnormalities	Total rate of abnormalities (%)
Wramsby and Liedholm (1984)	Not given	Non-inseminated oocytes	8	25.0	–	2	25.0
Michelmann and Mettler (1985)	22–40	IVF failure	51	2.0	35.3	–	37.3
Spielmann *et al.* (1985)	Not given	IVF failure	38	10.5	–	–	10.5
Martin *et al.* (1986)	24–35	Non-inseminated oocytes	50	30.0	–	4.0	34.0
Plachot *et al.* (1986)	Not given	IVF failure	35	17.1	2.8	5.7	25.7
Wramsby and Fredga (1987)	22–38	IVF failure	52	59.6	3.8	1.9	65.4
Veiga *et al.* (1987)	Not given	IVF failure	115	10.4	6.9	4.3	21.6
Wramsby *et al.* (1987)	Not given	Non-inseminated oocytes	21	57.1	–	–	57.1
Plachot *et al.* (1988)	Not given	IVF failure	120	25.8	–	1.6	27.4
Bongso *et al.* (1988)	27–42	IVF failure	251	21.1	2.0	4.0	27.1
Van Blerkom and Henry (1988)	32–40	IVF failure	135	8.1	–	–	8.1
Djalali *et al.* (1988)	24–39	IVF failure	96	27.1	11.5	–	38.6
Pellestor and Sèle (1988)	22–40	IVF failure	188	18.6	–	–	18.6
Papadopoulos *et al.* (1989)	Not given	IVF failure	30	20.0	26.6	20.0	66.6
Ma *et al.* (1989)	24–39	IVF failure	65	26.1	21.5	1.5	49.1
Pieters *et al.* (1989)	Not given	IVF failure	28	21.4	9.6	–	31.0
Michaeli *et al.* (1990)	27–41	IVF failure	60	46.6	13.3	–	59.9
Delhanty and Penketh (1990)	24–39	IVF failure	155	45.8	8.4	–	54.2
Macas *et al.* (1990)	24–38	IVF failure	55	25.4	9.1	3.6	38.2
Tarín and Pellicer (1990)	Not given	IVF failure	168	18.5	7.1	8.7	34.3
Kola *et al.* (1990)	Not given	Subzonal insemination	18	22.0	–	–	22.0
Kola *et al.* (1990)	Not given	IVF failure	30	30.0	–	–	30.0
Tarín *et al.* (1991a)	Mean: 32	IVF failure	168	18.5	7.1	8.3	33.9
Tarín *et al.* (1991b)	Mean: 31.5	Non-inseminated oocytes	69	21.7	7.2	5.8	34.7
Selva *et al.* (1991)	Mean: 32	IVF failure	132	30.0	8.1	18.0	56.1
Tejada *et al.* (1991)	23–40	IVF failure	334	19.2	3.9	–	23.1
Pieters *et al.* (1991)	Not given	IVF failure	86	26.3	–	–	26.3
De Sutter *et al.* (1991a)	Not given	IVF failure	110	49.1	4.5	–	53.6
De Sutter *et al.* (1991b)	Mean: 31.7	IVF failure	171	41.5	7.0	–	48.5
Gras *et al.* (1992)	27–39	Unstimulated oocytes	20	20.0	–		20.0
Gras *et al.* (1992)	27–38	Non-inseminated oocytes	68	34.0	–	–	34.0
Edirisinghe *et al.* (1992)	Not given	IVF failure	103	3.8	20.4	1.0	25.2
De Sutter *et al.* (1992)	Not given	IVF failure	256	32.8	–	–	32.8
Ma *et al.* (1994)	Not given	IVF failure	227	26.0	16.7	–	42.7
Zenzes *et al.* (1995)	22–43	IVF failure	286	38.5	8.4	–	46.9

hysterectomy. Sixteen metaphase I complements and 22 metaphase II complements were obtained either immediately or after *in vitro* incubation. The quality of the preparations was said to be good enough to allow identification of primary and secondary non-disjunction, but no estimate of aneuploidy was performed on this sample. The first attempt to classify human oocyte chromosomes was published by Chandley (1971) for a diakinesis metaphase I complement. Jagiello *et al.* (1976) reported the results of larger samples of cytogenetic observations made on *in vitro*-matured oocytes from various species, including human. For 411 human metaphases II scored, abnormalities were noted in six cases, all corresponding to 'additional bodies', unidentifiable with the staining procedure used (2% Toluidine Blue solution). In their conclusions, the authors pointed out the necessity to improve the chromosome identification on this material in order to be able to perform reliable analysis.

The rush of karyotyping studies

During the 1980s, the large development of IVF techniques made oocytes available for cytogenetic analysis and led to a renewed interest for this field of research. Two types of oocytes were used for chromosomal study: fresh, donated and non-inseminated oocytes, and oocytes remaining unfertilized after *in vitro* insemination. For the most part, these oocytes are at the metaphase II stage, and so they can be directly used for chromosome spreading. There were only a few reports on donated oocytes and the large majority of observations were performed on *in vitro*-unfertilized oocytes. Obviously, this population of rejected oocytes constituted a selected population of gametes, which might not be representative of the general population of human oocytes in the *in vivo* situation, and therefore it is important to bear this in mind when interpreting the reported data.

Following the first report of Wramsby *et al.* (1982), until 1996, 33 cytogenetic surveys based on the Tarkowski fixation technique were published (Table I). The mean frequency of chromosomal abnormalities derived from these pooled data was 35.9%, including 26.4% aneuploidies (15.5% hypohaploidies, 7.3% hyperhaploidies, 3.6% complex aneuploidies) and 6.1% structural aberrations (deletion, acentric fragment, chromosome fragmentation). No significant difference was noted between the population of 'rejected' oocytes and naturally ovulated oocytes or ovulation-induced but non-inseminated oocytes (Gras *et al.*, 1992). Nevertheless, the data displayed a wide variability in the incidence of aneuploidies (from 2.0 to 59.6%) and contradictory conclusions concerning the maternal age effect (Pellestor, 1991; Zenzes and Casper, 1992) and the correlation with various clinical parameters (Plachot, 1997).

The major reasons for these divergences seem to be the small size of analysed oocyte samples and the difficulty of performing accurate chromosome analysis on this material. The size of oocyte metaphase II samples ranged from eight to 334 (mean: 101), but most of the studies were based on small series (under 100 scored metaphases II) for which a greater variability in abnormality rates was observed. Because of the particular morphology of human oocyte chromosomes, with floating but condensed arms, it was difficult to obtain suitably spread chromosomes and subsequently an accurate identification of individual chromosomes. In most of the studies, chromosomes were only classified according to the Denver classification, after basic Giemsa staining. Only a few studies were performed using banding techniques to improve chromosome identification, but the quality of banding on human oocyte II metaphases remained poor (Martin *et al.*, 1986; Djalali *et al.*, 1988; Pellestor and Sele, 1988; Kola *et al.*, 1990).

Among aneuploidies reported using the Tarkowsky fixation technique, there appeared to be up to three times more hypohaploidies than hyperhaploidies. This variation strongly suggested artefactual loss of chromosomes because the Tarkowsky method can easily induce both displacement and loss of chromosomes during the fixation procedure. Consequently, conservative estimates of aneuploidy were usually calculated by doubling the number of observed hyperhaploidies. Therefore, although the artificial loss of chromosomes certainly constituted the cause of most hypohaploidies, it must be noted that various mechanisms inherent to oogenesis have been described, such as anaphase lag (Martin, 1984; Coonen *et al.*, 2004), alteration in the cytoskeleton (Eichenlaub-Ritter *et al.*, 1986) or the displacement of chromosomes (Ford and Lester, 1982; Williams and Fisher, 2003). They could also explain the loss of chromosomes in female meiosis, and must therefore be considered.

