Chromosomal abnormalities in human gametes

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Chromosomal abnormalities in human gametes

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Chromosomal abnormalities in human gametes

Proefschrift

ter verkrijging van de graad van doctor
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Prof. Dr. A. Nieuwenhuijzen Kruseman
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in het openbaar te verdedigen
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door

Elena Martini

geboren op 29 februari 1968 te Livorno, Italië

in memoria di GianLuigi Capitanio

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Chapter 1

General Introduction

1.1. Definition of Chromosomal Abnormalities

Chromosomal abnormalities are responsible for at least half of spontaneous miscarriages and are an important cause of congenital malformations (Hassold 1986; Edwards 1986; De Grouchy and Turleau 1984; Gardner and Sutherland 1989). Chromosome abnormalities may be numerical or structural.

The number of human chromosomes in a normal somatic cell is 46 (diploid or $2n$, n being the number of chromosomes in a normal haploid gamete) (Figure 1.1). Triploidy is an euploid situation ($3n$) and is a common chromosomal abnormality found in spontaneous abortions (van Lijnschoten *et al.*, 1993). Aneuploidy is any number of chromosomes that is not euploid (an exact multiple of n) and refers to either an extra chromosome, i.e. trisomy (as in trisomy 21, Down's syndrome), or the absence of a chromosome, i.e. monosomy (as in monosomy 45,X0, Turner's syndrome). The consequences of such numerical aberrations vary from early embryonic or fetal wastage, perinatal and infant mortality to mental retardation and congenital anomalies, depending on the chromosome involved. Some estimates suggest that up to 50% of all human concepti carry a chromosomal abnormality (Boue' *et al.*, 1975; Hassold *et al.*, 1980; Eiben *et al.*, 1990), and chromosomal aberrations which arise during gametogenesis and early embryonic development play a significant role in fetal loss (Chard 1991). In human live born infants, trisomy is commonly only seen for chromosomes 13, 18, 21 and the sex chromosomes (De Grouchy and Turleau 1984). Such trisomies occur in 4% of all clinically recognized pregnancies, 24% of spontaneous abortions and 0.5% of live births (Jacobs and Hassold 1995).

There is a process of natural selection whereby most trisomies and monosomies that arise during gametogenesis, fertilization or early embryonic development are expelled during the first stages of gestation. Trisomy has been observed for every chromosome in human spontaneous abortions (Hassold *et al.*, 1980), with the exception of chromosome 1, for which a trisomy has been seen only once in an 8-cell human pre-embryo (Watt *et al.*, 1987), suggesting that trisomy 1 is lethal very early in development. Embryos with trisomy 16 (the most frequent trisomy at conception) are lost at about 10 weeks of gestation, while embryos carrying a trisomy 13, 18 or 21 will eventually develop to term, but the majority of embryos with these abnormalities are expelled during the different stages of gestation (De Grouchy and Turleau 1984; Martin and Rademaker 1990; Warburton *et al.*, 1987). Structural abnormalities of chromosomes usually involve one or two chromosomes, but may involve more than two. They are categorised as (i) inversions, deletions, insertions or duplications of parts of the chromosome(s) or (ii) translocations. In the case of a balanced translocation (reciprocal or Robertsonian), usually no genetic material is lost and usually no genes are damaged, which means that individuals carrying such a translocation will be genetically, and thus clinically, normal (Jacobs 1977). However, they are at increased risk of having chromosomally unbalanced offspring (De Braekeleer and Dao 1990; Martin *et al.*, 1991; Goldman and Hulten 1993a; Goldman and Hulten 1993b; Estop *et al.*, 1995; Martini *et al.*, 1998a).

1.2. Gametes and Meiosis

Human gametes, spermatozoa and oocytes, originate from germ cells in the testis and ovary, respectively. The stem cells, spermatogonia and oogonia, are diploid and it is through the process of meiosis during spermatogenesis and oogenesis, that the resulting haploid gametes are produced (Figure 1.2). There are several causes of aneuploidy in gametes, depending on the stage at which the delicate process of meiosis is disturbed. Human meiosis, especially in females, is a process that seems to be prone to error (Warburton 1997). The major cause of aneuploidy is non-disjunction (NDJ) of the homologues during

meiosis, in which the homologues line up at the meiotic spindle but fail to separate at anaphase I. The resultant cells either lack a chromosome (hypohaploidy) or have an extra chromosome (hyperhaploidy) (Figures 1.4- 1.5). This error can occur either in meiosis I or meiosis II and such aneuploid gametes will produce abnormal embryos. Another meiotic error responsible for NDJ is anaphase lagging, as a result of which a single chromosome is lost because it lags behind the others during anaphase and does not migrate to the opposite pole of the spindle. Anaphase lagging at syngamy can lead to mosaicism, with one euploid and one monosomic cell population.

Figure 1.1. Schematic explanation of meiosis and mitosis.

The differences between male and female meiosis are illustrated in Figure 1.2. The principal difference is that male meiosis proceeds throughout adult life whereas female meiosis is arrested at prophase of the first meiotic division at the fetal stage and is only completed many years later in the adult ovary. Just before or shortly after birth, human oocytes undergo prophase of meiosis I and then arrest at the diplotene stage of prophase. At this point, the oocyte is characterized by the presence of a distinctive nucleus called a germinal vesicle (GV). This state seems the most appropriate for prolonged conservation of the

DNA. Meiosis I resumes after the pre-ovulatory surge of luteinising hormone (LH) during a process known as oocyte maturation. The GV disappears and the chromatin condenses into bivalents. The homologues separate and the first polar body (PB), containing the first set of haploid chromosomes (each consisting of two chromatids), is extruded into the peri-vitelline space. Meiosis then arrests again at metaphase II until after fertilization, at which time it resumes. A second PB is extruded during telophase II, leaving the oocyte with a haploid number of chromosomes (Figure 1.3).

MEIOSIS

Figure 1.2. Female and male meiosis are illustrated and as an example the sex chromosomes are visualised and followed through the meiotic process. (Polar body = PB)

While male meiosis produces four haploid gametes from one diploid germ cell, female meiosis yields only one haploid gamete. Since the length of meiosis I in mammals is directly correlated with maternal age, it was both expected and verified that meiosis I errors were associated with increased maternal age. The data support the hypothesis that, in older women, bivalents lacking peri-centromeric chiasmata separate prematurely, resulting in meiotic NDJ, whereas in young women the same oocyte is likely to be processed normally (Warburton 1997; Angell 1997; Plachot 1997) (Figure 1.4).

MEIOSIS & FERTILIZATION

Figure 1.3. The different sequences of female meiosis are illustrated, from oogonium to the formation of the zygote after fertilization.

Spermatogenesis is a process that serves to expand the number of germ cells and to permit their development into mature spermatozoa. This process takes place in the seminiferous tubule, a long tubular structure lined by a single layer of Sertoli cells.

During spermatogenesis, spermatogonia slowly evolve into spermatocytes, then spermatids and finally through spermiogenesis the complete maturation is achieved and spermatozoa are released into the lumen (Bellvé and O'Brien 1983; De Kretser and Kerr 1988; Moore 1995). Errors can occur during the first or second meiotic division and aneuploid spermatozoa are released into the lumen of the seminiferous tubule, ejaculated and may fertilize oocytes and give rise to abnormal embryos (Martin *et al.*, 1991; Goldman and Hulten 1993b; Williams *et al.*, 1993; Spriggs *et al.*, 1996) (Figure 1.5).

1.3. Medically Assisted Reproduction

During the last two decades, techniques for medically assisted reproduction (MAR) have been developed enabling couples to overcome infertility. A study by the World Health Organization (WHO 1993) found that infertility was due to female factors in 41% of the cases, male factors in 24%, a combination of female and male factors in 24%, and no cause

could be found in 11%. In vitro fertilization (IVF) was initially developed to overcome tubal blockage, but has also proven suitable for the treatment of other forms of infertility (Jones 1986; Thatcher and De Cherney 1989). One of the techniques is a technique which is used in conjunction with IVF to overcome male factor infertility is intracytoplasmic sperm injection (ICSI) (Palermo *et al.*, 1992).

Figure 1.4. Two examples of nondisjunction during female meiosis. MI = meiosis I, MII = meiosis II.

Parallel advances in MAR and molecular genetics have now made it possible to study meiotic and mitotic errors which arise during oogenesis, spermatogenesis, fertilization and early embryogenesis and to assess the contribution of these errors to chromosomal abnormalities in embryos and fetuses. Genetic studies on single gametes and embryos can provide valuable diagnostic information, which may help couples who require MAR or who are at risk of inheritable diseases (Harper *et al.*, 1994; Munné *et al.*, 1995).

1.4. Methodological Aspects

Spermatozoa

The earliest studies on chromosomes in human sperm used differential staining of specific regions of chromosomes. In 1970, Pearson and Bobrow (1970) used quinacrine to stain the distal part of the long arm of the Y chromosome, the so called "Y-body". Giemsa and Leishman's staining were used by Bobrow *et al.*, (1972), Pearson *et al.*, (1972) and

Figure 1.5. Nondisjunction during male meiosis.

Geraedts and Pearson, (1973) to study autosomes. However, due to non-specific staining of chromosomes, these techniques were considered unreliable and were therefore not developed further. Shortly thereafter, Yanagimachi *et al.*, (1976) and Rudak *et al.*, (1978) introduced the direct visualization of human sperm chromosomes after penetration of zona-free hamster oocytes. More than 20,000 sperm chromosome complements have been karyotyped to date using this method (Martin *et al.*, 1993; Brandriff *et al.*, 1985; Estop *et al.*, 1991; Pellestor 1991; Downie *et al.*, 1997 and Guttenbach *et al.*, 1997 for review). This yielded valuable data on the entire chromosome complement of sperm, and the presence of both numerical and structural chromosomal abnormalities can be studied (Martin and Rademaker 1990). However, sperm karyotyping is a laborious and time-consuming procedure and the results may be biased by the fact that only those spermatozoa which were able to fertilize hamster oocytes are karyotyped (Jacobs 1992; Martin *et al.*, 1993; and Guttenbach *et al.*, 1997 for review).

The drawbacks of these earlier methods are overcome by in situ hybridization (ISH) techniques, which permit analysis of both numerical and structural aberrations in metaphase chromosomes as well as in interphase nuclei (Lichter *et al.*, 1991; Tkachuk *et al.*, 1991; Hopman *et al.*, 1991). Since its introduction in 1969 (Gall and Pardue 1969; John *et al.*, 1969), ISH has been used to localize specific DNA sequences in interphase nuclei from many different cell types. This technique enables identification of specific parts of the ge-

nome (the target DNA) using chemically modified or labelled chromosome-specific DNA probes which hybridize to the target DNA. Following hybridization, the probes can be visualized using fluorochromes (fluorescence ISH) or cytochemical precipitation products (brightfield ISH) as reporter molecules (Speel *et al.*, 1995; Martini *et al.*, 1995).

The first probes used for ISH were labelled radioactively, but poor resolution and long exposure time soon led to their replacement by biotinylated probes (Langer *et al.*, 1981) and later by directly fluorochrome labelled probes. Autoradiography was replaced by fluorescence visualization (Manuelidis *et al.*, 1982) and hitherto routinely used. Although the information obtained by ISH is limited to the DNA targets examined, it has the advantage that virtually every cell is informative, in contrast to conventional karyotyping (Martin *et al.*, 1993; Martini *et al.*, 1995; Martini *et al.*, 1997).

Joseph *et al.*, (1984) reported the first radioactive ISH on spermatozoa, followed by West *et al.*, (1989) and many others (see Downie *et al.*, 1997 for review). Since then these methods were further developed and improved for application on mature spermatozoa. A delicate balance between hybridization efficiency and maintenance of sperm morphology has led to incongruent results (Martin *et al.*, 1993; Williams *et al.*, 1993; Martini *et al.*, 1995; Downie *et al.*, 1997 and Guttenbach *et al.*, 1997a for review). The crucial steps for ISH, in order to obtain efficient hybridization, are to achieve a proper decondensation of the protamines (the proteins which replace in human sperm the nuclear histones) and denaturation of the DNA of the specimen. However, after decondensation the morphology of sperm heads is partly distorted and the majority of the tails are lost. This leads to problems distinguishing hyperhaploid from diploid spermatozoa, and abnormal spermatozoa from somatic cells.

Oocytes

The chromosomal constitution of human oocytes retrieved for IVF has been studied extensively (see Plachot 1997 for review). For ethical reasons, the usual approach has been to study those oocytes that failed to fertilize and remained arrested at metaphase II. This should provide information on the frequency and types of errors that occur in oocytes during meiosis I. However, many of these studies relied on poor quality preparations in which accurate identification of individual chromosomes and the detection of abnormalities was very difficult, besides the spreading method which led to high rates of artefactual chromosomal loss (Warburton 1997; Angell 1997; Plachot 1997). The unusually condensed nature of the chromatids on the metaphase II spindle (Pellestor 1991) has hampered reliable analysis by karyotyping and necessitated the introduction of a modified protocol based on Tarkowski (1966) to spread the chromosomes. This method uses methanol:acetic acid (3:1) fixation and has major drawbacks such as loss of cells and/or loss of morphology, thereby reducing the efficiency, reliability and reproducibility. This is a great disadvantage in the study of aneuploidy, wherein the results must be reliable and accurate. Larger series of studies suggest that a high proportion of human oocytes (25-30%) are chromosomally abnormal (Benkhalifa *et al.*, 1990; Plachot *et al.*, 1988; Angell *et al.*, 1993; Angell *et al.*, 1994; Edirisinghe *et al.*, 1992; Martin *et al.*, 1991; Pellestor 1991; Roberts and O'Neill 1995).

