RESEARCH ARTICLE

Human oocyte chromosome analysis: complicated cases and major pitfalls

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Abstract

Human oocytes that remained unfertilized in programmes of assisted reproduction have been analysed cytogenetically for more than 20 years to assess the incidence of aneuploidy in female gametes. However, the results obtained so far are not indisputable as a consequence of difficulties in evaluating oocyte chromosome preparations. Because of the lack of guidelines, we decided to summarize for the first time, the possible pitfalls in human oocyte chromosome analysis. Therefore, we screened the material from our previous studies and compiled representative, complicated cases with recommendations for their cytogenetic classification. We point out that maturity and size of the oocyte are important parameters and that fixation artefacts, as well as the particular structure of oocyte chromosomes, may predispose one to misinterpretations. Moreover, phenomena related to oocyte activation and fertilization are illustrated and explained. This compilation may help to avoid major problems in future studies and contribute to a more precise, and uniform assessment of human oocyte chromosomes.

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Introduction

Approximately 15% of all clinically recognized human pregnancies end as spontaneous abortions and about half of them carry an abnormal karyotype, mainly autosomal trisomies and monosomy X chromosomes. The proportion of chromosomally abnormal zygotes inferred from such data is nearly 62% (De Braekeleer and Lin 2006), indicating a high rate of aneuploidy in human gametes. Consequently, cytogenetic investigations of spermatozoa and oocytes are of particular interest in order to verify this assumption and to elucidate the parental origin of the observed abnormalities.

Valuable information on the chromosomal constitution of female gametes has accrued due to the advent of assisted reproductive technologies, because oocytes that failed to become fertilized *in vitro* can be subjected to cytogenetic analysis. To date, several thousands of oocytes have been studied using a conventional approach to visualize the complete haploid chromosome set (Pellestor *et al*. 2005; Rosenbusch 2006a) or molecular cytogenetic techniques that primarily employed fluorescence *in situ* hybridization (FISH) for detection of selected chromosomes (Pellestor *et al*. 2005, 2006). Though the reported frequencies of numerical chromosome abnormalities vary considerably between individual studies, it is generally accepted that aneuploidy in human oocytes is not exclusively caused by a nondisjunction of bivalents. Another, possibly, even more important mechanism is premature centromere division (predivision) at meiosis I, leading to the loss or gain of single chromatids in mature gametes (Angell 1997).

Inconsistencies in the results published so far certainly originate from a multitude of factors. First, gametes were obtained from patients differing in age, hormonal stimulation regime and type of infertility. Second, technical shortcomings regarding oocyte fixation seem to be responsible for the frequently observed excess of hypohaploidy and for difficulties in the correct interpretation of structural chromosome

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abnormalities (Pellestor *et al*. 2005; Rosenbusch 2006a). Another issue repeatedly addressed by us (Rosenbusch and Schneider 2000, 2006; Rosenbusch 2007) is that the description and presentation of the cytogenetic data is still not standardized even after more than 20 years of research, making it difficult to draw meaningful comparisons and unequivocal conclusions. We believe that the minimum requirements for communicating the results should comprise: (i) the exclusive use of fully karyotyped complements for calculating the rate of aneuploidy, (ii) a complete list of karyotypes, (iii) a differentiation of metaphases affected by nondisjunction or predivision, and (iv) the use of a standardized nomenclature for the latter (Rosenbusch 2007).

It is evident that these requirements can only be met in case of an accurate analysis of the metaphases. To demonstrate the complexity of oocyte chromosome analyses, we screened the material of our previous studies (Kamiguchi *et al*. 1993; Rosenbusch 2000; Rosenbusch and Schneider 2006), compiled representative complicated cases and provided explications or recommendations for their cytogenetic evaluation. Because no general guidelines exist for human oocyte chromosome studies, we hope that this approach will help to avoid major pitfalls in future studies.