The approximate nature of these initial studies was highlighted by Angell's observation that premature separation of homologous chromatids through anaphase I might be a major class of segregation abnormalities in human meiosis, and might thus constitute the main mechanism for human aneuploidy (Angell, 1991, 1995, 1997). The emergence of this singular mechanism of malsegregation introduced an important new parameter in the investigation of meiotic non-disjunction occurrence, but also emphasized that in addition to the technical loss of chromosomes, the erroneous scoring of single chromatids as additional small chromosomes might have significantly biased the assessment of non-disjunction in the published studies of human oocytes (Figure 1). According to this malsegregation mechanism, two types of chromatid defects can be observed in oocyte metaphase II, in addition to conventional whole chromosome non-disjunction (Figure 2). These defects are the extra single chromatid and the balanced chromatid separation. Some authors suggested that this last type of chromatid defect could be

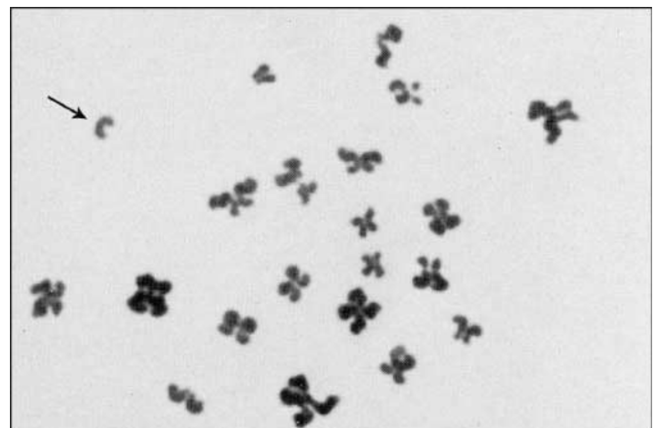

Figure 1. Example of oocyte metaphase II showing a free chromatid (arrow) which could be mis-scored as an extra small chromosome. Note the particular morphology of oocyte chromosomes with floating but highly condensed arms.

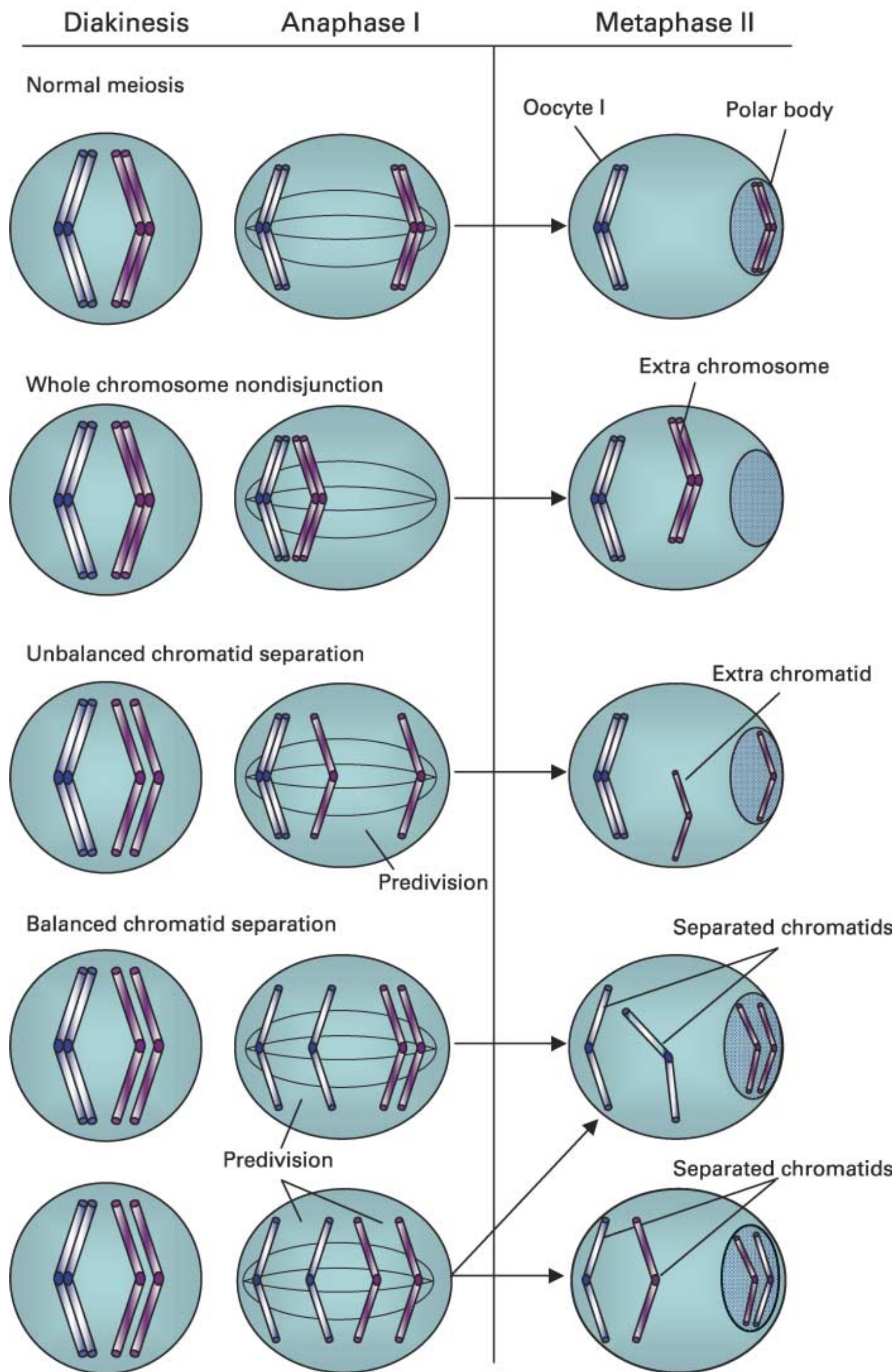

Figure 2. Schematic representations of normal female meiosis and the occurrence of the three types of malsegregation found in metaphase II preparations, i.e. the whole chromosome non-disjunction, the unbalanced chromatid separation and the balanced chromatid separation. The potential second meiotic errors are not represented in the figure.

an artefact related to the time of *in vitro* culture (Kamiguchi *et al.*, 1993; Dailey *et al.*, 1996). Although the possibility of an artefactual origin of balanced separation of sister chromatids could not be ruled out, the observation of such events in fresh, non-inseminated human oocytes contrasts with this technical explanation and indicates that the phenomenon must be taken into consideration in the analysis of aneuploidy occurrence.

The cohesion of chromatids in question

Premature chromatid separation is usually observed in chromosome instability syndrome, and studies on somatic cells have suggested that the basis of this phenomenon might be a spindle checkpoint defect (Matsuura *et al.*, 2000). Several proteins involved in spindle function and anaphase progression have been recently identified, involving a new class of nuclear proteins called cohesins. These highly conserved proteins provide

cohesion between sister chromatids, oppose the splitting force mediated by microtubules and are required for the association of chiasmate homologues during meiosis (Bickel *et al.*, 2002; Revenkova *et al.*, 2004). The sister chromatid cohesion is exerted mainly in the centromeric area, as well as via numerous sites within the chromosome arms (Michaelis *et al.*, 1997). At the onset of anaphase, sister-chromatid cohesion is disrupted by proteolytic cleavage of the cohesions, mediated by the anaphase promoting complex (APC). The gradual degradation or the loss of cohesins during meiosis I might be responsible for the premature sister chromatin separation observed in human oocyte metaphase II. This event might be maternal age dependent (Pellestor *et al.*, 2003a). With the loss of cohesion, an increased proportion of meiotic configurations could become unstable and preferentially adopt a linear configuration on the spindle (Wolstenholme and Angell, 2000). Such a configuration promotes equational segregation and separation of single chromatids, because each exposed kinetochore can easily capture microtubules over a large angle.

New approaches, new data

Improvements in the karyotype analysis of human oocytes were directly linked to the development of new procedures for chromosomal preparation. Mikamo and Kamiguchi (1983) described a soft and gradual fixation technique, allowing the artefactual loss of chromosomes and separation of chromatids during the fixation procedure to be avoided. As early as 1991, new cytogenetic studies were then conducted, based on this new fixation method. In addition to this technical improvement, these new assays were performed on larger samples of human oocyte karyotypes (from 44 to 1397; mean: 287) and took into consideration the mechanism of premature sister chromatid separation in the scoring and the analysis of chromosome abnormalities. Thus, these studies provided reliable and detailed data on aneuploidy occurrence in human female meiosis (Table II).