Coonen *et al.*, (1994b) presented an alternative method for preparing and isolating interphase nuclei from pre-implantation embryos using HCl and Tween 20 which offers high reproducibility, good cellular morphology and a high ISH efficiency (Coonen *et al.*, 1994a; Coonen *et al.*, 1994b; Harper *et al.*, 1994; Muggleton-Harris *et al.*, 1995). This technique can be adapted for the spreading of oocyte DNA and when combined with ISH offers an effective alternative to karyotyping (Martini *et al.*, 1997; Martini *et al.*, 1998b).

1.5. Aims of this study

This study was mainly initiated to develop reliable methods for estimating aneuploidy in human gametes. It was particularly focused on the application of ISH to human spermatozoa and oocytes.

The specific aims of this study were:

- ★ To improve the efficiency, sensitivity and reproducibility of ISH on human gametes.
- ★ To assess whether or not ISH is a valid alternative to estimate the incidence of aneuploidy in spermatozoa and unfertilized oocytes to karyotyping of human gametes, being less time consuming and less labour intensive.
- ★ To analyse the incidence of aneuploidy in spermatozoa and unfertilized oocytes.
- ★ To estimate the risk of transmitting chromosomal aberrations using gametes from males with karyotypic abnormalities.

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Chapter 2

Application of different in situ hybridization detection methods for human sperm analysis

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Abstract

The detection of some types of aneuploidy in human spermatozoa can be based on the use of fluorescence in situ hybridization technique (FISH). One of the crucial steps for FISH is to achieve a proper decondensation and denaturation of the DNA in the specimen, so as to obtain efficient hybridization results. However, after DNA decondensation the morphology of sperm heads is partly distorted and the majority of the tails is lost. This situation leads to problems in the distinction between disomic and diploid sperm, as well as between abnormal spermatozoa and somatic cells. Double- and triple-target FISH can partly solve this discrimination problem. To improve these procedures we adapted the steps of decondensation and visualization of the single sperm cells. Firstly, DNA decondensation with 25mM DTT in 1M TRIS at pH 9.5 resulted in sperm cells with intact morphology of both the head and the tail and allowed to perform efficient single-, double- and triple-target ISH. Secondly, we applied a novel detection method, based on enzyme immunocytochemical reactions, with coloured precipitation products. Thirdly, this ISH procedure was combined with Diff-Quik staining and the use of brightfield microscopy. This absorption method has the advantage of a permanent signal, and the adapted cytoplasmatic staining of the sperm plasma membrane allows the visualization of the outline of the single spermatozoa. Using this approach, therefore, it is possible to discriminate between disomic-, diploid-, and abnormal spermatozoa, somatic cells and spermatozoas that overlap, because the morphology of the cells is not distorted and the tails of the spermatozoa are intact and properly visualized.

Introduction

For several years the detection of numerical chromosome aberrations in human spermatozoa has been performed with karyotyping, a very laborious and time consuming procedure, involving the fusion of one single spermatozoa with a zona pellucida free hamster oocyte (Martin *et al.*, 1990). In recent years, with the advent of interphase cytogenetics, using in situ hybridization (ISH) with specific DNA probes, the interest has focused on the latter. With ISH it is possible to analyse a whole specimen within a few hours. In this respect, the fluorescence ISH (FISH) technique has become a powerful method used widely for the detection of numerical and structural chromosomal aberrations in metaphase spreads and interphase nuclei. It is used to study the effect of carcinogenic and teratogenic agents as well as in prenatal and preimplantation diagnosis. However, the use of fluorescent ISH on human spermatozoa is more complex, due to the inaccessibility of sperm DNA and the interpretation of the results. Joseph *et al.* (1984) reported the first ISH on spermatozoa. They used an autoradiographic method on testicular spermatozoa. Since then many other authors have performed fluorescence ISH on mature spermatozoa. Although the hybridization rate has increased, the morphology of the sperm cell, is often lost and the interpretation of the ISH signals has led to different results among different groups (Martin *et al.*, 1993; Williams *et al.*, 1993).

The DNA of human spermatozoa is tightly compacted in the nucleus due to small, arginine rich nuclear proteins, the protamines, which are specific for spermatozoa and replace the nuclear histones of somatic cells. These protamines neutralize the charge of DNA and form a network of molecules in mature spermatozoa interconnecting intra- and inter-protamine disulfide bridges. ISH requires denaturation of the chromosome specific DNA probe and of the target cell DNA. Therefore, a proper decondensation of the sperm chromatin, prior to the denaturation step is necessary for positive ISH results. From early studies, the denaturing agent, dithiothreitol (DTT) is known to be an efficient reducing agent (Calvin and Bedford, 1971). It breaks the disulphide bonds between the protamine

chains and allows proper DNA hybridization, and several different decondensation methods using DTT have been published (Perrault and Zirkin, 1982; West *et al.*, 1989; Wyrobek *et al.*, 1990; Coonen *et al.*, 1991; Han *et al.*, 1992; Holmes *et al.*, 1993; Vidal *et al.*, 1993; Williams *et al.*, 1993; Miharu *et al.*, 1994). Only one group (Guttenbach and Schmid, 1990) has claimed the ability of performing ISH without previous decondensation of their samples. Another difficulty of the ISH procedure is the recognition of somatic cells and their distinction from diploid, overlapping or morphologically abnormal spermatozoa. A specific marker that allows the visualization of the outline of the whole spermatozoon is needed.

The aim of this study was to improve ISH on sperm cells, replacing the diverse decondensation procedures by a standard, routinely applicable protocol. Furthermore, the morphology of the whole, single spermatozoa had to be retained in preparations that allowed a correct and reliable evaluation of the ISH signals.

The use of a detection method based on immunocytochemical reactions, using different enzymes as conjugates in combination with precipitating substrates, allows the detection of ISH signals with a brightfield microscope instead of a fluorescence microscope, and has therefore many advantages. No fading of the ISH signals occurs, the signals are permanently and accurately localized and can be examined in relation to cell morphology (Speel *et al.*, 1994). Counterstaining of these preparations with Diff-Quik highlights the outline of the single spermatozoa, and the clear, contrasting staining of the sperm tails, the sperm heads and the somatic cells makes the evaluation reliable (Kruger *et al.*, 1988).

Materials and methods

Preparation of sperm nuclei

Ejaculates from 7 normal males were used in this study. The samples were allowed to liquify at room temperature for 30 min and then routinely analyzed by the IVF-lab of our hospital. The number of sperm cells, their motility and morphology was determined for each sample. The semen samples were washed 3 times in phosphate buffered saline (PBS, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.2) and centrifuged for 10 min at 1200 rpm. The pellet was then resuspended carefully in 1 ml of fresh, cold fixative (-20°C), (methanol : acetic acid, 3:1) and stored at -20°C.

Decondensation of sperm nuclei

Sperm cells were cytospun on poly-L-lysine coated slides, washed in 2x SSC (0.3 M NaCl, 30 mM sodium citrate) to remove the remainders of fixative and incubated for 5 min in 1 M TRIS/HCl buffer, pH 9.5, containing 25 mM DTT. After decondensation the slides were washed once in 2x SSC, once in PBS, finally dehydrated through an ethanol series (70-96-96-100-100%) and air dried (Jones *et al.*, 1987).

DNA probes and labeling procedures

The DNA probes used in this study recognize the (peri)centromeric regions of the human chromosomes 1 (pUC 1.77, 1.77 kB; Cooke and Hindley, 1979), 7 (p7t1, 0.68 kB; Waye *et al.*, 1987) and 17 (p17H8, 2.7 kB; Waye and Willard, 1986). Further, the centromeric probes for chromosome X (pBam X5, 2.0 kB; Oncor, Gaithersburg, MD) and Y (DYZ1, 3.4 kB; Cooke *et al.*, 1982) were used. These DNA probes were labelled with biotin-11-dUTP (Enzo Diagnostics; New York, NY), digoxigenin-11-dUTP or fluorescein-12-dUTP (Boehringer; Mannheim, Germany) or tetramethylrhodamine-4-dUTP (Amersham International, Little Chalfont, UK) in a standard nick-translation reaction and used in single-, double- or triple-target ISH procedures.

ISH procedure

The DNA probes described above were used at a concentration of 0.4 ng/ μ l (pUC1.77) and of 1 ng/ μ l for all the other probes, in single- and double-target ISH. In triple-target ISH the autosomal probes p7t1 and p17H8 were used at a concentration of 0.25 ng/ μ l, to enable simultaneous localization of three ISH signals in the sperm head. The different probes were hybridized in different combinations (see below) in a hybridization buffer containing 60% formamide, 2x SSC, pH 7.0, 10% dextran sulfate, 0.2 μ g/ μ l herring sperm DNA as carrier DNA and 0.2 μ g/ μ l yeast t-RNA as carrier RNA. 5 μ l buffer was added to each slide under a coverslip (18x18mm). Denaturation was performed on the bottom of a metal box in a water bath at 73°C for 3 min and hybridization was performed for 4 hrs at 37°C. The slides were washed twice at 42°C with 60% formamide, 2x SSC, pH 7.0, containing 0.05% Tween 20, followed by 2x5 min washes with 2x SSC, pH 7.0 at 42°C and a 5 min wash with 4x SSC, pH 7.0, containing 0.05% Tween 20 at room temperature.

Cytochemical detection procedures

Detection of signals was performed as described previously (Speel *et al.*, 1994). Briefly, the detection procedures for the avidin conjugates were diluted in 4x SSC, pH 7.0, containing 5% non fat dry milk (NFDM), while all the antibody conjugates were diluted in PBS containing 0.05% Tween 20 and 2% normal goat serum (NGS). After each incubation step of 20-30 min at 37°C, the slides were rinsed twice in 4 xSSC pH 7.0/0.05% Tween 20 (Avidin conjugates) or PBS/0.05% Tween 20 (antibody conjugates).

Fluorescent ISH

Single target fluorescent ISH was performed with the biotinylated probe for chromosome 1 and detected with a 1:100 dilution of FITC-conjugated avidin (Vector, Brunswick Chemie, Amsterdam, The Netherlands). For double-target fluorescent ISH the directly labelled probes for the sex chromosomes (X-FITC and Y-TRITC) were applied simultaneously and could be detected directly. Slides were dehydrated and mounted in PBS/glycerol (1:9, v/v) containing 2.3% of the anti-fading reagent 1,4-diazobicyclo-(2,2,2)-octane (DABCO, Merck, Darmstadt, Germany) and 0.5 μ g/ml of DAPI for nuclear DNA staining. In triple-target FISH with the probes X-FITC, Y-TRITC and biotinylated pUC 1.77, the first two probes were detected directly and the biotinylated probe was detected by means of three subsequent incubation steps of AMCA-conjugated avidin (AV-AMCA, 1:100; Vector), biotinylated goat anti-avidin (BioG α A, 1:100; Vector) and again AV-AMCA. Slides were then embedded as described above without the DAPI counterstaining.

ISH using enzyme precipitates

Single-, double-, and triple-target ISH using enzyme-conjugated reagents in combination with precipitating substrates were performed as described previously (Speel *et al.*, 1994). Briefly, in single-target ISH the biotinylated pUC 1.77 probe was detected with horse radish peroxidase-conjugated avidin (AV-PO; 1:50; DAKO A/S; Glostrup, Denmark), followed by either the diaminobenzidine (DAB) or the tetramethylbenzidine (TMB) reaction. For alkaline phosphatase-Fast Red (APase-Fast Red) visualization, the probe was detected with two layers of mouse anti-biotin (M α Bio, 1:100, DAKO A/S) and APase-conjugated goat anti-mouse IgG (G α M-APase, 1:25; DAKO A/S).

In double-target ISH the biotinylated, chromosome X-specific probe and the fluoresceinated, chromosome Y-specific probe were detected respectively with AV-PO, mouse anti-FITC (M α FITC, 1:500; DAKO A/S) and G α M-APase, followed by first, the APase-Fast Red

reaction and finally the PO-TMB reaction; or AV-PO, M α FITC and PO-conjugated rabbit anti-mouse IgG (R α M-PO, 1:80; DAKO A/S). In this case the PO-DAB reaction was performed after the first detection layer, followed by an incubation of the slides in 0.01 N HCl for 10 min at room temperature to inactivate any rest activity of the PO enzyme. After the last incubation step with R α M-PO the PO-TMB reaction was performed.

In triple-target ISH the biotinylated X-probe, the FITC-labelled Y-probe and the digoxigenin-labelled 1-probe or biotinylated 1-probe, the digoxigenin-labelled 7-probe and the FITC-labelled 17-probe, were detected with three incubation steps of AV-PO, a mixture of mouse anti-digoxigenin (M α Dig, 1:2000; Sigma, St. Louis, MD) and rabbit anti-FITC (1:2000; DAKO A/S), and a mixture of G α M-APase and PO-conjugated swine anti-rabbit IgG (SW α R-PO, 1:100; DAKO A/S). After the first detection layer the PO-DAB reaction was performed followed by the 0.01 N HCl step. After the last incubation step first the APase-Fast Red precipitation reaction was performed, followed by the PO-TMB reaction. The individual enzyme reactions have been described previously (Speel *et al.*, 1994).