Materials and methods

As previously described (Kamiguchi *et al*. 1993; Rosenbusch 2000; Rosenbusch and Schneider 2006), oocytes were obtained from infertile patients undergoing treatment by *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). Appearance and the number of pronuclei (PN) were assessed for 16 to 20 h after insemination or ICSI. Oocytes judged as unfertilized were incubated at least for another 24 h and checked again for possible delayed formation of PN or unexpected cleavage. Uncleaved cells were fixed according to the gradual fixation air-drying (GFAD) method (Mikamo *et al*. 1994). Briefly, oocytes were incubated in hypotonic solution for up to 15 min and transferred into the first fixative (methanol : acetic acid : H2O = 5 : 1 : 4). The gametes were then placed on a grease-free glass slide and gently covered with fixative II (methanol : acetic acid = 3 : 1). After few minutes, the slide was dipped into fixative III (methanol : acetic acid : H2O = 3 : 3 : 1) for 1 min and dried in warm moist air. The chromosomes were stained homogeneously with Giemsa and photographed at X1000 magnification.

Our cytogenetic investigations of unfertilized human oocytes have been approved by the ethical committee of the University of Ulm.

Results

A variety of metaphases with true numerical abnormalities involving whole chromosomes and/or single chromatids have already been illustrated elsewhere (Kamiguchi *et al*. 1993; Rosenbusch and Schneider 2000, 2006). Therefore, it is not necessary to repeat corresponding examples here. Instead, the present report contains previously unpublished figures to demonstrate and explain problems that may arise during cytogenetic analysis.

The female gamete

The desired study material is the secondary oocytes that has completed the first meiotic division. These gametes are arrested at a stage termed as metaphase II (MII) that can be identified by the presence of the first polar body (PB) (figure 1). After normal progression of meiosis I, both the oocyte and the first PB have obtained a haploid chromosome set (23, X). The first PB is frequently lost during the fixation procedure. If not, it may happen that the oocyte and PB chromosomes lie close together or, at worst, intermingle on the slide (figure 2). Although PB chromosomes tend to degenerate earlier, and generally appear less distinct or more fluffy, confusions may occur and the oocyte will wrongly be judged as aneuploid.

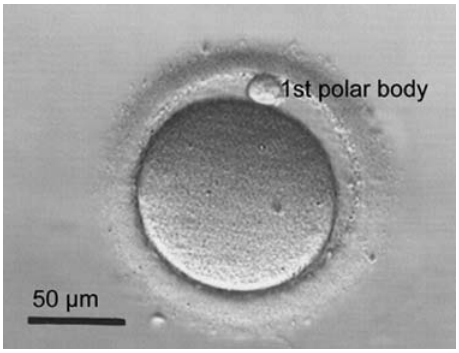

Figure 1. A human oocyte that remained unfertilized in our programme of assisted reproduction. It is arrested at metaphase II, characterized by the presence of the first polar body (PB).

As shown before, an oocyte can appear diploid at first sight due to a fixation artefact. However, there are also mechanisms leading to a truly diploid chromosome complement. This results most frequently from fixation of an immature (primary) oocyte, characterized by the absence of first PB. Consequently, 46 chromosomes will be observed that need not appear as bivalents if separation of the homologous chromosomes had already proceeded (figure 3), a situation that has been called arrest of first PB extrusion (Kamiguchi *et al*. 1993). However, diploidy also occurs in apparently mature oocytes. This peculiarity can be attributed to the presence of giant oocytes, rather than to nondisjunction of all 23 chromosome pairs. Giant oocytes are tetraploid up to meiosis and become diploid at MII. At the later stage, they can carry one or two first polar bodies (Balakier *et al*. 2002; Rosenbusch *et al*. 2002).

These pecularities demonstrate that the presence of PB and size of the oocyte are important criteria that should be