Except for the results of Angell (1997) and Angell *et al.* (1991, 1993) who observed only premature chromatid separation and concluded that chromatid errors were the unique cause of aneuploidy in female meiosis, all these reports indicated that both whole chromosome non-disjunction and chromatid predivision contribute to the formation of aneuploid oocytes. Also, these results showed more homogeneous data regarding the rate of aneuploidy (mean 17.0%) and the ratio between hypohaploidy and hyperhaploidy. The mean incidence of aneuploidy found in these new studies was much lower than previously reported value (17.0 versus 26.4%). It is very likely than this discrepancy is due to the mis-scoring of single chromatids and the artefactual loss of chromosomes in previous studies based on small numbers of oocytes fixed using the Tarkowski technique. However, as in previous cytogenetic studies, most identified abnormalities were numerical abnormalities. Such a high incidence of aneuploidy in female gametes contrasts with the distribution of chromosomal abnormalities found in human sperm (on average 7.0% structural aberrations and 3.0% numerical abnormalities) (Martin *et al.*, 1987; Guttenbach *et al.*, 1997). Both the morphology and the condensation of oocyte chromatids in metaphase II make the detection of structural abnormalities extremely difficult. Consequently, the estimated rate of structural abnormalities drawn from cytogenetic surveys is probably underestimated. For example, no chromosomal translocation was observed in oocyte metaphase II whereas these abnormalities are the most frequent structural rearrangements found in humans and the majority of *de novo* forms are of maternal origin (Page and Shaffer, 1997; Bandyopadhyay *et al.*, 2002).

In a recent report on a large sample of 1397 oocyte metaphase II karyotypes, we performed a detailed analysis of aneuploidy in human oocytes. We found that the numerical abnormalities due to single chromatids significantly exceeded conventional whole chromosome non-disjunction (5.9 versus 3.5%; $\chi^2 = 3.96$; $P < 0.05$). This finding clearly indicated that single chromatid defects constitute a major class of abnormalities in female meiosis. Some observed oocytes displayed extreme hypohaploidy with only 13 to 18 chromosomes, or presented complex aneuploidies by combining a chromosome aneuploidy and chromatid separation, indicating that various mechanisms can lead to a loss of chromosomes during female meiosis. Also, the presence of single chromatids at the first meiotic division could directly affect the segregation of other chromosomes (Hunt *et al.*, 1995;

Table II. Results of karyotyping studies on human oocytes, using gradual fixation techniques

Reference	Age of women (years)	Origin	No. metaphases II analysed	% aneuploidy	% polyploidy	% structural abnormality	Total rate of abnormality (%)
Angell *et al.* (1991)	Mean: 30.0	IVF failure	44	20.0	–	–	20.0
Rosenbusch *et al.* (1992)	24–43	IVF failure	260	14.9	10.2	2.6	27.7
Kamiguchi *et al.* (1993)	21–43	IVF failure	185	11.3	9.7	4.8	24.3
Angell *et al.* (1993)	Mean: 33.6	IVF failure	177	17.5	3.9	–	21.4
Almeida and Bolton (1993)	22–43	IVF failure	167	27.5	31.1	–	58.6
Nishino *et al.* (1994)	Meam: 32.1	IVF failure	149	10.1	3.4	6.0	19.5
Almeida and Bolton (1994)	25–39	IVF failure	215	33.0	14.0	–	47.0
Lim *et al.* (1995)	24–44	IVF failure	179	12.3	10.1	2.8	25.2
Robert and O'Neill (1995)	23–42	IVF failure	233	12.0	10.3	–	22.3
Wall *et al.* (1996)	Mean: 33.3	IVF failure	67	37.3	1.4	10.2	49.1
Angell (1997)	25_44	IVF failure	200	30.5	–	–	30.5
Sengoku *et al.* (1997)	26–39	IVF failure	147	12.9	5.4	–	18.3
Nakaoka *et al.* (1998)	22–44	IVF failure	388	8.5	6.4	3.9	18.0
Pellestor *et al.* (2002)	19–46	IVF failure	1397	10.8	5.4	2.1	18.3

Cheng *et al.*, 1998). These observations have suggested that the loss of chromosomes might be a natural propensity of mammalian female meiosis.

All chromosome groups displayed aneuploidies. However, some groups (A and C) showed lower aneuploidy frequency than expected, whereas the E and G groups exhibited a much higher frequency of non-disjunction than expected. This finding was in good agreement with previous data (Kamiguchi *et al.*, 1993; Lim *et al.*, 1995; Angell, 1997; Nakaoka *et al.*, 1998). An over-representation of aneuploidy in both E and G groups is also consistent with epidemiological data from spontaneous abortions and liveborns which pointed out the strong prevalence, and even the exclusivity, of a maternal origin in group E and G aneuploidies (Nicolaidis and Petersen, 1998; Hassold and Hunt, 2001). Previous assignment of aneuploidy by chromosome groups had also shown a similar tendency, but with an excess of non-disjunction for acrocentric chromosomes (D and G groups) (Pellestor, 1991; Zenzes and Casper, 1992). These former data might be largely biased by the mis-scoring of single chromatids.

The detailed analysis of non-disjunction events led to the comprehension of the impact of single chromatid defects in aneuploidy. No single chromatid was found for the chromosome A group, but chromatid abnormalities exceeded the number of whole chromosome aneuploidies in E and G groups (Pellestor *et al.*, 2002). This finding might be consistent with the mechanism of lack of chromatid cohesion discussed above, or might reflect some particular features in the conformation or in the DNA sequence of group E and G chromosomes, which favour meiotic malsegregation. To date, conventional non-disjunction, which also contributes to the elevated rate of aneuploidy in these two chromosome groups, has been associated with proximal reduced recombination. Consequently, it can be speculated that there might be particular features in the conformation or in the DNA sequence of group E and G chromosomes, which favours malsegregation. A similar tendency was also observed in human sperm where chromosomes 21, 22, and to a lesser extent chromosome 16, are more prone to non-disjunction (Egozcue *et al.*, 1997; Shi and Martin, 2000).

The loss of sister chromatid cohesion could be linked to the variation in centromeric DNA sequence or size. The existence of a direct relationship between small alphoid DNA domains and meiosis I non-disjunction has been suggested in several studies (Lo *et al.*, 1999; Maratou *et al.*, 2000). It could be speculated that the premature separation of sister chromatids reflects the fact that small alphoid arrays do not bind enough centromere-associated cohesins to durably maintain cohesion between homologous chromatids. In larger chromosomes, the presence of several chiasmata could prevent the occurrence of premature chromatid separation. This could provide a plausible explanation for the prevalence of aneuploidy for small chromosomes in aged oocytes. Also, the asymmetry of female meiosis division, resulting in only one functional gamete and two small polar bodies, might favour a non-random meiotic segregation of chromosomes and chromatids (Pardo-Manuel de Villena and Sapienza, 2001). In any case, such variability in non-disjunction occurrence indicates that the malsegregation was not a random event in female meiosis and that data drawn from the cytogenetic analysis of human oocytes provided valuable information on the mechanism of non-disjunction in female meiosis.