Counterstaining

The haematoxylin counterstaining was first used, but than substituted by the Diff-Quik staining procedure. Of the latter we used only the cytoplasmatic staining step, 1g/l xanthene in sodium azide preserved phosphate buffer, pH 6.6 (solution 1) and was obtained from Baxter, Utrecht, The Netherlands.

Embedding

Embedding dependent on the cytochemical reactions used:

after a single- or double-target ISH with brown PO-DAB and green PO-TMB precipitates, the slides were embedded in organic mounting media, such as Entellan (Merck, Darmstadt, Germany), since the PO-TMB reaction product is unstable in aqueous mounting media. When a APase Fast Red reaction in single- or double-target (combining PO-DAB and APase Fast Red) ISH was performed, the slides had to be embedded in aqueous mounting media, after counterstaining, since the APase Fast Red precipitation product is unstable in ethanol and organic-based mounting media (Speel *et al.*, 1992). When a triple target ISH was performed, the slides had to be embedded in a thin protein layer, after counterstaining (Speel *et al.*, 1993, 1994), to ensure a permanent stabilization of the enzyme reaction products and optimal visualization of color contrast. The preparations were stored permanently at 4°C.

Microscopy

FISH signals were analyzed with a Zeiss Axiophot microscope, equipped with appropriate filters for FITC, TRITC and DAPI fluorescence.

The evaluation of the enzyme precipitate ISH signals was done with a standard Zeiss brightfield microscope. Microphotographs were made on a Zeiss Axiophot microscope, using a Kodak Color Gold 100 ASA film in combination with blue and magenta filters.

Results

DNA decondensation

The original DNA decondensation protocol of Coonen *et al.*, (1991), where DTT is combined with 0.1 M trypsin treatment, was used for our initial studies. Over- and under-exposure to the denaturing agent influenced the ISH efficiency, as well as sperm morphology. Overexposure caused a complete loss of morphology and splitting of the ISH

signals, making evaluation difficult. On the contrary, underexposure resulted in a non-accessibility of sperm DNA and subsequent failure of the ISH procedure. To improve DNA decondensation without having to assess the correct denaturation time for each individual case, we developed a decondensation procedure with DTT at a high pH.

A proper decondensation of nuclear DNA and protamines was obtained after standard exposure to 25 mM DTT in 1M TRIS at a pH of 9.5, for 5 min (tested in a range of 3 to 10 min) at room temperature. No efficient hybridization was observed without pretreatment of the samples, since only a limited number of sperm cells reacted properly, their number varying between the different samples. With the protocol delineated above all the investigated samples showed an oval, compact shape of the sperm head and tail, together with efficient hybridization.

The problem of clustering and overlapping spermatozoa was partly solved by careful fixation in methanol : acetic acid (3:1), and by cytospinning the samples on poly-L-lysine coated slides. However, sometimes adjacent or overlapping sperm cells and somatic cells could not be easily evaluated.

Single-, double- and triple-target FISH-procedure with differently labelled probes has been used for these tests. Additional counterstaining of the slides with DAPI allowed a better evaluation of the signals and a more reliable recognition of the tail. The FISH spots were discretely localized and bright, while the sperm tail was distinguishable (data not shown). In triple-target FISH a counterstaining was unnecessary since background-fluorescence was strong enough for a faint counterstaining.

Brightfield microscopical analyses

For brightfield microscopical detection of ISH signals, the enzymatic precipitation reactions were optimized for single target ISH. The optimisation involved variation of reaction times and composition of the reaction media in order to obtain a proper localization of the ISH signal to enable a reliable evaluation.

Figure 1a illustrates a single target ISH reaction on a sperm preparation with an autosomal probe, performed with a PO-DAB reaction (brown precipitate). Figure 1b shows the APase Fast Red reaction (red precipitate), and the PO-TMB reaction (green precipitate) in a double-target sex chromosome ISH. In single-target ISH the oval shape of the head and the tail is clearly visible (Figure 1a). In this case the counterstaining was performed with haematoxylin. Aneuploidies could be detected in the single-target ISH procedure in properly counterstained sperm cells (see Figure 1a). Although in double- and triple-target ISH the different colors and the shape of the sperm-head were recognizable, the staining of the tail was normally inefficient, even after haematoxylin counterstaining (Figures 1b-d).

Combination of ISH and Diff-Quik sperm counterstaining

In the first experiments counterstaining was performed with haematoxylin (Figures 1a-d), but it showed to be important to dilute the dye solution properly to allow a correct staining of the sample without covering the ISH signals and prevent mixing with the colored reaction products. However, it was still not possible to distinguish overlapping sperm heads or twisted tails because of the weak and non-contrasting staining that is obtained with haematoxylin.

To overcome these problems and to enable a simultaneous visualization of ISH signals and of the whole spermatozoon, we applied the Diff-Quik staining Kit, routinely used in the IVF lab for the evaluation of sperm morphology (Kruger *et al.*, 1988; Enginsu *et al.*, 1991). It could be combined with all ISH detection systems tested in this study, and we only reduced the standard staining to a 2 sec cytoplasmatic staining step. This was sufficient for a clear

and contrasting staining of the plasma membrane. Figures 1e-g show single-target ISH with brown DAB signals, while in Figure 1h an example of the green PO-TMB precipitation product is shown. Examples of morphologically abnormal spermatozoa showing abnormalities of chromosome 1 are indicated in Figure 1f and Figures 1g-h, respectively. In Figure 1i a double-target ISH with the sex chromosomes is shown, in spermatozoa next to a somatic cell. The sperm head has a small cytoplasm, and is therefore less stained as compared to the tail and to the somatic cells. A reliable evaluation of the ISH signals together with cytogenetic and morphological abnormalities is now possible with the Diff-Quik method.

Numerical data

Sperm samples from 7 normal males were analyzed with the centromeric probe for chromosome 1. For each donor an average of 1600 sperm cells were counted (range from 1200 to 1800). The average ISH efficiency was 98.7% (range from 98.0% to 99.3%). The detected aneuploidy rate was 0.69% (range from 0.40% to 1.40%). The percentage of morphologically abnormal spermatozoa ranged from 3.71% to 8.21%, with an average of 5.68%.

Discussion

This study was initiated to develop a standardized method for an efficient detection of numerical chromosomal aberrations in human spermatozoa by ISH. The decondensation of the chromatin of the sperm head appeared to be a crucial and essential step for such analyses. Only after proper decondensation the DNA probe is able to penetrate and hybridize to the DNA of the specimen. The denaturation of DNA, both of the probe and of the target cells, concedes the annealing of the first to the complementary sequences of the latter.

DNA Denaturation

Different denaturation methods are used, based on the reducing quality of DTT. Several buffers and exposure times have been reported, and the methodological approach occasionally varied per sample (for example time of digestion, time of denaturation prior to ISH) in order to obtain optimal results (Coonen *et al.*, 1991; Robbins *et al.*, 1993).

Our study started from a protocol that was based on exposure of the sperm samples to DTT during a carefully assessed denaturation time (Coonen *et al.*, 1991). This crucial timing involved the use of time sequences of the reducing agent and was too much time consuming to be useful for a routine analysis. The exposure to 25 mM DTT and 0.1 M Trypsin resulted in a partial loss of morphology and of the tail. The dilution of DTT in a TRIS/HCl buffer gave a compact morphology, but the pH of the buffer strongly influenced DTT's reducing efficiency. Our experiments revealed a more compact head structure the higher the pH of the DTT containing buffer was (in a range that varied from pH 6.0-9.5) (data not shown). Skipping of the enzymatic treatment of spermatozoa resulted in a retainment of the tails. At a pH of 9.5, where DTT is almost completely ionized, 5 min of exposure at room temperature was enough for a proper decondensation of the DNA, without an excessive swelling of the sperm head. The hybridization efficiency in single target ISH showed to be 98.7% (in a range from 98.0% to 99.3%) with this decondensation protocol. In conclusion, this decondensation method keeps the morphology of the whole spermatozoon intact and results in efficient DNA hybridization.

Figure 1. (magnification for Figures a,b,d,e,f,g,h,i: 1000X; Figure.c: 2000X) (a.) Single-target ISH with a biotinylated probe for chromosome 1, detected with the DAB reaction. The arrow indicates a spermatozoa with 2 ISH signals for chromosome 1. (b.) Double-target ISH with a biotinylated probe for chromosome X and a FITC-labelled probe for chromosome Y. Detected with the Fast-Red and the TMB reactions, respectively. (c-d.) Triple-target ISH with a biotinylated probe for chromosome 1, a digoxigenin-labelled probe for chromosome 7 and a FITC-labelled probe for chromosome 17. Detected with DAB, Fast-Red and TMB, respectively. In Figure 1c the arrow indicates the tail. (a-d.) Counterstaining with haematoxylin. (e-g.) Single-target ISH with a 1-bio probe, detected with DAB. The arrow in (f.) indicates a spermatozoa with 2 ISH signals for chromosome 1. The arrow in (g.) indicates a morphologically abnormal sperm cell, with two heads, two signals, two midpieces, but one tail. (h.) Single-target ISH with 1-bio probe, detected with TMB. The arrow indicates a morphologically abnormal sperm cell with one head but with two ISH signals and two tails. (i.) Somatic cell and double-target ISH with a X-bio probe and FITC-labelled probe for chromosome Y, detected with DAB and TMB, respectively. (e-i.) Counterstaining with Diff-Quik. For colour illustration see page 113.

Brightfield microscopical analyses

The different and partly contrasting ISH results on sperm cells published so far can be explained by the complexity of the FISH procedure, by signal interpretation as well as the morphological evaluation (West *et al.*, 1989; Coonen *et al.*, 1991; Guttenbach and Schmid,

1994; Martin *et al.*, 1993; Robbins *et al.*, 1993; Miharu *et al.*, 1994). In particular the proper combination of efficient ISH with good morphology of the sperm cells is still a problem and not very well documented in the literature. Some authors do not consider and count morphologically abnormal spermatozoa, overlapping spermatozoa and spermatozoa without an hybridization signal (Martin *et al.*, 1993; Bischoff *et al.*, 1994). This hampers a correct evaluation as much as a controversial signal interpretation does, because aneuploid spermatozoa are not easily distinguishable from abnormal spermatozoa (two tails, but one head or vice versa), or from diploid somatic cells (see Figures 1a,f-i), especially in fluorescent ISH.

Our data show an aneuploidy rate for chromosome 1 of 0.69%, with a range that varies from 0.40% to 1.40%. This value fits in the range of the published data, where the detected aneuploidy rate with the centromeric probe for chromosome 1 is in the range from 0.06% to 0.96% (Holmes and Martin, 1993; Pang *et al.*, 1994). The percentages of morphologically abnormal spermatozoa (2 heads one tail or viceversa) were in between 3.71% and 8.21%, with an average value of 5.68%. This high value of morphologically abnormal spermatozoa can now reliably be excluded from aneuploidy analysis.

The exposure to a reducing agent and detergents leads to swelling of the sperm head. In a single color probe system, disomic and diploid sperm nuclei will produce both two signals for particular chromosomes. In spermatozoa Carothers and Beatty (1975) and Miharu *et al.* (1994) observed no correlation between cell size or morphology at the one hand and ploidy levels on the other. As a result it is not feasible to differentiate a monosomic spermatozoa with a split signal from a disomic or a diploid one on basis of nuclear size with a single target ISH. Secondly, if the tail is lost, it is extremely difficult to separate sperm cells from somatic cells on basis of morphological criteria. Diploid somatic cells are normally present in an ejaculate and are not completely removed during washing steps designed to discard seminal plasma, which are applied before fixing the samples. These cells can contribute to an erroneous estimation of the ploidy level, or to interpretation problems between different groups, also if care is taken in scoring of the samples (Holmes and Martin, 1993; Miharu *et al.*, 1994). Some authors have tried to solve this problem with triple-target FISH (Williams *et al.*, 1993). Such a procedure with the sex chromosomal probes and one autosomal DNA probe permits the easy detection of diploid somatic cells. Although multiple target FISH is feasible, the interpretation of the signals, in relation to the morphological recognition of the single spermatozoa, is difficult and therefore brightfield microscopy with multiple color detection procedures is to be preferred.

Moreover the FISH signals, though localized and bright, can be at different levels of focus, due to the size of the interphase nuclei (Miharu *et al.*, 1994), and proper focusing is therefore needed. Added to a splitting of the signal, an inhomogeneous fluorescence intensity, and minor binding sites of the signals, this can lead to misestimations. Furthermore, fading of the fluorescent signals requires a rapid evaluation.

The absorption protocol for multiple ISH is routinely used in our lab for the analysis of numerical and structural genomic aberrations of somatic cells (Speel *et al.*, 1994). It is based on immunocytochemical and enzymatic detection reactions, that allow the evaluation of ISH results with a brightfield microscope. This has the advantages of avoiding fading of the signal, partly solves the focusing problem, gives well localized signals and the procedure is relatively easy to perform. We used different enzymatic detection reactions in combination with different substrates, which resulted in stable precipitates with contrasting colors. The use of these different detection colors, allows a correct evaluation of the signals in single-, double- and triple-target ISH reactions.