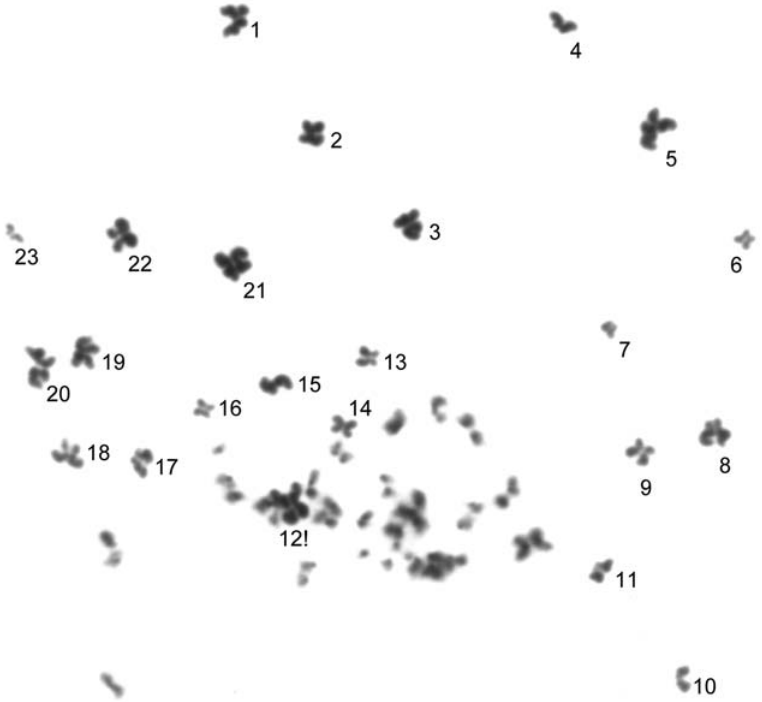

Figure 2. The first PB has not been lost during fixation and its chromosomes have mixed with those of the oocyte, thus pretending diploidy. However, the chromosomes of the oocyte (numbered; $n = 23$) show a more distinct morphology.

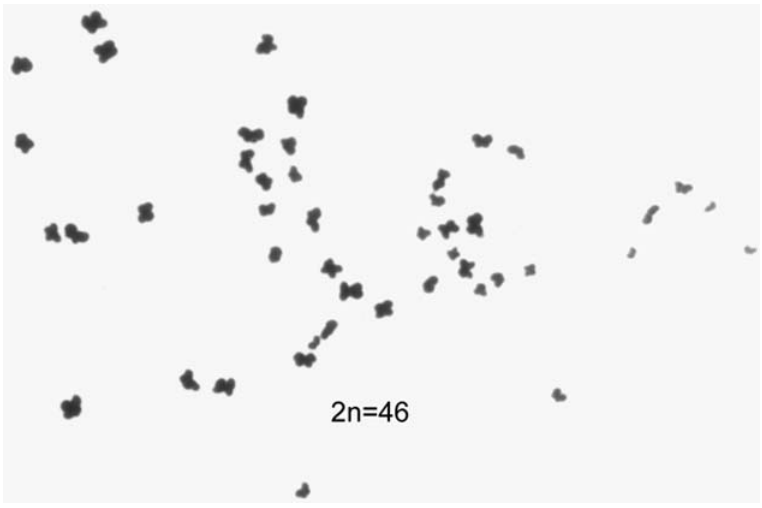

Figure 3. A diploid chromosome set from an oocyte that failed to extrude the first PB. Note that in this case the chromosomes are not morphologically different.

considered before fixation, because they may contribute to the interpretation of unexpected cytogenetic findings.

Chromosome structure

Oocyte chromosomes are characterized by a particular morphology with highly condensed arms. The two chromatids are loosely associated and tend to separate, most probably under the impact of fixation (figure 4,a), thus facilitating a change in their orientation (figure 4,b). These are normal features that should not be confused with a balanced predivision of chromatids (Angell 1997) or true structural chromosome abnormalities. Moreover, an association of two acrocentric chromosomes has occasionally been observed by us (figure 4,c&d). The two associated elements can be

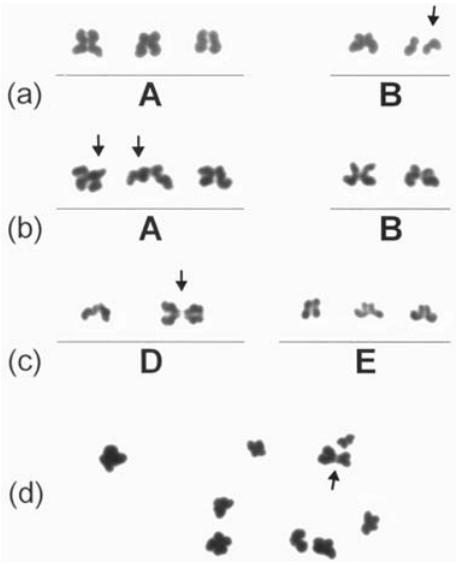

Figure 4. Normal features of oocyte chromosomes that should not be confused with real aberrations. (a) Partial karyotype showing slight chromatid separation in a B-group chromosome. The p arm of one chromatid (arrow) faces downward. (b) Partial karyotype showing A-group chromosomes in which one chromatid has rotated (arrows). (c) Partial karyotype with association of two D-group chromosomes (arrow). (d) Part of a metaphase with association of a D-group and a G-group chromosome (arrow).