A significant proportion of analysed oocytes exhibited polyploid chromosome sets (from 3.4 to 31.1%) (Table II). The lack of first polar body extrusion appears to be the main causal mechanism for diploidy occurrence. Also, giant metaphase II oocytes, twice the normal size and sometimes with two distinct spindles, have been observed in Chinese hamster oocytes and human oocytes (Funaki and Mikamo, 1980; Pellestor *et al.*, 2002; Rosenbusch *et al.*, 2002). However, with an estimated incidence of 0.06–0.2%, giant diploid oocytes remain rare events in human female gametes (Gougeon, 1981; Balakier *et al.*, 2002). The arrest of the first polar body extrusion has been associated with cytoplasmic immaturity (Calafell *et al.*, 1991; Almeida and Bolton, 1993). Experiments on mouse oocytes have highlighted the importance of synchrony between nuclear and cytoplasmic maturation to ensure the correct order of events through fertilization. In particular, a disturbance in the synthesis of proteins involved in spindle formation and cytokinesis may be responsible for the production of diploid metaphase II oocytes (Soewarto *et al.*, 1995). One can speculate that defects in the completion of maturation may be due to various parameters, whether physiological, hormonal, genetic or environmental (Tejada *et al.*, 1992; Asch *et al.*, 1995; Zenzes *et al.*, 1995). Thus, Van Blerkom *et al.* (1995) reported a correlation between oocyte maturity, spindle structure and ATP content in human oocytes. Several studies suggested that reduced pH or oxygen may directly affect the spindle during resumption of maturation (Gaulden, 1992; Tarín, 1996). Also, mutations in cell cycle-regulating genes such as c-mos may cause altered spindle formation and then altered meiotic progression (Colledge *et al.*, 1994; Araki *et al.*, 1996). The activity of such kinase complexes is driven by protein phosphorylation/dephosphorylation. Analysis of protein phosphorylation in mouse oocytes has provided evidence for a causal relationship between altered phosphorylation, cell cycle regulation and predisposition to segregation errors (Eichenlaub-Ritter, 1998).

In the particular case of *in vitro*-unfertilized oocytes, additional factors linked to the IVF procedure might affect the maturation kinetics. Thus, in previous assays, correlations with the rate of diploid oocytes were reported for temperature fluctuation under *in vitro* conditions (Almeida and Bolton, 1995), cytoplasmic dysmorphism (Van Blerkom and Henry, 1992) or ovarian stimulation regimens (Tarín and Pellicer, 1990). In contrast, no significant variations in the frequency of aneuploidy were associated with the IVF indications or the ovulation induction protocols. However, the complete innocuousness of IVF processes cannot be established with certainty because of the multiplicity of technical parameters. All these data might have interesting implications for the understanding and diagnosis of various forms of infertility.

Molecular cytogenetic studies of human oocytes

During the last few years, molecular cytogenetic technology has been applied to human gametes and blastomeres. The direct *in situ* chromosomal analysis of isolated cells has constituted an important challenge, especially in conjunction with the clinical development of human gamete investigations and preimplantation genetic diagnosis (PGD). Several techniques have been

Table III. Results of fluorescent *in situ* hybridization (FISH) studies on human oocytes

Reference	Age of women (years)	Origin	No. metaphases II analysed	No. polar bodies analysed	FISH procedure used	% aneuploidy	% polyploidy	Observations
Wall *et al.* (1996)	25–41	IVF and ICSI failures	8	–	Consensus centromeric probe, alpha-satellite probe for chr. 18, and cosmid probe for chr. 21	Not given	–	FISH was only used for re-analyse the karyoptype of oocytes with known abnormalities
Dyban *et al.* (1996)	Not given	IVF failures	156	156	Satellite probes for chr. 18 and X	36.8	–	Five abnormalities scored, leading to an estimated overall aneuploidy rate of 36.8%
Dailey *et al.* (1996)	25–45	IVF and ICSI failures, non-inseminated oocytes	275	188	Satellite probes for chr. 13/21, chr. 18 and X	4.9 to 29.8 according to the age	11.0	Estimate of 10.6% FISH errors
Martini *et al.* (1997)	21–40	ICSI failures	65	–	Satellite probes used in two rounds of labelling: (chr. 1, 7, 15) and (chr. 1, X, Y)	38.4 aneuploidy, 35.4 ambiguous	–	Ambiguous chromosome complements due to an abnormal number of DNA fragments
Benzacken *et al.* (1998)	22–44	IVF failures	100	–	Painting probes for chr. 21 and X	13.0	–	
Mahmood *et al.* (2000)	Mean: 33	IVF and ICSI failures	127	57	Satellite probes and locus-specific probes used in three rounds of labelling: (chr. 13, 21), (chr. 16, 18, X) and (chr. 1, 9)	7.1 hyperhaploidy	–	
Martini *et al.* (2000)	26–39	IVF and ICSI failures	57	–	Satellite probes and locus-specific probes used in two rounds of labelling: (chr. 1, 13, 21) and (chr. 7, 18, X)	44.0	8.3	The oocytes were classified according to the number of DNA fragments and arrangement of hybridization signals
Honda *et al.* (2002)	Not given	IVF failures	183	93	Satellite probes and locus-specific probes for chr. 18, 21 and X	3.0	6.0	Chromosome spreads prepared by gradual fixation technique
Anahory *et al.* (2003)	21–42	IVF and ICSI failures	54	89	Satellite probes and locus-specific probes used in two rounds of labelling: (chr. 13, 16, 18, 22) and (chr. 1, 15, 17, X)	47.5	–	
Pujol *et al.* (2003a)	18–45	IVF failures and non-inseminated oocytes	54	89	Satellite probes and locus-specific probes used in two rounds of labelling: (chr. 13, 16, 18, 21, 22) and (chr. 1, 15, 17, X)	47.5	–	
Cupisti *et al.* (2003)	22–44	IVF and ICSI failures	203	88	Satellite probes and locus-specific probes used in two or three rounds of labelling: (chr. 1, 9, 13, 16, 18, 21, 22, X)	3.8	–	Only gain of whole chromosome or chromatid scored
Eckel *et al.* (2003)	22–40	IVF and ICSI failures	108	–	Locus-specific probes for chr. 13 and 21 used in two rounds of FISH and multi-locus FISH	19.4	–	Chromosome spreads prepared by gradual fixation technique by multi-locus FISH

successfully adapted for the chromosomal screening of human oocytes, each presenting advantages and disavantages.

The fluorescence in situ hybridization (FISH) procedure

Because of its relative simplicity and the commercial availability of numerous labelled DNA probes and *in situ* hybridization kits, FISH has gained acceptance in laboratories and has become the standard technique for aneuploidy assessment. On human oocytes, the FISH method appears to be a significant improvement over karyotyping because it overcomes the difficulty of chromosome spreading and can sometimes allow parallel analysis of the first and second polar bodies.

Typically, sequential FISH procedures in which two or three rounds of multicolour FISH reactions are performed on a same oocyte preparation are used. Sets of two to nine probes were thus utilized. In the majority of studies, both satellite repeat probes and locus-specific probes were used, including probes specific for the chromosomes most frequently involved in chromosomal abnormalities (Table III). Between 1996 and 2003, 12 FISH studies have been reported on a total of 1467 metaphase II oocytes and 727 first polar bodies, demonstrating the feasibility of sequential FISH procedure on isolated oocytes. All these studies have confirmed the co-existence of whole chromosome non-disjunction and homologous chromatid predivision as mechanisms of aneuploidy occurrence in human oocytes, which is in good agreement with recent karyotyping studies.

When successfully performed, parallel analysis of the first polar body constitutes a good internal control and provides interesting additional data regarding the origin of aneuploidy. Thus, in several oocytes from the same woman, the observation of a discrepancy between metaphase II and polar body analysis, in the form of extra chromosomes without corresponding lack of material in complementary complement, has provided evidence for the existence of gonadal mosaicism (Mahmood *et al.*, 2000; Pujol *et al.*, 2003a).

The incidences of aneuploidy in the reported FISH studies display considerable variations, with values ranging from 3 to 47.5% (Table III). These differences in aneuploidy rates are similar to the variations observed in karytotyping studies. Consequently, one can be sceptical about the relevance of some FISH results. Various parameters have been evoked to explain variations in the reported aneuploidy rates, involving the type of infertility, the origin of oocytes or patient age. However, it seems difficult to admit that such extensive variations could be attributed to only these parameters, which were often similar in the reported studies. The main reasons for these variations could actually be some technical aspects of the FISH procedure used on human oocytes.

Notwithstanding the questions of the limited number of targeted chromosomes and of the small oocyte sample size (from eight to 275 oocytes; mean: 103), several factors may affect both results and interpretations of FISH assays on human oocytes.