Counterstaining

The time of counterstaining proved to be a crucial point in case of brightfield microscopy. If this was done too long, the ISH signal was not distinguishable from the general counterstaining, because of the mixing of the different colors. On the other hand, if counterstaining was too weak, no correct morphological evaluation of the single spermatozoa was feasible. The presence of the tail was essential for correct evaluation of the single spermatozoa, especially in case of morphologically abnormal spermatozoa. It is important to be able to distinguish an abnormal spermatozoa (with two heads, two midpieces but one tail or two tails but one head) from a diploid somatic cell or from a disomic spermatozoa itself (see Figures 1a, 1f-i). The cytoplasmatic component of the Diff-Quik protocol stains the membrane that covers the whole spermatozoon. Human sperm has a relatively small amount of cytoplasm, and therefore the head piece is less stained than the tail or than somatic cells, thus allowing a reliable estimation of the aneuploidy rate. Some problems regarding its usefulness in abnormal sperm cells persist, however, for example when the tail is single but thick, when the ISH spot is double but two midpieces and two head membranes are recognizable or when one sperm head shows two tails. In these cases the single sperm has to be classified on basis of morphological criteria. Our procedure makes it possible to discriminate between morphologically abnormal or immature spermatozoa on one hand, and aneuploid mature spermatozoa on the other.

Our new approach will be applied to evaluate the cytogenetic abnormalities in sperm samples of normal, healthy donors of proven fertility in single target ISH with a repeated probe for the telomeric region of chromosome 1, and a double target ISH using centromeric probes.

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Chapter 3

Constitution of semen samples from XYY and XXY males as analyzed by in situ hybridization

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Abstract

A brightfield microscopical in situ hybridization (ISH) technique was applied to semen samples of two 47,XYY males, one 46,XY/47,XXY male having fertility problems, and two normal 46,XY men, who served as controls. The use of a standardized nuclear DNA decondensation method, together with double-target ISH and morphological staining, allowed an accurate study of the sex chromosomal content and morphology of spermatozoa. In the males carrying an extra sex-chromosome, we detected X- and Y-bearing spermatozoa in a ratio which did not differ significantly from the 1:1 ratio found in normal males. Aneuploidy for the sex-chromosomes was found in approximately 15% of the sperm cell population of both XYY males and in 3% of the XXY male. The most striking finding was the relatively low percentage of sperm cells in these patients, with an average of 65% in the XYY males and 84% in the XXY male. The other cells represented immature germ cells (IGC), like spermatogonia and spermatocytes arrested at various stages of spermatogenesis. Apparently, in XYY or XXY men, these IGC are shed into the semen to an increased extent as compared to normal, fertile men. The sex chromosome constitution of these IGC was heterogeneous. However, the finding that the majority of spermatozoa in semen of 47,XYY and 47,XXY males carried a single sex chromosome, strengthens the hypothesis that a 46,XY germ cell line must be present, apparently with a proliferative advantage over the 47,XYY or 47,XXY cells.

Introduction

The estimated incidence of chromosomal abnormalities in cells of subfertile men is 4% to 5% compared with 0.5% to 0.7% in the general population. It has been found that up to 11.9% of azoospermic- and 4.4% of oligospermic patients carry a constitutional chromosomal abnormality (Harari *et al.*, 1995). In a study done on 496 infertile males an overall incidence of 7.1% constitutional abnormalities suggests that a selection criterion based on chromosome studies as routine analysis of infertile men might be advisable (Retief *et al.*, 1984).

The most frequent anomalies found in subfertile males are sex-chromosome aneuploidies, predominantly 47,XYY and 47,XXY.

Virtually all cases of XYY are due to paternal meiosis II nondisjunction. The frequency of XYY conceptuses and liveborns (0.1%) coincides with the observed frequency of YY sperm in ejaculates of normal males (0.06 - 0.11%) (Rothwel, 1983; Bischoff *et al.*, 1994). The first fluorescent quinacrine staining on testicular biopsies of XYY males was performed in 1971 by Hulten and Pearson. In 1988, Benet and Martin analyzed 75 sperm complements of one 47,XYY male by karyotyping and observed no increase in the percentage of spermatozoa with sex-chromosome abnormalities. In 1994 fluorescence in situ hybridization (FISH) was applied to spermatozoa of a 47,XYY patient (Han *et al.*, 1994). The ratio of X- and Y-bearing spermatozoa appeared not to differ from the normal 1:1 ratio (Han *et al.*, 1994; Benet and Martin, 1988). The offspring of 47,XYY men does not show an increased frequency of congenital abnormalities, nor a higher frequency of 47,XYY or 47,XXY children (Stoll *et al.*, 1979).

The 47,XXY karyotype is associated with the Klinefelter Syndrome (KS), and is the most common form of male hypogonadism. It occurs in 1 out of 600 new-born males, and arises from maternal or paternal nondisjunction at meiosis. Jacobs *et al.*, (1988) found no effect of parental age on the conception of 47,XXY males of paternal origin, but a very marked association between increasing maternal age and 47,XXY males of maternal origin. Although males with KS are usually sterile, patients with mosaic karyotypes may have tu-

bules consisting of cells with a normal chromosomal constitution that produce spermatozoa. Cozzi *et al.*, (1994) studied 543 sperm karyotypes of an XY/XXY mosaic patient and found a significantly increased incidence (0.9%) of hyperhaploid 24,XY chromosome sets, in absence of the expected corresponding hypohaploid cells. The results of Cozzi *et al.*, (1994) support the observations of previous studies (Berthelsen *et al.*, 1981; Vidal *et al.*, 1984; Speed *et al.*, 1991) which indicated that 47,XXY cells may be able to undergo meiosis and produce 24,XY spermatozoa. These results are supported by a recent study of Chevret *et al.*, (1996), where FISH analysis was performed on semen of a 46,XY/47,XXY mosaic man, and a significantly increased frequency of hyperhaploid 24,XY spermatozoa was found, suggesting that a few 47,XXY germ cells might be able to produce mature spermatozoa.

This study was performed to investigate the morphology and chromosomal constitution of cells present in the semen of infertile men carrying an extra sex chromosome. The ISH procedure is based on an enzymatic-cytochemical detection procedure, and resulted in a stable and non-fading precipitate that can be analysed by brightfield microscopy. The use of Diff-Quik in combination with haematoxylin as a morphological stain allowed the discrimination between morphologically normal and abnormal spermatozoa, as well as IGC (Martini *et al.*, 1995b).

The combination of an efficient ISH method with an adequate morphologic staining procedure for sperm cells has been introduced recently (Martini *et al.*, 1995a). Until now, most authors did not take the morphological criteria into account when analyzing aneuploidy in semen. As a result, abnormal spermatozoa (e.g. double heads with one tail) or overlapping spermatozoa could have been confused with somatic cells or diploid sperm cells (Martin *et al.*, 1993; Bischoff *et al.*, 1994; Wyrobek *et al.*, 1994). Therefore, in our opinion, a correct evaluation of the aneuploidy rate is hampered in assays where morphological parameters are not incorporated.

Materials and methods

Case histories

Case 1 concerns a 29 years old man, who was referred for intracytoplasmic sperm injection (ICSI) because of oligoasthenospermia. Lymphocyte karyotyping was done, and showed a 47,XYY constitution in all cells. Before an ICSI appointment was made, his wife conceived on natural way.

Case 2 concerns a 33 years old man, who was referred for ICSI because of severe oligospermia. As part of the clinical work-up for ICSI, karyotyping was done, and revealed a 47,XYY constitution in all cells.

Case 3 is a 45 years old man, who was referred for ICSI treatment because of previous diagnosis as a 46,XY/47,XXY mosaic. After FISH analysis of 200 peripheral blood cells, 5 cells with an XXY constitution were detected.

Control samples were obtained from 2 men with a normal 46,XY karyotype.

Semen specimens were analyzed using WHO (1993) criteria for concentration and motility, and Kruger's strict criteria for morphology analysis (Kruger *et al.*, 1988). The analysis showed the semen of the patients to have a concentration of $2 \times 10^6/\text{ml}$, $3.6 \times 10^6/\text{ml}$ and $11.8 \times 10^6/\text{ml}$ respectively for case 1, 2 and 3. Motility percentages were 59%, 50% and 1% respectively for the three cases while the semen of the control men was within the normal value range.

All three patients and the two healthy volunteers gave written consent for the semen samples to be used for research purposes.

Preparation of Sperm Nuclei

After routine semen analysis, sperm from patients and controls was prepared for the ISH procedure as described below.

Sperm samples were washed 3 times in phosphate buffered saline (PBS), pH 7.4, and centrifuged at 1200 rpm for 10 min, and then fixed in methanol: acetic acid (3:1). The specimens were stored at -20°C until slide preparation. Sperm cells were cytospun on poly-L-Lysine coated slides and were kept at +4°C for 7-10 days. Then the slides were washed in 2x standard saline citrate (SSC) and incubated for 5 min in 1 M TRIS buffer, pH9.5, containing 25mM dithiothreitol (DTT) (Martini *et al.*, 1995a). After decondensation the slides were washed once in 2xSSC, once in PBS and finally dehydrated (Jones *et al.*, 1987).

DNA Probes and Labelling Procedures

For the detection of the centromeric region of the X-chromosome, we used the repetitive DNA probe pBamX5, 2.0 Kb (Oncor, Gaithersburg, MD) and for detection of the Y-chromosome, the repetitive DNA probe DYZ1, 3.4 Kb (Cooke *et al.*, 1982). The probes were labelled with biotin-11-dUTP (Enzo Diagnostic, New York, NY) or fluorescein-12-dUTP (Boehringer, Mannheim, Germany) in a standard nick-translation reaction following the manufacturer's instructions.

ISH Procedure

Double target ISH with the biotinylated centromeric probe for the X-chromosome and the FITC-labelled probe for the Y-chromosome was performed. The probes were used at a concentration of 0.4 ng/ μ l. Hybridization and detection procedures were performed as described previously (Martini *et al.*, 1995a). Briefly, after overnight hybridization, the biotinylated probe was detected with avidin-peroxidase (AV-PO, 1:50; DAKO A/S, Glostrup, Denmark), followed by the diaminobenzidine reaction (DAB, Sigma, St. Louis, MO, USA). The slides were then incubated for 10 min at room temperature in 0.01N HCl to inactivate peroxidase activity. Subsequently, the fluoresceinated probe was detected with mouse anti-FITC antibodies (1:500; DAKO A/S), and peroxidase-conjugated rabbit anti-mouse IgG (1:80; DAKO A/S). After this last incubation step, the peroxidase-tetramethylbenzidine (TMB, Sigma, St. Louis, MO, USA) reaction was performed (Speel *et al.*, 1994).

Counterstaining and embedding

The slides were first dehydrated, then briefly counterstained in haematoxylin, again dehydrated and finally counterstained with Diff-Quik (DADE AG, Dudingon, CH) as described before (Martini *et al.*, 1995b). We utilized exclusively the cytoplasmic staining step of the Diff-Quik staining procedure, which consists of Eosin G in phosphat buffer, pH6.6 (1.22 g/l; solution 1).

Embedding was accomplished with Entellan (Merck, Darmstad, Germany), and slides were stored at +4°C until evaluation.

Microscopy

The ISH signals were evaluated with a standard Zeiss brightfield microscope. Microphotographs were made on a Zeiss Axiophot microscope, using a Kodak Color Gold 100 ASA film, in combination with blue and magenta filters.

Evaluation criteria

After ISH and haematoxylin/Diff-Quik staining, the slides were used to evaluate sperm

morphology and their sex chromosome constitution. Spermatozoa showing one head and one tail were defined as morphologically normal. The morphological evaluation according to Krugers strict criteria was not possible after ISH, due to the exposure of the spermatozoa to DTT prior to ISH. Likewise, morphologically abnormal spermatozoa, characterized by 2 heads and 1 tail, or vice versa, or showing any other major morphological abnormality were recognized as abnormal, but could not be evaluated properly for their chromosome content (Martini *et al.*, 1995a).

The big, round cells, without tail and brightly stained with the cytoplasmatic component of Diff-Quik, seen in this study are scored as immature germ cells (IGC). Although it cannot be excluded that these cells are somatic cells, it is very unlikely because in routine semen analysis of these samples, the amount of epithelial cells is comparable to normal semen samples, which is very low. A high amount of small, round and brightly coloured cells, which did not show ISH signals were not included in the evaluation.

Results

The results of the routine semen analysis and ISH of all samples are presented in table 1. The average ISH efficiency of all samples was 97.3%. X- and Y- bearing spermatozoa were present in the regular 1:1 ratios in all the samples analyzed. The results of the routine analyses and ISH of control semen specimens were all within the normal range.

Table 1. Constitution of semen samples of controls, XYY and XXY males.