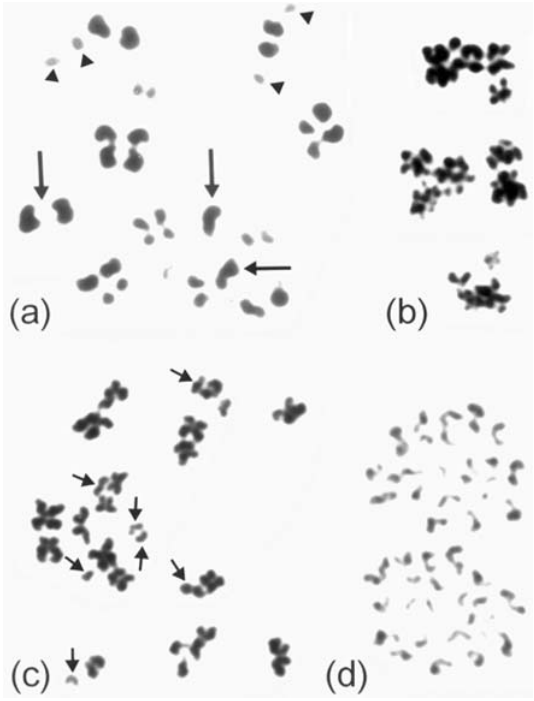

Figure 5. Chromatid separation and fragmentation of chromosomes. (a) Part of metaphase with detached sister chromatids (arrows) and p arms (arrowheads). (b) Fragmentation and clumping of chromosomes. (c) Metaphase with seven chromatids (arrows). (d) Early anaphase II.

confused with a single large chromosome, leading to an incorrect total chromosome count.

As already explained in materials and methods, most oocytes available for analysis are aged *in vitro* for two to three days after their retrieval. This extended time in culture can induce complete chromosome fragmentation with a detachment of chromatids and a separation of p and q arms which scatter on the slide (figure 5,a). Another possibility is fragmentation and subsequent clumping of chromosomal elements (figure 5,b). Such preparations inevitably must be classified as 'not analysable'. There are, however, metaphases with intact chromosomes and several single chromatids which represent borderline cases because they may or may not allow an unequivocal classification. These complements also raise the question of origin because they could be ascribed to actual predivision, beginning degeneration or partial progression to anaphase II (Rosenbusch and Schneider 2006). We have illustrated an example with apparently 19 chromosomes and seven chromatids (figure 5,c). Establishing a definite karyotype was not possible because of the small size and indistinct morphology of the chromatids. Therefore, we only described this case as '19 chromosomes +7 chromatids (counted)'.

Sometimes all the 23 chromosomes are divided into chromatids. Normally, they scatter on the slide but very rarely we found oocytes in which the chromosomes obviously were in early anaphase II (figure 5,d). In case of second PB extrusion, chromatid sets in the haploid range will be observed, mostly associated with prematurely condensed sperm chromatin (see below, participation of the spermatozoon; figure 7,b). Depending on the quality of the preparation, diploid and haploid sets of single chromatids may allow counting or karyotyping. However, attention should be paid to the fact that it is not possible to distinguish between an error during meiosis I and II, if an aneuploid set of single chromatids remains in the oocyte after second PB extrusion (Rosenbusch and Schneider 2006).

Because of the above-mentioned compact appearance of oocyte chromosomes, true structural abnormalities are difficult to evaluate. In homogeneously stained preparations, acentric fragments (figure 6,a), chromatid breaks (figure 6,b), chromosome breaks (figure 6,c) and deletions (figure 6,d) can be detected. Breaks mostly occur in the centromeric region and should be limited to one or few chromosomes for being classified as structural abnormality because excessive breakage rather points to cell degeneration (compare figure 5,a). It is our experience that larger fragments are sometimes difficult to distinguish from single G-group chromatids. Finally, parts of chromosome or chromatid arms can be folded and hidden, thus mimicking a deletion.