First of all, the particular morphology of metaphase II chromosomes, with floating arms and frequent lack of close contact between homologous centromeres, must be considered, since this feature can facilitate both the *in situ* mixing and overlapping of chromosome territories, and subsequently of fluorescent signals. The success of cytogenetic analysis of human oocytes is greatly dependent on the chromosome preparation, even in FISH assays. The simple fact that the FISH procedure has allowed results to be obtained from poor quality chromosome spreads which would be discarded in karyotyping studies, has introduced a significant bias, since such chromosome sets might preferentially result from atretic or degenerated oocytes. Degenerative oocytes frequently display aberrations such as dispersion of chromosomes, clumping of chromatin or degeneration of the first polar body (Balakier and Casper, 1991). Unfortunately, the majority of these abnormal oocytes appear morphologically normal after culture (Racowsky and Kaufman, 1992).

Another critical issue is the efficiency and the reliability of *in situ* hybridization on oocytes. FISH errors cannot be ruled out and have been estimated to be ~10% in human oocytes (Dailey *et al.*, 1996). In their recent report, Pujol *et al.* (2003a) estimated that 25.8% of their FISH data were artefactual, on the basis of the metaphase II/first polar body comparison. In fact, mistakes in assigning fluorescent spots to a chromosome or a chromatid could probably be more frequent in human oocyte preparations than currently believed because of the lack of well-defined chromosomal morphology (Warburton, 1997). The impact of a weak or artefactual signal on the interpretation of FISH results may be insignificant when hundreds of cells are evaluated, but it becomes an evident source of inaccurate interpretation when arising on isolated cells such as oocytes, which are potentially subjected to various mechanisms of non-disjunction.

Inefficient or artefactual hybridization cannot be excluded when only one specific probe is used per chromosome. Unfortunately, the great majority of FISH studies on oocytes have been performed using a unique type of probe per chromosome, i.e. centromeric probes or locus-specific probes, leading to the *in situ* visualization of only single dots per chromatid. This could be a source of artefact and miscoring, especially in a sequential FISH procedure where the re-probing and washing steps inevitably decrease the hybridization efficiency and the morphological quality of the chromosome spreads (Harper and Wells, 1999).

To overcome this potential source of error, two cytogenetic teams have developed alternative FISH strategies for the accurate identification of both chromosomes and chromatids. Eckel *et al.* (2003) combined dual colour FISH analysis using commercial locus specific probes (LSI 13 and LSI 21 from Vysis) with the re-analysis of metaphases with abnormal patterns by using two further locus-specific BAC or YAC probes located within the distal regions of both chromosomes 13 and 21. Thus, on 17 abnormal re-analysed metaphases, they found seven cases of normal signal patterns and three metaphases with a different abnormal signal pattern than in the first FISH assay. This sequential approach has provided evidence for artefactual loss or ‘drop out’ of *in situ* FISH signals.

Another approach improving the reliability of FISH analysis on human oocytes has been reported by our group (Anahory *et al.*, 2003). The procedure involves the simultaneous *in situ* visualization of specific domains (centromeric or locus-specific) and chromosome arms of each targeted chromosome (Figure 3A–C). This combined use of centromeric (or locus-specific) probes and whole chromosome painting probes has been tested in a sequential FISH study for the detection of

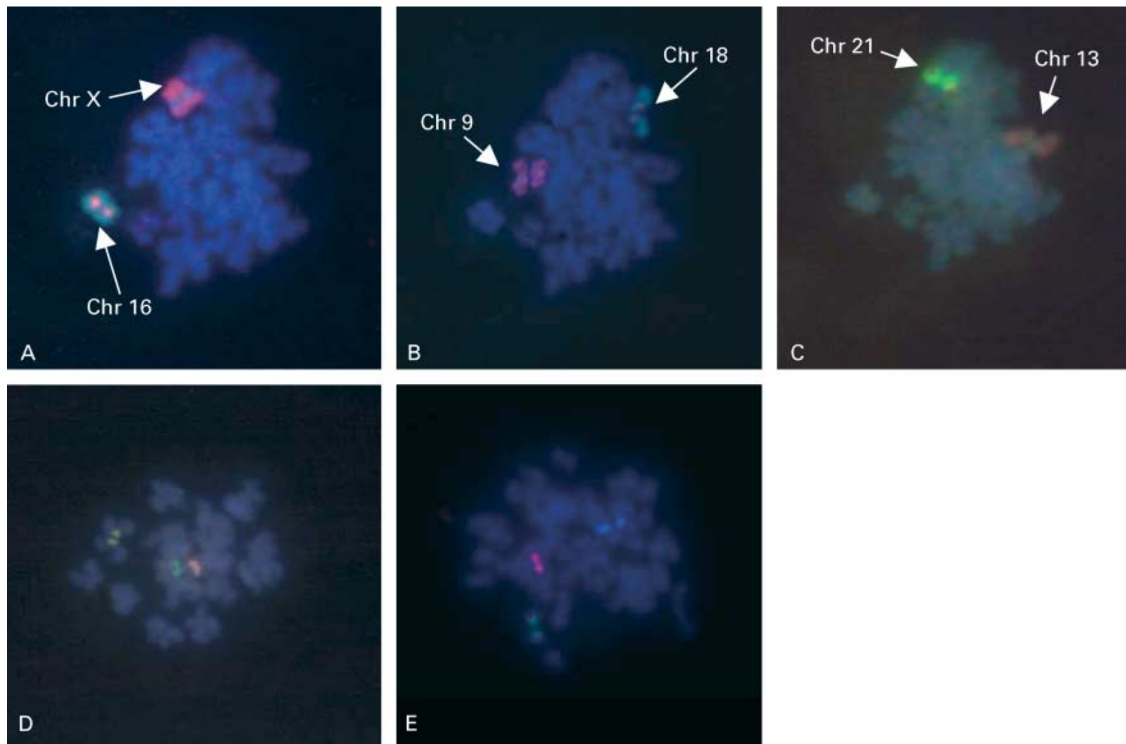

Figure 3. (**A–C**) Specific labelling of six chromosomes on an oocyte metaphase II, obtained by using the double-labelling fluorescent *in situ* hybridization procedure. The three combinations of centromeric (or locus-specific) probes and whole chromosome painting are presented. (**D**) Triple-colour labelling of an oocyte metaphase II by primed *in situ* labelling. Chromosome 1 is labelled in yellow, chromosome 7 in green and chromosome 16 in red. (**E**) Triple-colour labelling of an oocyte metaphase II by peptide nucleic acid technique. Chromosome 1 is labelled in blue, chromosome 4 in green and chromosome 16 in red.

chromosomes 9, 13, 16, 18, 21 and X, on 104 human oocytes and 56 first polar bodies. By allowing a clear and accurate identification of both chromosomes and single chromatids, this procedure allows clarification of 18% of ambiguous chromosome labelling results due to weak or artefactual signals on specific domains, and thus to avoid both miscoring and overestimating of chromosomal abnormalities in human oocytes.

These two recent studies have pointed out some important limitations of FISH assay on human oocytes and their potential consequences in the interpretation of results. Although these procedures were a little more time-consuming than the conventional FISH sequential reaction, the benefit in terms of interpretation is evident since these strategies can lead to less approximate estimates of aneuploidy in human oocytes. Thus the rates of aneuploidy found in these studies are consistent with the values obtained in the more recent and reliable karyotyping studies, as well as a few previous FISH studies. Taken as a whole, these data seem to indicate that the overall incidence of aneuploidies in human oocytes will not be as high as earlier studies have suggested.

In accordance with karyotyping data, the distribution of aneuploidy observed in FISH studies argues for the frequent involvement of small chromosomes in non-disjunction events. Thus, in their analysis of 230 oocytes, Cupisti *et al.* (2003) found 14 hyperhaploidies, 13 of which affected small chromosomes (chromosomes 13, 16, 18, 21). Also, Pujol *et al.* (2003a) and Anahory *et al.* (2003) reported the prevalence of whole chromosome and single chromatid segregation errors in small chromosomes. These findings are consistent with the assumption of altered recombination or loss of chromatid cohesion in small size human chromosomes during female meiosis. Certain distinct types of chromosomal configurations seem to have a high risk for non-disjunction, and this is correlated with maternal age in a chromosome-specific manner.