	Control 1 XY	Control 2 XY	Case 1 XYY	Case 2 XYY	Case 3 XXY mosaic
cells scored	2820	2993	3300	3500	3800
ISH Efficiency (%)	99.4	99.4	94.5	97.3	98.7
Morph. Normal Sperm (%)	100	99.8	58.6	66.7	79
Morph. Abnorm. Sperm (%)	0	0.1	3.4	3.3	5.0
IGC (%)	0	0	38	30	16

The most revealing finding in the patients' semen samples was that next to mature spermatozoa, showing a normal morphology after ISH, a population of large, round cells was seen, which we have interpreted as IGC (Figure 1b,c). These cells were present in a substantial proportion: in case 1, 38% of cells were classified as IGC, in case 2 we found 30%, and in case 3, 16% IGC. The percentage of IGC in the control semen samples was only 0.02%.

The ISH analysis for sex chromosome constitution in spermatozoa and IGC are given in table 2 and examples of ISH staining results are depicted in Figure 1. In control samples 98.4% of spermatozoa carried either one X- or Y- chromosome and 1.1% were aneuploid. For the patients the frequency of aneuploid cells ranged from 4% to 15%, with the highest percentage in both the 47,XYY males (Figure 1d, e, f, g).

Morphologically abnormal sperm was present in the patients in a range of 3% to 5% (Figure 1h,i), while in control samples only 0.13% of spermatozoa was classified as being morphologically abnormal.

Table 2. Sex chromosome content of sperm and IGC of control, XYY and XXY males* as analyzed by ISH

Freq. (%)	Control 1 XY		Control 2 XY		Case 1 XYY		Case 2 XYY		Case 3 XXY- mosaic	
	Sperm	IGC	Sperm	IGC	Sperm	IGC	Sperm	IGC	Sperm	IGC
X	51.3	0.0	49.0	0.0	43.5	6.7	45.4	7.9	46.7	10.6
Y	47.7	0.0	49.5	0.0	37.5	6.6	35.0	7.6	43.5	5.7
no signal	0.6	0.0	0.6	0.0	8.2	-	3.8	-	1.5	-
XY	0.4	0.0	0.4	0.0	2.3	36.2	5.4	28.1	1.3	40.8
XYY	-	0.0	-	0.0	0.8	24.4	0.6	39.3	-	10.7
XXY	-	0.0	-	0.0	-	5.9	-	2.0	-	10.0
XX	-	0.0	0.1	0.0	2.0	9.3	2.7	4.9	0.5	8.8
YY	-	0.0	0.2	0.0	0.8	4.5	2.3	6.6	0.7	8.3
XXYY	-	0.0	-	0.0	0.1	5.2	0.1	1.6	-	5.5
XYYY	-	0.0	-	0.0	-	1.2	-	2.0	-	-
total aneuploid	0.4		0.7		6.0		11.1		2.5	

* Only the chromosome constitution of apparently normal spermatozoa and IGC was evaluated. The morphologically abnormal sperm cells in these preparations could not be analyzed reliably for their chromosome content.

Discussion

Based on ISH, combined with a morphological staining procedure, a distinction between morphologically normal sperm cells with a normal sex-chromosome constitution on the one hand, and morphologically and/or chromosomally abnormal spermatozoa, somatic cells and immature germ cells (IGC) on the other hand is possible (Martini *et al.*, 1995a). We have applied this technique to the semen samples of three patients with sex chromosome abnormalities and two normal males in order to achieve a reliable estimate of the fraction of normal sperm cells in the semen of these patients. For this purpose we have assessed the proportion of morphologically as well as chromosomally abnormal sperm cells.

Summarizing our findings we can state that (i) the estimated risk of abnormal chromosomal complement in morphologically normal spermatozoa is 14.5% in XYY and 4% in XXY patients (depending on the percentage of mosaicism); (ii) the incidence of IGC in semen samples of patients with XYY and XXY-mosaic are higher than in normal controls; and (iii) further studies are needed to identify the stages of spermatogenesis, where chromosomally abnormal germ cells are segregated.

Several previous studies showed that 47,XYY males indeed present this sex chromosome constitution in all their somatic cells, but that a 46,XY/47,XYY mosaicism is found in testicular biopsies (Tettenborn *et al.*, 1970; Berthelsen *et al.*, 1981; Han *et al.*, 1994). XYY cells can have difficulties in completing spermatogenesis, resulting in diminished sperm production (Speed *et al.*, 1991). The offspring of 47,XYY men is mostly 46,XY (Stoll *et al.*, 1979), which is in agreement with the results obtained from karyotyped sperm cells (Benet and Martin, 1988), sperm cells analysed by means of FISH (Han *et al.*, 1994) and also with the data of the present study, where no real shift in the X:Y ratio was apparent. In our study the evaluation of the sex chromosomal constitution of the sperm cells in both XYY patients gave comparable results and revealed that the majority of cells was normal for their sex-chromosome constitution, however, the data might indicate a slight shift in favour of the X-bearing spermatozoa, but our results are too small to perform a reliable statistical analysis. FISH analysis on spermatozoa of a 47,XYY man did not show any major abnormality or aneuploidy (Han *et al.*, 1994). In contrast, in our study, high percentages of the IGC

Figure 1. Diff-Quik and Haematoxylin stained semen preparation to which double-target ISH was applied with an X-probe (brown) and a Y-probe (green). (a) Control sample showing an XX-bearing spermatozoon. (b, c) Immature germ cells from an XYY male (b) and an XXY male (c) showing a XYY- and XY- constitution respectively. Morphologically normal spermatozoa from an XXY male (d, e) and an XYY male (f, g) showing a normal sex-chromosome constitution and YY constitution respectively. (h, i) Morphologically abnormal spermatozoa from the XXY patient. (Magnification x 1000). For colour illustration see page 114.

carried an XY, XYY, XX or YY constitution. This supports the hypothesis that the extra Y-chromosome is lost during spermatogenesis and that the 46,XY germ cell epithelium has a proliferative advantage as compared to the 47,XYY germ cell line (Berthelsen *et al.*, 1981). Studies of spermatogenesis in XYY male confirm the tendency for the germ cells to lose the second Y chromosome but for some XYY cells to reach meiosis I. The degenerate category of pachytene and meiosis I spermatocytes seen in the study of Speed *et al.*, (1991) represent germ cells with an X univalent or cells with an XY+Y genotype, where the presence of an Y univalent has disrupted cell progression. Probably, spermatogonia and spermatocytes with a 47,XYY constitution are shed into the seminal fluid and detected as IGC in our study. Indeed, the ISH analysis on IGC confirm a high frequency of combined XY and XYY constitution. Also in the study of Han *et al.* (1994), a high proportion of IGC and somatic cells are found, though not histologically or cytogenetically confirmed. It might be obvious that a distinction between different stages of germ cell development and somatic

cells is important for future ISH studies on spermatogenesis and for semen diagnosis, and additional identification methods are thus needed (Wyrobek *et al.*, 1994).

47,XXY men are usually azoospermic, but patients with an XXY/XY mosaicism occasionally may show seminiferous tubules, consisting of cells with a normal chromosomal constitution, that produce spermatozoa (Harari *et al.*, 1995). The XY/XXY patient studied, showed the XXY cell line in a very small proportion of cells in peripheral blood and the semen contained a lower percentage of IGC (16%) than the semen samples of the XYY patients. This lower percentage of IGC concurs with the explanation of Terzoli *et al.*, (1992), who found that KS patients are fertile when mosaicism is confined to testicular tissue. Studies in patients with a mosaic 46,XY/47,XXY constitution suggest that only normal XY-germ cells can complete meiosis (Luciani *et al.*, 1970; Vidal *et al.*, 1984). Cozzi *et al.*, (1994) and Chevret *et al.*, (1996) using sperm-karyotyping the first and triple-FISH on spermatozoa the latter, found a significantly increased incidence (0.9% and 2.09% respectively) of hyperhaploid 24,XY sets in absence of the expected corresponding hypohaploidy. The results of these studies support the suggestion that some 47,XXY cells are able to go through meiosis and to form spermatozoa. This suggestion correlates with the relatively high frequency of XY bearing spermatozoa (1.3%) found in the XXY patient, as compared to the percentage found in normal males in our study (0.4%), and by Chevret *et al.* (0.36%), (1996).

Our results show that an average of 80%-90% of the morphologically normal spermatozoa of XYY- and XXY-males carry a normal sex chromosome constitution. Based on the results of the present study, the average estimated risk of morphologically normal sperm cells having an abnormal sex chromosomal content is 8.6% in the XYY and 2.5% in XXY patients, including spermatozoa cells without sex-chromosome. In this respect it should be kept in mind that our criteria to define morphologically normal cells in ISH/Diff-Quik/haematoxylin stained samples, are not the same as those used in routine semen analyses. Whenever these patients apply for artificial reproductive techniques (ART) the relatively high percentage of aneuploid spermatozoa should be kept in mind. These patients have to be evaluated individually and the risks of ART procedure should be weighted on basis of the ISH results. It is our opinion that after fertilization pre-implantation diagnosis should be performed.

Furthermore, to study the relationship between morphological appearance and genetic/chromosomal constitution of sperm cells, also cytogenetic studies should be performed. In this respect an additional approach would be to apply ISH analysis to testicular biopsies, in order to study cells with a XYY or XXY genotype, their difficulties in completing spermatogenesis. The possibility that germ cell loss in certain chromosomally abnormal situations could be explained by genetic imbalance (Speed *et al.*, 1991), and whether the stages of spermatogenesis where chromosomal abnormalities are introduced are more likely filtered out, is still a challenging area. Since the number of such studies on ejaculates of 47,XYY and 47,XXY men is still limited, more studies of larger populations should be performed.

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Chapter 4

Comparison of gonosomal aneuploidy in spermatozoa of normal fertile men and those with severe male factor detected by in situ hybridization

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Abstract

The purpose of the study was to analyse the frequency of sex-chromosome numerical abnormalities in human sperm of infertile men by using a standardized experimental protocol of double target in-situ hybridization (ISH). The experiments were performed on decondensed sperm heads from 15 infertile patients (six cases of unexplained infertility and nine cases of severe oligo-astheno-teratozoospermia). Three men of proven fertility were used as controls. The probes employed recognized the centromeric regions of human X chromosome and the long arm of the Y chromosome. In a smaller number of cases, additional experiments of double ISH were performed using centromeric probes for chromosomes 1 and 17. Signal detection was based on protocols of enzymatic cytochemical reactions. A total of 24508, 24679 and 42285 cells were scored in the control, unexplained infertility and severe male factor groups of patients, respectively. In all the patients ISH efficiency result was ~ 98%. In controls, unexplained infertility and severe male factor patients, the frequency of morphologically normal sperm cells carrying an abnormal chromosome constitution (XX or YY or XY or > 2 sex chromosomes signals) was 0.86%, 0.75% and 1.35%, respectively. The value of this last group of patients (severe male factor) was significantly higher than in the other two groups of patients ($p < 0.008$). The same findings were made using the autosomic probes. Our preliminary data support the possibility of an increased risk from paternal origin sex chromosome aneuploidies in children born after intracytoplasmic sperm injection (ICSI). Further investigation of the cytogenetic constitution of spermatozoa from severe male factor patients is warranted.

Introduction

With the advent of assisted reproduction technology (ART) and especially intracytoplasmic sperm injection (ICSI) combined with epididymal and/or testicular spermatozoa retrieval, men presenting a severely impaired spermatogenesis are currently able to be treated for infertility. However, available data from cytogenetic analysis on chromosome constitution of newborns from ICSI show that, in comparison with the general population, these liveborns may be at increased risk for sex chromosome aneuploidy (1.0% rather than 0.2%) (Liebaers *et al.*, 1995; In't Veld *et al.*, 1995). Since aneuploidy is unlikely to be the product of the ICSI procedure itself, it has been speculated that the incidence of trisomy in embryos and children born following ICSI may be related to a direct injection of disomic spermatozoa (Chandley and Hargreave, 1996). This may be of concern particularly for sex chromosomes since the vast majority of gonosomal aneuploidies in human liveborns and spontaneous abortions are the consequence of meiotic non disjunction during spermatogenesis (100% of 47,XYY males, 80% of 45,X0 females, 47% of 47,XXY males and 10% of 47,XXX females) (Lorda-Sanchez *et al.*, 1922; MacDonald *et al.*, 1994; Jacobs and Hassold *et al.*, 1995; Griffin *et al.*, 1995). Although applying data valid for normal newborns (with normal fathers), to infertile males may be questionable, from a theoretical point view, a paternal origin of gonosomal aneuploidy could be estimated in at least 54% of the ICSI-liveborns (6/11 sex chromosomes abnormalities in 506 newborns) (Liebaers *et al.*, 1995; In't Velde *et al.*, 1995).

In recent years, fluorescent in situ hybridization (FISH) has been increasingly used as an alternative to sperm karyotyping, being less laborious, less time consuming and allowing the analysis of a whole specimen in few hours. However, despite the abundance of studies using in situ hybridization (ISH), the exact aneuploidy rate for sex chromosomes in spermatozoa of normal healthy men remains controversial (Wyrobek *et al.*, 1994; Martin and Rademaker, 1995). Moreover, in spite of general concern, little is known until now in

regard to the frequency of aneuploidy in spermatozoa of infertile and OAT patients (Moosani *et al.*, 1995; Chevret *et al.*, 1995).