Participation of the spermatozoon

A considerable number of oocytes examined after failed attempts of IVF or ICSI contain chromatin contributed by the

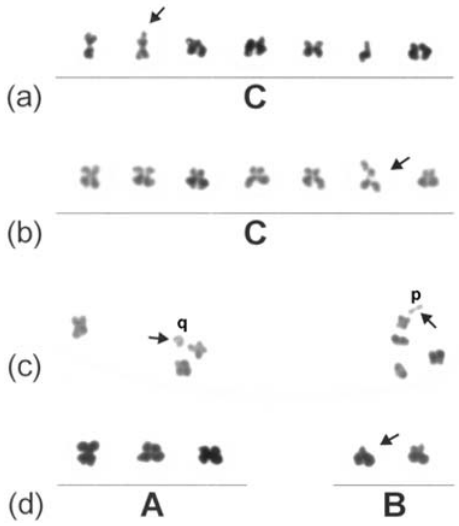

Figure 6. Structural chromosome abnormalities. (a) Partial karyotype with an acentric fragment (arrow) lying close to a C-group chromosome. Note that the structure of the first chromosome is not clear and that this group also contains a single chromatid. (b) Partial karyotype with a chromatid break in the centromeric region (arrow). (c) Part of a metaphase with a chromosome break in the centromeric region (arrows). (d) Partial karyotype with a deleted p-arm in group B (arrow).

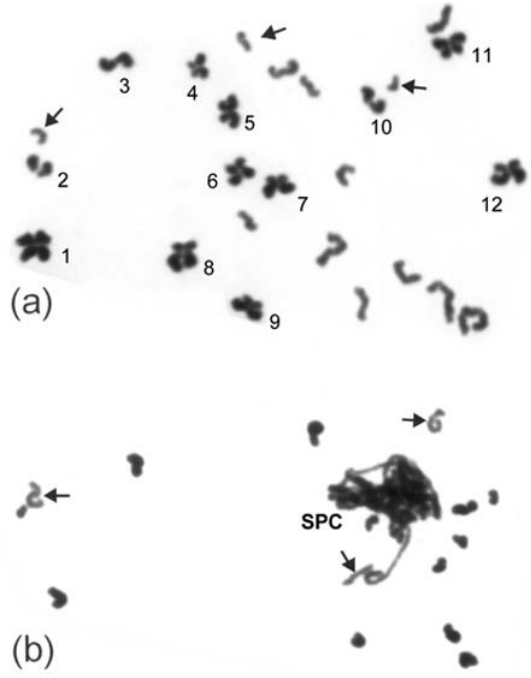

Figure 7. Prematurely condensed sperm chromatin in uncleaved oocytes. (a) Part of metaphase with 12 oocyte chromosomes (numbered) and sperm chromatids of which the smaller ones (arrows) resemble oocyte chromatids. (b) Part of metaphase with single oocyte chromatids and a rather compact mass of sperm chromatin (SPC) from which only few chromatids (arrows) have separated.

spermatozoon. This phenomenon has been ascribed to a lack of oocyte activation and the continuing presence of cytoplasmic chromosome-condensing factors inducing a premature chromosome condensation (PCC) of the sperm nucleus (Schmiady *et al*. 1986). Different degrees of PCC can be distinguished (Rosenbusch 2000), the final step being the appearance of 23 so-called 'prematurely condensed sperm chromosomes' that are in fact single chromatids. They can be associated with oocyte chromosomes (figure 7,a) or oocyte chromatids (figure 7,b) and might be confused with the latter.

Finally, oocytes displaying no pronuclei during examination and failing to cleave may nevertheless contain mitotic chromosomes, suggesting undetected fertilization followed by developmental arrest for unknown reasons (Tejada *et al*. 1991). The mitotic metaphases are frequently in the diploid range (Macas *et al*. 1990; Tejada *et al*. 1991) but we also observed a case with an approximately tetraploid count (figure 8). It is comparable to the example depicted by Schmiady and Kentenich (1993), found in arrested zygotes with preceding formation of two distinct pronuclei. Most probably, tetraploidy is the result of arrested cytokinesis (Schmiady and Kentenich 1993).