The polar body FISH analysis

This approach provides the opportunity for a direct analysis of chromosome abnormalities originating from both first and second meiotic divisions. This constitutes a significant advantage over conventional analysis of metaphase II, which takes into account only first meiotic errors. The procedure has been actively investigated by the group of Dr Verlinsky in Chicago and has been used for the screening of age-related aneuploidy in human oocytes. A few assays were also performed using domain-specific probes and whole chromosome painting probes, for the segregation analysis and PGD analysis of translocations in female carriers (Munné *et al.*, 1998, 2000; Durban *et al.*, 2001; Pujol *et al.*, 2003b).

Extensive data have been reported by Verlinsky *et al.* (1996, 1998, 1999) and Kuliev *et al.* (2003) for IVF patients aged $\geq$34 years (Table IV). The FISH procedure used commercial probes for chromosomes 13, 16, 18, 21 and 22. These studies reported high rates of aneuploidy (from 32.1 to 52.1%), which could reflect the effect of maternal ageing on aneuploidy occurrence. However, as pointed out by the authors, overestimates due to limitations of the multi-FISH technique, the fragmentation and *in vitro* ageing of polar bodies, or the low quality of

Table IV. Results of fluorescent *in situ* hybridization (FISH) analysis on polar bodies

Reference	Age of women (years)	Origin	No. polar bodies analysed	FISH procedure used	% aneuploidy	% metaphase I errors	% metaphase II errors
Verlinsky *et al.* (1996)	>34	IVF	648	Satellite probes for chr. 13/21, 18 and X	32.1		
Verlinsky *et al.* (1998)	>34	IVF	2952	Satellite probes and locus-specific probes for chr. 13, 16 and 21	43.1	35.8	26.1
Verlinsky *et al.* (1999)	>35	IVF	3217	Satellite probes and locus-specific probes for chr. 13, 18 and 21	43.1	35.7	26.1
Kuliev *et al.* (2003)	>35	IVF	6733	Satellite probes and locus-specific probes for chr. 13, 16, 18, 21 and 22	52.1	41.7	30.7

chromosome spreading, cannot be excluded. Thus, the authors have indicated that the reported incidences of chromosome abnormalities could be reduced to ~28% (Verlinsky *et al.*, 1998). These studies have demonstrated that both first and second meiotic errors contribute to the occurrence of aneuploidy, with a prevalence of abnormalities in meiosis I (42%) and a significant proportion of cells (29%) displaying both meiotic division abnormalities. An unexpected finding concerns the rate of aneuploidy observed in second polar bodies, since almost 30–35% of second polar bodies were found to be aneuploid. In contrast to first polar bodies where the majority of aneuploidies were represented by missing chromatids (48 versus 15.4% of extra chromatids), the ratio between missing and additional chromatids was balanced in second polar bodies (36 and 41% respectively) (Kuliev *et al.*, 2003). In addition, ~50% of meiosis II errors were found in oocytes with prior meiosis I errors, leading to apparently chromosomally normal zygotes. The relationship between errors in the first and the second meiotic division was thus questioned by Kuliev *et al.* (2003) and Kuliev and Verlinsky (2004). Since a significant proportion of oocytes displayed both segregation errors in first and second polar bodies, they suggested that non-disjunction in the second meiotic division might be due to errors in the first meiotic division, and that a hypothetical mechanism of aneuploidy rescue could explain the formation of balanced zygotes through sequential errors in the first and second meiotic female divisions (Kuliev and Verlinsky, 2004).

In contrast to the previous oocyte studies, but also to the DNA polymorphism studies, these studies have reported low rates of whole chromosome non-disjunctions (6.4%) in comparison with chromatid errors (63.5%) in meiosis I, and no prevalence for small chromosome non-disjunction (Kuliev *et al.*, 2003). Consequently, although this approach provides interesting data and has a real predictive value for PGD, the reported results concerning the incidence of abnormalities in human oocytes must be considered with caution, because of the difficulties in handling such small cells and obtaining good quality preparations (Durban *et al.*, 2001).

The primed in situ (PRINS) labelling and the peptide nucleic acid (PNA) techniques

These two techniques constitute alternatives to FISH for *in situ* labelling of nucleic acid sequences, and have also been tested on human female gametes.

Based on the *in situ* annealing of specific oligonucleotide primers and their extension by a Taq polymerase in the presence of labelled nucleotides, the PRINS technique exhibits a high specificity for the identification and the discrimination of repeat DNA sequences (Pellestor *et al.*, 1994; Serakinci and Koch, 1999) and has thus been used for the assessment of aneuploidy in various types of cells (Hindkjaer *et al.*, 1994; Orsetti *et al.*, 1998; Pellestor *et al.*, 1999). However, only a few experiments have been performed on human oocytes to date. The multicolour PRINS procedure has been successfully tested on human oocytes (Pellestor *et al.*, 1996). On a small series of 11 oocytes, two numerical abnormalities were found (Table V). Another assay was performed in 1999 on 118 oocytes, using specific primers for chromosomes X and Y, in order to evaluate the reliability of PRINS procedure for PGD (Findlay *et al.*, 1998). Recently a new ultra-fast multicolour PRINS protocol has been tested on human oocytes and polar bodies (Figure 3D). Various combinations of three or four chromosome-specific primers were used on a sample of 16 oocytes and seven polar bodies. Two cases of supernumerary chromatid 16, and two cases of balanced chromatid separation were detected in this sample (Pellestor *et al.*, 2004a). Although PRINS displays several features that make it very attractive for cytogenetic purposes (specificity of primers, rapidity and low cost of the reaction), the technique is always limited to the detection of repeat sequences and requires the use of a thermocycler for sequential reactions.

The peptide nucleic acids (PNA) are a new family of probes with remarkable properties. PNA are synthetic DNA analogues in which the phosphodiester backbone is replaced by a noncharged polyamide backbone (Pellestor and Paulasova, 2004), which confers great stability and affinity to PNA probes. During the last decade, PNA have been incorporated into an expanding variety of hybridization-based procedures, involving antigene therapy, genome mapping and mutation detection (Nielsen and Egholm, 1999). Recently, this new class of probes has been introduced in cytogenetics. The unique physicochemical properties of PNA allowed the development of fast and robust *in situ* assays. Studies have reported the successful use of chromosome-specific PNA probes on human lymphocytes, amniocytes and sperm (Lansdorp *et al.*, 1996; Taneja *et al.*, 2001; Pellestor *et al.*, 2003b). The procedure has also been tested on isolated human oocytes, polar bodies and blastomeres, in order to assess the possibility of using it for preimplantation diagnosis of aneuploidy (Paulasova *et al.*, 2004). Sequential multicolour PNA labelling procedures have been experimented on 34 oocytes and 17

Table V. Results of primed *in situ* labelling (PRINS) and peptide nucleic acid (PNA) studies on human oocytes

Reference	Age of women (years)	Origin	No. metaphases II analysed	Procedure used	% aneuploidy
Pellestor *et al.* (1996)	Not given	IVF	11	Double PRINS with specific primers for chr. 1, 8, 9, 12, 13, 16, 18, 21, X	18.2
Findlay *et al.* (2004a)	Not given	IVF	118	Single PRINS with specific primer for chr. X	Not given
Pellestor *et al.* (2004)	Not given	IVF	16	Multicolour PRINS with specific primers for chr. 1, 7, 9, 16, 18	12.5
Paulasova *et al.* (2004)	Not given	IVF	27	Multicolour PNA with specific synthetic probes for chr. 1, 4, 9, 16, 18, X	23.5

polar bodies (Table V), using centromeric PNA probes specific for chromosomes 1, 4, 9, 16 and X (Figure 3E). Satisfactory results were obtained with a short hybridization timing of 60 min. Eight oocytes (23.5%) displayed numerical abnormalities for the targetted chromosomes, which is in agreement with results of FISH studies. These preliminary results have indicated that PNA might provide an interesting adjunct to FISH for aneuploidy screening. However, further studies are needed to validate this new technology in cytogenetics and an extended variety of PNA probes must be produced to allow the rapid development of the PNA technique within the field of *in situ* chromosomal analysis (Pellestor *et al.*, 2004b).