Our study was undertaken with the purpose to attempt a comparison between fertile and infertile men in terms of gonosomal DNA ploidy (disomy plus diploidy) in spermatozoa using an ISH approach. The ISH method was based on a protocol of immunocytochemical reactions resulting in precipitation of signal products of different colour and counterstaining of the cells with Diff-Quik (Martini *et al.*, 1995). This has allowed the correct recognition and distinction of roundish cells (germ cells, somatic cells) and overlapping spermatozoa and/or morphologically abnormal spermatozoa. Additionally, in order to provide a preliminary estimation of diploidy, experiments of double ISH were performed in a subgroup of patients using probes for autosomes 1 and 17.

Materials and methods

Semen samples

Semen samples were obtained from three healthy fertile men, used as controls (C) and 15 patients. Six out of these patients were referred to our laboratory presenting normal semen parameters, normal endocrine profiles, normal fertility work-up in their female partners and were considered cases of unexplained infertility (U). Three patients of this group underwent ART treatment. Medical records showed for two of them a previous episode of spontaneous abortion. In two cases, despite the achievement of normal in vitro fertilization (IVF) rates (17/25 and 8/12) and embryos available for cryopreservation (12/17 and 3/8), no pregnancy ensued. For the third patient, two successive gamete intra-fallopian transfer (GIFT) cycles were attempted resulting in a biochemical pregnancy on both occasions. Nine other infertile patients were cases of severe oligo-astheno-teratozoospermia (O) (severe male factor patients). Informed consent was obtained from all the patients. Semen parameters and clinical profiles for the 3 groups of patients are shown in Table 1.

Table 1. Semen parameters and clinical characteristics of patients. Ranges are given in parentheses.

No. cases	age (years)	Total no. normal motile spermatozoa ($\times 10^6$)	Normal forms	Diagnosis
3	36 (33-37) ^a	77 (42-117)	> 40 (60-70)	Normal fertile controls (C)
6	34 (29-44) ^a	50 (13-122)	> 20 (45-70)	Unexplained infertility (U)
9	37(30-44) ^a	4.5 (0.01-20)	> 20 (0-20)	Oligoasthenoteratozoospermia (O)

a: Not significant

Sperm nuclei preparation

Semen samples were routinely analyzed at the Andrology Laboratory of our hospital. Sperm cell count, motility and morphology were determined and total normal motile count (TNMC) was estimated for each pre-processed sample (total count $\times$ % of motile $\times$ % normal forms) (Ord *et al.*, 1993). Sperm preparation prior to ISH followed the protocol of Martini *et al.* (1995). Samples were washed 3 times in phosphate buffered saline (PBS, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.2) and centrifuged at 280g for 10 minutes. The pellet was then resuspended carefully in 1 ml of fresh, cold fixative (methanol : acetic acid, 3:1) and stored at -20°C. Sperm cells were cytospun on poly-L-lysine coated slides, washed in 2 x SSC (0.3 M NaCl, 30 mM Na citrate) to remove excess fixative and incubated for 5 minutes in 1 M Tris/HCl buffer, pH 9.5, containing 25 mM dithiothreitol (DTT). After decondensation the slides were washed once in 2 x SSC, once in PBS, dehydrated through an ethanol series (70-96-96-100-100%) and air dried.

Double target in situ hybridization (ISH) procedure

The DNA probes used in this study included an alphoid probe specific for the centromeric region of the human X chromosome (pBam X5, 2.0 kb; Willard *et al.*, 1983), a satellite probe specific for the long arm of the human Y chromosome (DYZ1, 3.4 kb; Cooke *et al.*, 1982) and two other probes recognizing the (peri) centromeric regions of the human chromosome 1 and 17 (pUC 1.77, 1.77 kb; Cooke and Hindley, 1979; p17H8, 2.7 kb; Wayne and Willard, 1986). Probes were labelled by either nick translation using biotin-11-dUTP (Enzo Diagnostics; New York, NY, USA) for the X- and 1- specific probes or fluorescein-12-dUTP (Boehringer; Mannheim, Germany) for the Y- and 17- specific probes. Probes were dissolved in hybridization mixture containing 60% formamide, 2 x SSC, pH 7.0, 10% dextran sulfate, 0.2 µg/µl herring sperm DNA as carrier DNA and 0.2 µg/µl yeast t-RNA as carrier RNA. A final volume of 5 µl (corresponding to a concentration of 0.4 ng/µl for each probe) was added to each slide under a coverslip (18 x 18 mm). Denaturation was achieved at 70°C for 3 minutes and hybridization was performed for 4 hours at 37°C in a moist chamber. The slides were washed twice for 5 minutes at 42°C with 60% formamide, 2 x SSC, pH 7.0, containing 0.05% Tween 20, followed by two 5 minutes washes with 2 x SSC, pH 7.0 at 42°C and one 5 minutes wash with 4 x SSC, pH 7.0, containing 0.05% Tween 20 at room temperature. The detection of signals was performed as described previously (Speel *et al.*, 1994; Martini *et al.*, 1995). Briefly, the biotinylated probes for chromosome X or 1 were detected with horseradish peroxidase-conjugated avidin (AV-PO, Dako A/S, Glostrup, Denmark), amplified with biotinylated goat anti-avidin (BioGαA, Vector, Burlingame, CA, USA), followed by a second AV-PO layer and the diaminobenzidine (DAB) reaction. The slides were then incubated for 10 min at room temperature in 0.01 N HCl to inactivate peroxidase activity. Subsequently, the fluoresceinated chromosome Y- or 17- specific probes were detected with mouse anti-FITC (MαFITC, Dako A/S) and PO-conjugated rabbit anti-mouse IgG (RαM-PO, Dako A/S). After this last incubation step, the peroxidase-tetramethylbenzidine (TMB, Sigma) reaction was performed (Speel *et al.*, 1994). Avidin conjugates were diluted in 4 x SSC, pH 7.0, containing 5% non-fat dry milk, and the antibody conjugates were diluted in PBS containing 0.05% Tween 20 and 2% normal goat serum. After each incubation step of 20-30 min at 37°C, the slides were rinsed twice in 4 x SSC, pH 7.0, 0.05% Tween 20 (avidin conjugates) or PBS, 0.05% Tween 20 (antibody conjugates). Counterstaining was achieved by a combined use of haematoxylin and Diff-Quik (DADE s.p.a., Milan, Italy) as previously published (Martini *et al.*, 1995). We utilized exclusively the cytoplasmic staining step of the Diff-Quik consisting of Eosin G in phosphate buffer, pH 6.6 (1,22 g/l; solution 1).

Slides were embedded in Entellan (Merk, Bracco s.p.a., Milan, Italy), an organic mounting medium and stored at +4°C until evaluation. The evaluation of the ISH signals was performed with a standard Zeiss bright-field microscope. Microphotographs were obtained using a Leica CCD camera.

Scoring criteria

Sperm-slides were scored by two independent observers according to the recommendations of Wyrobek *et al.* (1994) and Martin and Rademaker (1995). Both the investigators were blinded as to the origin of the slides being analysed. Results represent the average of the values obtained by the two observers. Sperm nuclei were scored when morphologically preserved, not clumping or overlapping, with well-defined outline and tail and sperm-head decondensed to no more than twice the size of normal non-decondensed spermatozoa. The presence of the tail was considered essential for a reliable evaluation. The differentiation of the morphologically abnormal sperm cells (spermatozoa with two heads, two

midpieces but one tail, two tails but one head, micro-heads) from somatic cells or normal spermatozoa was made possible by Diff-Quik staining and their number omitted from the count of the aneuploid cells. The big, brightly stained, round cells showing efficient ISH signals were recorded as immature germ cells (IGC) (Martini *et al.*, 1996). Three main types of morphologically normal sperm cells carrying an abnormal sex chromosome constitution were observed: (i) sperm cells with both the brown and green signal-products (XY); (ii) sperm cells with two brown signal-products (XX); (iii) sperm cells with two green signal-products (YY). For spermatozoa to be recorded as XX or YY, two signals of the same color and equal size had to be located at the distance of approximately one diameter of the signal-domain. These criteria were not taken into account for sperm cells with XY signals. Sperm cells showing normal decondensation but no signals were scored and considered valid for calculations of ISH efficiency. The experimental conditions of this study did not allow to differentiate between nullisomic spermatozoa and cells with hybridization failure. Therefore, in order to minimize the subjectivity of the individual observations, our scoring values were compared with the nullisomy rates considered as theoretically equivalent to the incidence of all the aneuploidies (Blanco *et al.*, 1996). In morphologically normal spermatozoa, the evaluation of diploidy and disomy was based upon additional experiments of double target ISH for autosomes 1 and 17. This was possible, within each group, only for a smaller number of patients. An attempt at a realistic estimation of sex chromosomes disomy rate (aneuploidy) was finally achieved by subtracting the diploidy rate, obtained with double ISH for autosomes 1 and 17, from the total DNA ploidy rate for sex chromosomes (see table 2).

Data analysis

Among the three groups of patients, separate comparisons of the proportions observed for single variables (DNA ploidy, nullisomy, immature germ cells, abnormal cells) were performed using univariate statistical analysis by Chi Square test and analysis of variance (ANOVA). In addition, in order to obtain the total information from the data set test, the Principal Component Analysis (PCA) (Forina *et al.*, 1988) was applied for multivariate statistical analysis. PCA is an exploratory and display technique of the data matrix which allows: (i) to reduce the dimensionality of the problem; (ii) to find similarities between samples and/or variables; (iii) to identify directions corresponding to a property or characteristics having a biological meaning. In our case, data matrix was constituted by 18 patients (samples) and 5 variables (DNA ploidy, immature germ cells, abnormal sperm cells, morphology and total motile count). Geometrically, each individual sample in the data matrix was represented by a symbol (C or U or O) in a space of 5 dimensions (one dimension for each variable). PCA generates a set of axes called principal components. In this way, the maximal amount of variance contained in the starting data set (information) is concentrated in the first principal component. The coefficients of the original variables defining each principal component are called "loadings", while the projections of the samples on the new axes are called "scores". Data were auto-scaled (subtraction of the mean and division by standard deviation for each variable) before principal component computation in order to assign the same numerical weight to each variable (Massart *et al.*, 1988).

Results

Figure 1a shows morphologically normal sperm cells with normal hybridization signals (green for spermatozoa carrying Y chromosome and brown for spermatozoa carrying X chromosome). In figure 1b,c and d morphologically normal spermatozoa, carrying an excess of sex

chromosomes are shown (XY, XX and YY, respectively). Fig. 1e shows two different types of abnormal sperm cells (a spermatozoon with one tail and two heads and vice versa one head and two tails). In figure 1f the image of an immature germ cell (IGC) is shown.

Figure 1. Double target hybridization of semen samples with biotinylated X probe (brown) and fluorescent isothiocyanate (FITC)-labelled Y probe (green). **(a-d)** Morphologically normal spermatozoa. **(a)** Control sample showing either X or Y signal; **(b)** spermatozoa showing an XY sex chromosome constitution; **(c)** Sperm cell with an XX sex chromosome constitution; **(d)** Sperm cell with an YY sex chromosome constitution. **(e)** Morphologically abnormal sperm cells, top left with two heads and two chromosome X-signals; bottom right spermatozoon with two tails and an XY chromosome constitution. **(f)** Immature germ cell with hybridization signal for chromosomes X and Y. For colour illustration see page 115.

Table 2 lists the frequencies of morphologically normal cells with an abnormal chromosome constitution (XY, XX, YY and > 2 sex chromosomes) among the groups of controls (C), unexplained infertility (U) and severe male factor patients (O). A total of 24508, 24679 and 42285 cells were scored for C, U and O groups, respectively. Within these groups, an average of 7500, 3100 and 3900 cells were scored per patient respectively. Excluding both nullisomy and diploidy rates from the analysis, the sex chromosome disomy rate (true

aneuploidy) for the C, U and O groups was 0.46%, 0.45% and 0.82% respectively. Inter-observers differences in counting were small and similar findings were independently made by both the observers. The data shown in Table 2 and discussed in the text represent an average of the values recorded by the two observers. Total DNA ploidy and disomy frequencies for sex chromosomes among the 3 groups were significantly different (ANOVA: $p < 0.008$). In particular, the value of disomy found in the severe male factor patients (O group) was higher than in the other two groups of patients. No apparent differences in gonosomal disomy rates were noted between unexplained infertility and control patients ($p < 0.42$).

Table 2. Constitution of semen samples in controls (C), unexplained infertility (U) and oligoastheno-teratozoospermic (O) patients analysed by in situ hybridization (ISH).

Groups of patients	C	U	O
N° cells/group	24508	24679	42285
N° cells/patient	7500	3100	3900
ISH efficiency %	99.7	98.8	97.8
X:Y ratio %	49.7 : 48.9	50.2 : 48.7	49.5 : 47.1
XY %	0.45 (0.4 - 0.52)	0.42 (0.24 - 0.77)	0.70 (0.27 - 1.15) *
XX %	0.15 (0.07 - 0.22)	0.19 (0.09 - 0.30)	0.36 (0.18 - 0.48) *
YY %	0.25 (0.14 - 0.4)	0.14 (0.07 - 0.28)	0.27 (0.13 - 0.43)
> 2 sex chrom %	0.012	0.006	0.025*
DNA ploidy %	0.86	0.75	1.35
Estimated disomy % ^b	0.46	0.45	0.82

* $O > C : P < 0.0001$ and * $O > U : P < 0.0001$

b: Disomy obtained by subtraction of the diploidy rate (achieved using double ISH for autosomes 1 and 17) from total DNA ploidy (see table 3).