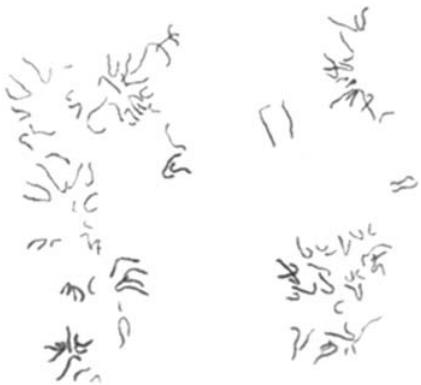

Figure 8. Mitotic chromosomes in the tetraploid range in an oocyte that did not display pronuclei after ICSI.

Discussion

For the cytogenetic analysis of human oocytes, some particular features of these cells have to be taken into account. First, every gamete is a unique product of meiosis. In contrast to mitotic cell cultures, the assessment of chromosomal abnormalities cannot be confirmed by testing other cells. Second, the already unsatisfactory morphological quality of meiotic chromosomes may become even worse owing to a prolonged *in vitro* culture. Third, oocytes were obtained after apparently failed attempts of IVF but unrecognized sperm penetration may trigger events that lead to unexpected findings, e.g. partial or complete oocyte chromatid separation or appearance of mitotic chromosomes.

In the present report, we summarize for the first time, the possible pitfalls in human oocyte chromosome analysis because we noticed that pertinent guidelines are still missing in the literature. It has been shown that maturity and size of the gametes are important parameters and that fixation artefacts as well as the structure of oocyte chromosomes may predispose to misinterpretations. Therefore, it is not astonishing that some questionable results have been published. For instance, the deletion of the long arm of a G-group chromosome (Gq-) reported by Plachot *et al*. (1986) may actually represent a G chromatid and absence of the q arms in chromosome 1 (Bongso *et al*. 1988) does not represent a structural abnormality. A closer look at the karyotype shows that the 'deleted' chromosome 1 has been confused with a B-group chromosome that has been arranged with the p arms facing downward. The fragmented oocyte chromosomes described by Papadopoulos *et al*. (1989) strongly resemble the first PB chromosomes and chromosomal translocations observed by Kumar and Khuranna (1995) must be ascribed to diploid arrested zygotes rather than unfertilized oocytes. It is evident that the compact morphology of oocyte chromosomes does not allow the detection of complex rearrangements such as translocations and inversions. As suggested by Zhivkova *et al*. (2007), further frequent errors may comprise the confusion of single chromatids and small acrocentric chromosomes and the interpretation of chromatid separation as hyperhaploidy.

Different authors (Papadopoulos *et al*. 1989; Michaeli *et al*. 1990) considered prematurely condensed sperm chromatin as a structural abnormality. However, it is not advisable to list sperm PCC as an anomaly of the oocyte chromosome complement because oocyte chromosomes are in fact not involved. We have already pointed out that excessive chromosomal fragmentation is not a true structural abnormality, because it results most probably from degenerative effects. Therefore, the distinction of chromatid separation and chromosomal fragmentation needs international standardization (Rosenbusch 2006a).

Pellestor *et al*. (2005) mentioned that the frequent scattering or overlapping of chromosomes and chromatids is a significant problem and that the lack of a well-defined chromosomal morphology during meiosis will also affect the results of studies performed with other techniques, e.g. FISH. Possibly, the incompatibility of data obtained from oocyte and first PB analysis regarding the distribution of nondisjunction and predivision (Rosenbusch 2006b) is caused at least in part by artefacts described in the present overview. In conclusion, the assessment of human oocyte chromosome complements remains a challenging task. Currently, it is not clear whether additional techniques will completely supersede the conventional approach of homogeneous staining. The latter may indeed have advantages for the analysis of compact chromosomes and the detection of fragments (Zhivkova *et al*. 2007). However, to improve the scientific value of corresponding studies, strict and uniform criteria should be followed, not only when analysing cytogenetic preparations but also in presenting the results (Rosenbusch 2007).

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