Comparative genomic hybridization (CGH)

Although CGH was not strictly speaking a cytogenetic method but rather a DNA-based technique, its use gives a good illustration of a complete karyotype and its results provide valuable data on chromosomal constitution. Its adaptation to single cells has constituted a technological challenge, because of the necessity to amplify the whole genome of the single cell before performing CGH (Voullaire *et al.*, 1999; Wells *et al.*, 1999). The first applications of CGH for the chromosome analysis of isolated oocytes or blastomeres have been recently reported (Voullaire *et al.*, 2000; Wells *et al.*, 2002; Wilton *et al.*, 2003; Trussler *et al.*, 2004). These preliminary assays performed in conjunction with FISH analysis have indicated that CGH could provide a more extensive and efficient chromosomal screening than FISH, since it enables the enumeration of all chromosomes and the identification of chromosomal abnormalities which would not be detected by FISH. However, CGH also displays major limitations such as the duration of the procedure and the incapability of detecting balanced rearrangements or ploidy. Consequently, its use for preimplantation embryo screening is still under discussion (Hill, 2003; Munné and Wells, 2003; Verlinsky and Kuliev, 2003). The technique has been tested on a small sample of 10 polar bodies from a 40 year old IVF patient with ovarian dysfunction. Only one polar body was found to be chromosomally normal and nine displayed aneuploidy with a predominant involvement of small chromosomes in imbalances (Wells *et al.*, 2002). More recently, Gutiérrez-Mateo *et al.* (2004) evaluated the reliability of using CGH on a series of metaphases II and first polar bodies from 30 oocytes. CGH results were obtained in 84% of cells. A high 48% aneuploidy rate was found, and the authors pointed out that ~33% of these abnormalities would remain undetected if a current FISH procedure with nine chromosome-specific probes had been used. The CGH analysis was also tested on metaphases II and polar bodies of oocytes from carriers of balanced reciprocal and Robertsonian translocations, demonstrating the ability of CGH to detect unbalanced segregations of translocations (Gutiérrez-Mateo *et al.*, 2004).

Further improvement of the CGH technology might contribute to increasing the efficiency of the procedure. Thus, CGH has been combined with microarray (array CGH) and this new approach could offer the sensitivity and the rapidity required for clinical application and the reliable assessment of aneuploidy on isolated cells (Bermudez *et al.*, 2004; Shaffer and Bejjani, 2004).

Spectral karyotyping

The 24-colour FISH painting techniques, using different combinations of five fluorochromes, have been recently introduced as M-FISH (multi-fluorochrome karyotyping) or SKY (spectral karyotyping) (Schröck *et al.*, 1996; Speicher *et al.*, 1996). Considering the above-mentioned limitations of the other molecular cytogenetic techniques applied to human oocytes, spectral karyotyping appears to be the most appropriate method for the cytogenetic analysis of female gametes, since ideally all the chromosomes are simultaneously and distinctively labelled and identified. To date, four studies have been published on small oocyte samples (from two to 60 metaphase II oocytes), demonstrating the feasibility of the technique on human oocytes and polar bodies (Table VI).

Except for the study of Clyde *et al.* (2001) reporting on only two abnormal oocytes from a patient with polycystic ovary syndrome, the other three studies have reported incidences of aneuploidy (from 20 to 39%) within the range of aneuploidy rates currently recorded in the published data, as well as the observation of the three types of numerical abnormalities previously described in human oocytes, i.e. chromosome non-disjunction, extra-chromatid and balanced chromatid separation. The observation of balanced chromatid predivision in fresh, non-inseminated metaphase II oocytes is an interesting finding (Sandalinas *et al.*, 2002), which clearly supports the notion that chromatid

Table VI. Results of spectral karyotyping on human oocytes

Reference	Age of women (years)	Origin	No. metaphase II analysed	Procedure used	% aneuploidy
Marquez *et al.* (1998)	30–42	IVF failures	60	SKY probes and SKYVision spectral imaging system	20.0
Clyde *et al.* (2001)	33	Non-inseminated oocytes	2	M-FISH with Spectra Vysion probes	100.0
Sandalinas *et al.* (2002)	24–48	Non-inseminated oocytes	47	SKY probes and SKYVision spectral imaging system	38.0
Clyde *et al.* (2003)	25–41	IVF and ICSI failures	67	M-FISH with Spectra Vysion probes	39.0

predivisions contribute to the occurrence of aneuploidy in human oocytes. Also, in the three studies, chromatid predivisions preferentially involved the smaller chromosome. This confirms previous findings in karyotyping and FISH studies.

These preliminary results have demonstrated the great potential of spectral karyotyping for the chromosomal screening of human oocytes. However, all the authors agree that spectral karyotyping also has limitations. Firstly, the quality of chromosome spreading is essential for the success of the spectral analysis. In oocyte preparations, the frequent scattering or overlapping of chromosomes and chromatids is a significant problem. Consequently, a high proportion of oocyte preparations are rejected because of their insufficient quality. The limits of resolution can also constitute a drawback since no small or intra-chromosomal rearrangement can be detected using chromosome paints. In addition, the whole procedure is time-consuming and remains expensive. Further improvements in fixation methods and the application of news sets of multicolour-banding probes (Chudoba *et al.*, 2004) could help to overcome these limitations and increase the efficiency of spectral karyotyping for chromosomal analysis in human oocytes.

Mechanisms and aetiology of aneuploidy in human oocytes

According to data summarized in the present review, the human oocyte appears to be particularly prone to meiotic segregation errors. Altogether, these data provide evidence for several processes leading to aneuploidy, including conventional whole chromosome non-disjunction, premature separation of homologous chromatids and gonadal mosaicism. Both altered recombination and advancing maternal age have been identified as essential aetiological factors, but the causative mechanisms underlying the occurrence of meiotic malsegregation are poorly understood.

Over the past decade, molecular studies of parental origin in trisomies have indicated that most aneuploidies were of maternal origin, and preferentially occurred during the first meiotic division (Hassold and Jacobs, 1984; Hassold *et al.*, 1993; Nicolaidis and Petersen, 1998). However, there is significant variability among chromosomes concerning the stage of meiotic non-disjunction. Thus, all cases of trisomy 16 seem to be due to MI non-disjunction (Hassold *et al.*, 1995). For trisomies 15, 21 and 22, meiosis I errors predominate (Antonarakis *et al.*, 1991; Lamb *et al.*, 1996; Robinson *et al.*, 1998), whereas most trisomies 18 result from metaphase II non-disjunctions (Kondoh *et al.*, 1988; Eggermann *et al.*, 1996). Both lack of and reduction of recombination have been reported for maternal trisomies, indicating that the pattern of chiasmata is an important predisposing factor to meiotic non-disjunction. In addition to their number, the location of recombinational events along the chromosome arms also appears to be an essential parameter. Both chiasmata too close or too far from the centromere might increase the risk of non-disjunction (Hassold and Hunt, 2001). Consequently, the relationship between recombination and non-disjunction is certainly highly chromosome specific (Warburton and Kinney, 1996).