Similar observations were made from results achieved in the same patients for autosomal aneuploidy (Table 3).

Within all three groups inter-individual and inter-chromosome differences of DNA ploidy frequency were observed. In the control, unexplained infertility and severe male factor patient groups frequencies of nullisomic spermatozoa of 0.75, 0.67 and 1.05%, respectively, were scored. These values were not much different from that hypothetically expected on the basis of total DNA ploidy (disomy plus diploidy). Overall, the number of spermatozoa showing no hybridization was small (0.29-1.05%), emphasizing the level of hybridization efficiency.

Table 3. Cytogenetic constitution of semen samples in a subset of patients using ISH for autosomes 1 and 17.

Groups	C	U	O
	<i>n</i> = 2	<i>n</i> = 4	<i>n</i> = 4
Cells /patient	> 3000	> 3000	> 3000
Disomy 1	0.4	0.38	0.54
Disomy 17	0.35	0.37	0.74
Diploidy	0.4	0.3	0.53
Total	1.15	1.05	1.80 *

* $p < 0.001$

In figure 2, the relative proportions of DNA ploidy, abnormal spermatozoa and IGC, for each group of patients, are shown. Overall, in the severe male factor patients (O)(normal morphology < 20%), the frequencies of gonosomal DNA ploidy, abnormal spermatozoa and number of immature germ cells were higher than in the other groups of patients (chi square test: $p < 0.0001$). Within the group of severe male factor patients (O), those having the poorest semen quality (total normal motile count < 1 million)($n=3$) also had an almost 10 times higher proportion of immature germ cells (6% versus 0.6%) along with a significantly higher DNA ploidy rate for sex chromosomes (1.6% versus 1.2%)(data not shown).

Figure 2. Percentage frequency of DNA ploidy (closed bars), abnormal spermatozoa (ABN; grey bars) and immature germ cells (IGC; open bars) in control groups of patients (C; normal forms >40%), and groups of patients with unexplained infertility (U; normal forms >20%), and severe male factor (O; normal forms <20%). Significant difference between C and U groups ($p < 0.001$).

In figure 3 the results of the PCA are shown. The entire data set can be visualized simultaneously (separate presentation of individual patients according to the 5 variables). The first two principal components retained 77% of the variance (total information). The first

principal component represents the direction from the high to the low values of the DNA ploidy rates. Overall, on the PCA graph, the gonosomal DNA ploidy, presence of abnormal spermatozoa and immature cells were distributed in parallel on the area plot and displayed as opposed to the total normal motile count and normal morphology variables. A similar PCA-scatter plot was achieved also when data from double ISH for autosomes were analysed.

Figure 3. Scatter plot of the principal component analysis (PCA) of sample projections (C= control; U= unexplained infertility; O= patients with severe male factor) and coefficients of the original variable: TNM= total normal motile count; IGC= immature germ cells; ABN= abnormal spermatozoa; aneuploidy= DNA ploidy.

Discussion

In this study we applied a standardized method of double in situ hybridization to study frequency of sex chromosome aneuploidy in different populations of men. The validity of this technique is based on a protocol of immunocytochemical detection reactions allowing combination of cytogenetic analysis with a morphological evaluation. As previously reported, this offers several advantages (Martini *et al.*, 1995).

Overall, our values of DNA ploidy for sex chromosomes in spermatozoa of control (0.86%) and unexplained infertility (0.75%) individuals are within the range of those found in the literature for multiple colour FISH [0.60%; 0.29-1.06 confidence interval (CI)] (Williams *et al.*, 1993; Schattman *et al.*, 1993; Bischoff *et al.*, 1994; Chevret *et al.*, 1995; Spriggs *et al.*, 1995; Martin and Rademaker, 1995; Moosani *et al.*, 1995; Griffin *et al.*, 1995; Robbins *et al.*, 1995; Martin *et al.*, 1996a; Martin *et al.*, 1996b; Chevret *et al.*, 1996; Spriggs *et al.*, 1996). Other studies using double colour ISH, have reported, on average, lower sex chromosome DNA ploidy rates [0.45%; 0.14-0.7 CI] (Han *et al.*, 1993; Goldman *et al.*, 1994; Wyrobek *et al.*, 1994; Chevret *et al.*, 1994; Pang *et al.*, 1995; Martini *et al.*, 1996; Mercier *et al.*, 1996). Methodological differences between all these studies include the number of cases, the scoring criteria, the decondensation methods, the use of a different Y probes and the numbers of scored spermatozoa.

We are aware of the fact that our approach has implicit limitations as it cannot distinguish

either between diploid and disomic spermatozoa (46,XY or 24,XY) or between nullisomic spermatozoa and ISH efficiency failure. In order to accurately distinguish between diploidy and disomy a multi-probe FISH including a probe for autosomes would be the best approach (Martin *et al.*, 1996b). This notwithstanding, we believe that our protocol of ISH, based on a proper staining of the sperm tail combined with brightfield-microscope evaluation of permanent signals, guarantees, as compared to FISH, a more correct recognition of the normal spermatozoa among all the other sperm cells. According to the literature, baseline diploidy rate in normal human spermatozoa corresponds to 0.20 ± 0.13 out of 0.60% (total aneuploidy) (Williams *et al.*, 1993; Schattman *et al.*, 1993; Bischoff *et al.*, 1994; Chevret *et al.*, 1995; Spriggs *et al.*, 1995; Griffin *et al.*, 1995; Robbins *et al.*, 1995; Martin *et al.*, 1996a; Martin *et al.*, 1996b; Chevret *et al.*, 1996; Spriggs *et al.*, 1996). However, it remains to be determined whether, in spermatozoa of infertile men a differential contribution to the total DNA ploidy rather than disomy exists. Our preliminary results achieved using probes for chromosomes 1 and 17 suggest that this contribution should be ascribed mainly to the disomy rates. Further data from our laboratory support this concept (Bernardini *et al.*, 1996).

We attempted a comparison of the aneuploidy rate between different groups of patients estimating the relative proportions rather than providing absolute rates. Though final conclusions cannot be drawn yet, our data suggest that the frequency of DNA ploidy for chromosomes in severe OAT patients is almost twice as common as that found in normal fertile men. Results achieved using autosomic probes indicate that this is most probably, valid also for other chromosomes and suggest that in spermatozoa of OAT patients a "generalized chromosomal pairing disruption" may occur (Egozcue *et al.*, 1983).

The value of increasing the size of our control group seems questionable as this would help at the most to achieve baseline aneuploidy rates closer to those found in some of the unexplained infertility patients (0.5%), simply reinforcing the level of discrepancy between results.

In men who are partners of couples suffering from unexplained infertility, the chances of finding gonosomal aneuploidy increased above the baseline rates seem reduced. However, this is a heterogeneous group of men including perhaps some who are completely healthy (misclassified due to failure of female fertility work up) as well as, at the other extreme those who carry specific types of sperm aneuploidy. In our study, few probes were analysed in a small sample size of patients, so that final conclusions on this regard are definitely hampered.

Other investigators also have reported the frequency of aneuploidy in sperm cells of infertile men. Mihar *et al.*, (1994), using FISH, did not find an increase of gonosomal disomy in a group of 12 infertile men. However, the patient population of this study was quite heterogeneous and the utilization of a single-probe FISH did not allow the determination of the number of spermatozoa carrying a XY content. In the experience of our own group and that of others, this type of hyperhaploid spermatozoa is prevalent. A significant increase of disomic spermatozoa was also found in 4 out of 5 infertile patients described by Moosani *et al.*, (1995). In the same study, the comparison of sperm karyotyping results achieved in 12 sperm donors and 5 infertile patients yielded a conservative estimate of total aneuploidy of 0.84% and 3.1%, respectively (no changes were seen in terms of structural chromosomal abnormalities).

Very likely, chromosome aneuploidy increases in cases with greater severity of infertility. Pang *et al.*, (1995) studying sex chromosomes aneuploidy frequencies by FISH in 9 severe male factor patients (normal morphology < 4%) in comparison with 4 normal men, found a 10 fold difference (5.0% versus 0.5%, respectively).

Our data also suggest a positive correlation between aneuploidy and the number of morphologically abnormal spermatozoa and immature germ cells observed in cases of decreasing sperm quality. This agrees with the finding of high percentages of immature germ cells (28%) and normal spermatozoa carrying abnormal sex chromosomal content in semen of XYY and XXY males (8.6% and 2.5%, respectively) (Martini *et al.*, 1996). While these patients are well known to be generally affected by severe oligozoospermia, much less is known about the chromosomal constitution of their spermatozoa. The supposition is of a prevalent population of 24, XY disomic cells (Mercier *et al.*, 1996; Chevret *et al.*, 1996). It has been suggested that, in some cases, the phenotype of azoospermic men may be normal, while a mosaicism confined to the germ tissue and leading to aneuploid cell lines may occur (Persson *et al.*, 1996). However, even when the karyotype is normal, as in the patients analysed in this study, an elevated frequency of aneuploid spermatozoa still occurs. This finding agrees with the concept that alterations of the recombination and/or pairing processes might concomitantly lead to a higher rate of aneuploidy along with the decreasing quality of semen (Hassold *et al.*, 1991; Martin, 1996c).

In conclusion, our data show a significantly increased aneuploidy rate for sex chromosomes in OAT patients compared with men with normal semen parameters. These data should be taken into account, and relative risks estimated, whenever an OAT patient is a candidate to undergo ICSI.

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Chapter 5

Detection of structural abnormalities in spermatozoa of a translocation carrier t(3;11)(q27.3;q24.3) by triple FISH

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Abstract

Structural chromosome abnormalities in spermatozoa represent an important category of paternally transmittable genetic damage. A couple was referred to our centre because of repetitive abortions and the man was found to be a carrier of a reciprocal translocation $t(3;11)(q27.3;q24.3)$. A tailored fluorescence in situ hybridization (FISH) approach was developed to study the meiotic segregation patterns in spermatozoa from this translocation carrier. A combination of three DNA probes was used, a centromeric probe for chromosome 11, a cosmid probe for chromosome 11q, and a YAC probe for chromosome 3q. The frequency of spermatozoa carrying an abnormal chromosome constitution was compared with baseline frequencies in control semen specimens, and it was found that a significantly higher percentage of spermatozoa carried an abnormal constitution for the chromosomes involved in the translocation. A normal or balanced chromosome constitution was found in 44.3% of the analyzable spermatozoa, while the remainder exhibited an abnormal chromosome constitution reflecting different modes of segregation (15.9%, adjacent I segregation; 6.5%, adjacent II segregation; 28.9%, 3:1 segregation; 0.8% 4:0 segregation; 3.6%, aberrant segregation). The frequency of aneuploidy for chromosomes X, Y, 13 and 21 was assessed using specific probes but there was no evidence of interchromosomal effects or variations in the sex-ratio in spermatozoa from the translocation carrier. In conclusion, structural aberrations can be reliably assessed in interphase spermatozoa using unique DNA probe cocktails, and this method provides insight into the genetic constitution of germ cells and enables evaluation of potential risks for the offspring.

Introduction

Balanced chromosome translocations result from an exchange of fragments between two non-homologous chromosomes, without any gain or loss of genetic material. Translocation carriers, although they do not exhibit any particular phenotypes, are often affected by reproductive failures, either due to spermatogenic arrest or to unbalanced progeny. Indeed, during meiosis I, the association of both translocated chromosomes and their normal homologues can lead to an unbalanced pattern of segregation for these chromosomes. Statistical data from imbalances observed at birth have been applied to determine segregational models for the assessment of reproductive risks (Jalbert *et al.*, 1980; Cans *et al.*, 1993). Studies on liveborn offspring or fetuses do not provide accurate information about meiotic segregations, as lethal segregations have already been lost through spontaneous abortions, hence the true mechanisms responsible for determining how structural rearrangements segregate remains unknown. In addition, an increase of aneuploidies involving chromosomes unrelated to the translocation, termed an "interchromosomal effect", has also been suggested, though never proven (Aurias *et al.*, 1978).

To date, 30 different reciprocal translocations in men have been studied by karyotyping sperm after penetration of zona-free hamster oocytes (see Rousseaux *et al.*, 1995; Estop *et al.*, 1995 for review of literature). High baseline frequencies (5-10%) of structural abnormalities were reported for human spermatozoa (Martin *et al.*, 1987; Estop *et al.*, 1991), however, 90% of the observed aberrations were unstable chromosome and chromatid aberrations suggesting high degrees of post-meiotic damage or possibly artefacts of inter-species fertilization (Martin 1992). Aneuploidy was estimated to be 1-2% and hypoploidy was observed at higher frequencies than hyperploidy (Martin and Rademaker 1990). However, this technique is time consuming, labor intensive and does not allow analysis of large numbers of cells (Martin and Rademaker 1990).