Even if altered recombination or lack of recombination are essential predisposing factors for non-disjunction, additional events are required to explain maternal age-related non-disjunction. A ‘two-hit’ model has been proposed, in which the first hit is the prenatal establishment of a susceptible meiotic configuration, and the second hit is the abnormal processing of the susceptible bivalent. The second hit could involve any element of the meiotic apparatus and would be the age-dependent element of the process (Lamb *et al.*, 1996). A number of models exploring factors that could promote meiotic non-disjunction according to maternal age have been suggested. Since most of meiosis is completed in the ovary, models related to hormonal imbalances (Warburton, 1989), accelerated follicle maturation (Eichenlaub-Ritter and Boll, 1989), depletion of oocyte pools (Zheng and Byers, 1992; Kline *et al.*, 2000), perifollicular microcirculation (Gaulden, 1992), reduced oxygen supply (Van Blerkom *et al.*, 1997), chromosome coiling and decondensation (Hultén, 1990), pre-meiotic non-disjunction (Hale, 1995) or deficiency in the maintenance of sister chromatid cohesion (Wolstenholme and Angell, 2000) have been proposed in relation to ageing. Subtle changes in the paracrine or endocrine regulation of folliculogenesis could also impact the meiotic process (Hodges *et al.*, 2002). In essence, all these models sustain the concept of an age-dependent deterioration of some cellular factors required for proper spindle function and chromosome progression through meiosis. Hawley *et al.* (1994) suggested that in oocytes the ability to form a normal spindle decreases with increasing maternal age and that damaged spindles favour the malsegregation of achiasmatic and distally chiasmatic homologues, and subsequently the precocious separation of sister chromatids. Support for this hypothesis has been provided by several reports describing increased aberrations in meiotic spindle formation and chromosome misalignment, or precocious separation of sister chromatids in oocytes from advanced age women (Hunt *et al.*, 1995; Battaglia *et al.*, 1996; Volarcik *et al.*, 1998). In the case of IVF,

the post-retrieval ageing prior to fertilization may also compromise the meiotic process (Racowsky and Kaufman, 1992; Eichenlaub-Ritter, 1998) and cause early pregnancy failure (Wilcox *et al.*, 1998). Recent studies on mice have also suggested that slight alterations of *in vitro* conditions might exacerbate a predisposing risk to non-disjunction (Ohno *et al.*, 2001; Bean *et al.*, 2002). Disturbances of the meiotic process do not seem to delay the anaphase onset (Hodges *et al.*, 2002), suggesting that in oogenesis of older women, the regulatory checkpoint mechanisms that monitor both the spindle assembly and chromosome movements could also be particularly ineffective (Le Maire-Adkins *et al.*, 1997; Steuerwald *et al.*, 2001). This is in contrast with male meiotic cells where the altered chromosome behaviors cause delay or arrest of meiotic progression (Eaker *et al.*, 2001).

Numerous other genetic or environmental risk factors have also been suggested, involving maternal smoking, oral contraceptives, irradiation, diabetes, folate metabolism, polymorphism or allelic combination (for review Hassold and Hunt, 2001), but results have often been contradictory and none of these correlations has been firmly established.

In summary, there is no clear evidence for any simple explanation of the relationship between maternal ageing and the occurrence of aneuploidy. The maternal age effect is without doubt multi-factorial. As discussed above, environmental and intrinsic factors may affect the meiotic segregation of chromosomes according to the maternal age. In this context, data provided by the chromosomal analysis of human oocytes might provide valuable baseline information on the effect of maternal ageing, since this direct approach allows elimination of the bias of viability present in studies of pregnancy losses and liveborns. As a result, karyotyping surveys have provided contradictory data. Among initial studies, six studies reported an increased rate of aneuploidy in oocytes from women aged >35 years (Bongso *et al.*, 1988; Plachot *et al.*, 1988; Ma *et al.*, 1989; Delhanty and Penketh, 1990; Macas *et al.*, 1990; Michaeli *et al.*, 1990), whereas a similar incidence of aneuploidy within different age groups was indicated in several other cytogenetic studies (Djalali *et al.*, 1988; Pellestor and Sele, 1988; Selva *et al.*, 1991; Tejada *et al.*, 1991). Similar lack of maternal age effect was also noted in oocytes derived from normal cycles (Gras *et al.*, 1992). In the most recent karyotyping studies, based on the gradual fixation technique and considering the mechanism of sister chromatid predivision, a similar heterogeneity in results is observed. Angell *et al.* (1991), Kamiguchi *et al.* (1993), Lim *et al.* (1995) and Nakaoka *et al.* (1998) found no direct correlation between maternal age and aneuploidy, but Angell (1997) and Pellestor *et al.* (2003a) reported a significant increase of aneuploidy rate with maternal ageing. Roberts and O'Neill (1995) also reported an increasing proportion of diploid oocytes with advancing maternal age. Most of these data must be considered as highly speculative because of the limited number of oocytes processed or the risk of chromosomal miscoring. In our recent karyotyping survey of 1396 oocyte II metaphases, the large size of this sample and the use of an adapted R-banding method (Pellestor *et al.*, 1993) have allowed us to perform an accurate and detailed study of maternal ageing effect, and to distinguish this effect on both whole chromosome non-disjunction and premature chromatid separation. We have clearly identified a positive relationship between maternal age and the global rate of aneuploidy, and the detailed analysis revealed the most marked correlation between age and chromatid separation events (Pellestor *et al.*, 2003a). This finding indicates that this process is an essential factor in the age-dependent occurrence of non-disjunction in human oocytes. Confirmatory data have been provided by some of the FISH analyses of human oocytes and polar bodies (Dailey *et al.*, 1996; Sandalinas *et al.*, 2002; Kuliev *et al.*, 2003), although some discrepancies can still be noted in the types of aneuploidy (whole chromosome non-disjunction, unbalanced or balanced chromatid separation) concerned by maternal age effect (Dailey *et al.*, 1996; Verlinsky *et al.*, 1999; Pujol *et al.*, 2003; Kuliev and Verlinsky, 2004).

Conclusions

Because it makes possible a direct approach to the female meiotic segregation process, the cytogenetic study of human oocytes has provided unique insight into some of the mechanisms of non-disjunction.

The source of the oocytes under study, i.e. essentially IVF or ICSI failures, is an important parameter to keep in mind, since this population constitutes a selected population of cells from a selected population of women that might not fairly represent the general population of reproducing women. This should always be remembered when interpreting the reported data, even if a large body of evidence has failed to demonstrate any significant correlation between chromosomal abnormalities and different parameters of IVF procedure or some type of infertility (Plachot, 1997, 2003). It is also important to distinguish between an unselected population of oocytes that fail to fertilize following insemination with dysfunctional sperm and a selected population of unfertilizable oocytes obtained from a cohort in which sibling oocytes are fertilized, because this difference in origin could affect the result of the chromosomal analysis. Technical factors such as the duration of the *in vitro* oocyte culture before spreading or the ovulation induction treatment have been suspected to affect the rate of chromosomal abnormalities. The principal effect of *in vitro* ageing is spindle instability and chromosome scattering but some investigations have proven the integrity of oocytes cultured *in vitro* up until 72 h (Gifford *et al.*, 1987; Payne *et al.*, 1997). However, the complete innocuousness of IVF processes cannot be established with certainty because of the multiplicity of technical parameters.

Ideally, cytogenetic surveys would involve a full karyotype analysis on metaphase chromosomes. The review of reported data points out the difficulties in attaining this objective in karyotyping studies of human oocytes, and the significant impact of interpretation errors on the assessment of chromosomal abnormalities.

The alternative approaches based on molecular cytogenetic technology have offered a wide scope for the detection of chromosomal abnormalities in human oocytes. The last decade has thus witnessed rapid progress in the application of multicolour *in situ* hybridization techniques on human gametes and embryo. Prime examples of the power of *in situ* hybridization approaches are the direct testing of polar bodies and the adaptation of CGH and spectral karyotyping on isolated oocytes. However, all these procedures display limitations, which can subject

the performance and the interpretation of *in situ* labelling on oocyte preparations to criticism.

Both karyotyping and molecular cytogenetic studies have reported significant variations in the incidence of chromosomal abnormalities found in human oocytes. This indicates that no approach can be definitively considered more reliable and efficient than another for the cytogenetic investigation of human oocytes. In accordance with the most dependable estimates, possible incidence of chromosomal abnormalities in metaphase II oocytes could be ~20%. Another point of consensus concerns the modes of malsegregation and the distribution of aneuploidies, since all the recent studies point out the contribution of both chromosome non-disjunction and chromatid separation in the occurrence of aneuploidy, with a predominant implication of small chromosomes.

Maternal ageing is also an essential factor in the analysis of the occurrence of aneuploidy in female gametes. Most previous cytogenetic studies have failed to confirm any relationship between maternal ageing and aneuploidy rate in human oocytes, whereas most of the more recent reports have provided evidence for a direct correlation between increased aneuploidy and advanced maternal age.

These data provide direct and valuable information on chromosomal abnormalities. This indicates that cytogenetic analysis contributes significantly to our understanding of the mechanisms and aetiology of aneuploidy in human female meiosis, in combination with molecular investigations. The improvement of the actual procedure and the adaptation of new methodologies would increase the reliability of the chromosomal analysis of human oocytes.

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