In situ hybridization (ISH) and fluorescence ISH (FISH) have been used to study sperm chromosomes in single, double and triple target procedures (see Downie *et al.*, 1997 for

review). This technique is limited to the detection of abnormalities for the specific chromosomes analyzed, but it allows rapid screening of many spermatozoa. Only few studies exist on men carrying a numerical chromosomal abnormality, like XYY and XXY karyotypes (Han *et al.*, 1994; Martini *et al.*, 1996; Mercier *et al.*, 1996; Guttenbach *et al.*, 1997; Chevret *et al.*, 1997) and on men carrying a structural chromosomal abnormality (Lu *et al.*, 1994; Spriggs and Martin 1994; Rousseaux *et al.*, 1995; Mercier and Bresson 1997). These studies were all based on the detection of meiotic segregation modes using centromeric probes.

The present paper reports a specific FISH approach, developed in order to simultaneously investigate the frequencies of structural and numerical chromosome aberrations in spermatozoa of a translocation carrier $t(3;11)(q27.3;q24.3)$. Two probes specific to DNA loci on the segregational fragments (a cosmid probe for chromosome region 11q25 and a YAC probe for chromosome region 3q29) and a centromeric probe for chromosome 11 were hybridized simultaneously. Furthermore, the sex ratio and aneuploidy rate for the sex-chromosomes was assessed, and the frequency of aneuploidy for chromosomes 13 and 21 was studied in order to exclude interchromosomal effects.

Materials and Methods

Case history

Spermatozoa from a 33-year-old man who carries a reciprocal translocation $t(3;11)(q27.3;q24.3)$ were studied. His sister, mother and maternal grandmother were also carriers of the same translocation. His wife had at least seven early miscarriages. In the fifth pregnancy an amniocentesis was performed at 16 weeks of gestational age. The karyotype of the foetus was abnormal: 46,XX,-11,+ der(11), $t(3;11)(q27.3;q24.3)$, resulting in partial monosomy 11q and partial trisomy 3q. On request of the parents the pregnancy was interrupted. A stillborn girl with multiple congenital anomalies was delivered. The younger sister of the proband also had several spontaneous abortions. Her third pregnancy ended at 24 weeks with the birth of an hydropic foetus (Willekens *et al.*, 1994). A Parvovirus B19 infection and an unbalanced karyotype were found in the abortion material. Reproductive history of the mother and the grandmother of the proband were unremarkable, with no miscarriages or stillbirths known. Both couples asked for a risk evaluation in using their own gametes and pre-implantation diagnosis of embryos.

The study was approved by the Research Ethics Committee of the Academic Hospital Maastricht. The patient and the three healthy control males gave written consent for the semen samples to be used for research purposes.

Sperm preparation

Semen analysis (WHO, 1992) revealed parameters in the normal range (volume, 4.5ml; sperm concentration $129 \times 10^6/\text{ml}$, progressive motility 35%, morphology 49%). A swim-up fraction was prepared and the sperm were spread on Super Starfrost Plus slides (Maenzel Glaeser®, Braunschweig, Germany). Slides were air dried at room temperature, fixed in methanol/acetic acid (3:1) and stored at -20°C . Before processing for FISH, the slides were washed in 2x standard saline citrate (SSC) and incubated for 5 minutes in 1M Tris buffer, pH 9.5, containing 25mM dithiothreitol (DTT) (Martini *et al.*, 1995). After nuclear decondensation, the slides were washed once in 2x SSC, once in 1x phosphate buffered saline (PBS), dehydrated and air dried. Finally, they were fixed in 1% paraformaldehyde/0.1M PBS (pH 7.3) for 5 minutes at 4°C , rinsed three times in PBS, dehydrated and air dried (Martini *et al.*, 1997).

Semen from 3 normal, healthy men with a 46,XY karyotype were used as controls. These men were partners of women with an IVF indication and normal IVF results and had semen parameters in the normal range (WHO, 1992). These samples were analysed and processed in an identical manner.

Lymphocyte preparation

Interphase nuclei obtained from methanol-acetic acid (3:1) fixed preparations of peripheral blood cells of the patient and from a healthy 46,XY control were used to test the quality of the DNA probes, the efficiency of the labelling and the FISH procedure.

DNA probes

Seven different DNA probes were used in this study (Table 1).

Three probes were required for the translocation study: (i) CEP11 labelled with Spectrum Orange® (Vysis, Inc., Downers Grove, IL), specific for the repetitive centromeric region of chromosome 11; (ii) a cosmid probe, D11S-477, specific for chromosome 11q25, insert size approximately 40 kb, (Tokino *et al.*, 1991, remapped - personal observations), labelled by nick translation with digoxigenin-12-dUTP (Boehringer Mannheim, Mannheim, Germany; (iii) a YAC clone, TYAC162, specific for chromosome 3q29, insert size 250 kb (Vocero-Akbani *et al.*, 1996), Alu-PCR-labelled with biotin-11-dUTP (Enzo Diagnostic, New York). Sex-chromosome probes were used: (i) pBamX5, alphoid, insert size 2.0 kb, (Willard *et al.*, 1983), specific for the centromeric region of human chromosome X; (ii) DYZ1, satellite II, insert size 2.1 kb (Cooke *et al.*, 1982), specific for the long arm of human Y chromosome. These probes were direct-labelled with FITC-12-dUTP (Boehringer Mannheim) and Rhodamine-4-dUTP (TRITC) (Amersham, Buckinghamshire, UK), respectively.

In order to study the baseline frequencies of aneuploidy and to exclude an interchromosomal effect, probes specific for chromosome 13q14 (LSI13) and chromosome 21q22.13-21q22.2 (LS21) were used (Vysis Inc.). The probes were direct-labelled with Spectrum Green® and Spectrum Orange®, respectively.

Table 1. Specificity, labelling, signal color and usage of probes.

Probe	Specificity	Label	Color	Combination (controls)	Combination (carrier)
CEP 11	alphoid 11	Spectrum Orange	orange		triple
TYAC162	3q29	Biotin	green	double	
D11S-477*	11q25	Digoxigenin	red	triple	
LSI 13	13q14	SpectrumGreen	green	double	double
LSI 21	21q22.13-21q22.2	SpectrumOrange	orange		
pBamX5	alphoid X	FITC	green	double	double
DYZ1	long arm Y	TRITC	red		

*remapped on 11q25, personal observations

FISH procedure

Triple target FISH was performed with the probe cocktail for the translocation chromosomes (figure 1a-e). Sperm were denatured by immersion in 70% formamide (FA)/2xSSC at 70°C for 3 minutes, dehydrated through a cold ethanol series and air dried. A 10 µl sample of hybridization mix (60% FA, 10% dextran sulfate) containing 200 ng of cosmid 11q25, 10

ng of CEP11, 100 ng of YAC 3q29, 40 µg of salmon sperm DNA and 20 µg of human Cot DNA (Gibco, BRL) was heated at 70°C for 5 minutes and then pre-annealed at 37°C for 20 minutes. The mixture was then applied to the specimen. The slides were covered with a 20x20 mm coverslip and hybridized overnight in a moist chamber at 37°C. Coverslips were then removed and slides were washed twice in 2xSSC/0.05% Tween 20 for 5 minutes at 42°C, once in 0.1xSSC for 5 minutes at 60°C and twice in 4xSSC/0.05% Tween 20 for 5 minutes at room temperature. Specimens were then pre-incubated for 15 minutes in 4% non-fat dry milk (NFDM). To detect the biotinylated probe, the following series of incubations was used: (i) 20 minutes at 37°C with avidin-FITC, (ii) 20 minutes at 37°C with anti-avidin, (iii) 20 minutes at 37°C with avidin-FITC. Between incubations, slides were washed for 5 minutes in 4x SSC/0.1% Tween 20 at room temperature. Slides were washed 5 minutes in PBS/0.1% Tween 20 at room temperature, then incubated for 30 minutes at 37°C with mouse anti-digoxigenin followed by 30 minutes at 37°C with Cy3-labelled goat anti-mouse to detect the digoxigenin labelled probe. Slides were rinsed in PBS, dehydrated through an ethanol series and mounted in Vectashield antifade (Vector laboratories, Burlingame, CA, USA) containing 0.01 mg/ml 4,6-diamidino-2phenylindole (DAPI) to counterstain the DNA.

Double target FISH using probes for the sex-chromosomes and chromosomes 13 and 21 was performed as described above except that only one 5 minute wash was used between each step.

Microscopy

Slides were analyzed with a Leica DMRBE fluorescence microscope equipped with separate filters for FITC, Rhodamine and DAPI. Microphotographs were taken using a Metasystem Image Pro System (CCD S30 camera, Sandhausen, Germany). Images were captured using green, red and blue filters and processed for sharpness in Adobe Photoshop 3.0 (120 pixels/inch) (figure 1).

Evaluation criteria

Strict scoring criteria from Hopman *et al.*, (1988) and Martin *et al.*, (1995) were used. Only individual and well delineated spermatozoa were scored. Two signals of the same color were scored as two when they were clearly distinct and of the same size and intensity. In the triple FISH, the centromeric probe for chromosome 11 (CEP11) and the cosmid probe for 11q25 (D11S-477) were of very different sizes (figure 1a-e) and were therefore distinguished although both yielded red signals. Coded slides (2 per sample) were scored by two observers (E.M. and A.v B.).

Statistical analysis

A chi-squared test was performed to compare the hybridization efficiencies of CEP11, TYAC162 and D11S-477. Statistical significance was considered when P values were <0.01.

Results

Triple FISH on lymphocytes

Probes were tested on lymphocytes to determine the characteristics and hybridization efficiencies of the probes. Once the probes worked efficiently in single and double FISH (data not shown), triple FISH was performed (figure 1a-b-c) and 600 control and carrier lymphocytes were scored. Overall, 97.2% of the cells displayed the expected, normal diploid constitution while the remaining cells showed either loss (hypoploidy or technical artifact) or gain (hyperploidy or split spots) of signals (figure 2a).

Figure 1. FISH results on peripheral blood cells (a-c) and spermatozoa (d-g) of the patient. (a) Triple target hybridization to metaphase spreads with the probes CEP11, TYAC 162 and D11S-477, respectively in orange, green and red. (b) Particular of the chromosomes involved in the translocation and the probes applied. (c) Lymphocyte, hybridized with triple FISH, showing the difference in signal size for the centromere and the locus specific probe used. (d) Spermatozoa of the patient hybridized with triple FISH (for explanation see figure 3). (i) Adjacent II segregation mode, signals for CEP11 and TYAC 162; (ii) 3:1 segregation mode, signals for CEP11 and D11S-477; (iii) Alternate segregation, normal or balanced sperm with signals for CEP11, TYAC 162, D11S-477; (iv) Aberrant segregation mode, two signals for CEP11. (e) Spermatozoa of the patient hybridized with triple FISH (for explanation see figure 3). (i) Adjacent I segregation, signals for CEP11 and twice for TYAC 162; (ii) and (iii) Alternate segregation mode, normal or balanced spermatozoa; (iv) 3:1 segregation mode, signals for CEP11, TYAC 162 and twice for D11S-477. (f): double FISH with chromosomal probes for chromosomes 13 (green) and 21 (red). All spermatozoa show a normal, haploid, chromosomal constitution. (g): double FISH with the sex-chromosomal probes. In green the X-probe, in red the Y-probe. At the bottom, right corner, a sperm cell carrying an XY constitution is visible. For colour illustration see page 116.

Double FISH on control spermatozoa

Probes were tested on control spermatozoa using a double FISH and different probe combinations (CEP11 and TYAC162; CEP11 and D11S-477; TYAC162 and D11S-477). A total of 3000 spermatozoa were scored for each combination. The results were as follows: (i) CEP11 and TYAC162: 98.87% of cells exhibited a signal for both chromosomes. More loss (CEP 11, 0.03%; TYAC162, 0.7%) than gain (CEP 11, 0.07%; TYAC162, 0.33%) was registered. (ii) CEP11 and D11S-477: 98.67% of spermatozoa were haploid, with loss (CEP 11, 0.03%; D11S-477, 0.83%) and gain (CEP 11, 0.20%; D11S-477, 0.20%) and 0.07% diploidy. (iii) TYAC162 and D11S-477 98.73% of cells exhibited signals for both probes with loss (TYAC162, 0.07%; D11S-477, 0.7%) and gain (TYAC162, 0.17%; D11S-477, 0.20%). Loss and gain for individual probes is illustrated in figure 2b.

Figure 2. Efficiencies of the single probes (CEP11, TYAC162, and D11S-477) on lymphocytes as determined by triple FISH (2a) and double and triple FISH on spermatozoa of controls (2b and 2c, respectively). Loss and gain of the probes is shown in the bars. Description in the text. *: $p < 0.01$

Triple FISH on spermatozoa from controls and translocation patient

A total of 8316 control spermatozoa were scored. The hybridization efficiencies were as follows: CEP11, 99.81%; TYAC162, 98.5%; D11S-477, 96.01%. The frequency of normal spermatozoa (carrying one signal for each probe) was 91.55%, and diploidy occurred at a frequency of 0.08%. Loss and gain for individual probes is illustrated in figure 2c. Moreover, 1.05% of the cells displayed an aberrant chromosome constitution, not classifiable in any of the above categories.

Analysis of 4029 spermatozoa from the translocation carrier by triple FISH yielded a hybridization efficiency of 99.6%. Spermatozoa resulting from the different segregation modes were present at different frequencies (figure 1d-e, 3). During meiosis, a quadrivalent is formed and, depending of the segregation mode, the resultant gametes carry different chromosomal segregations. Of these, only the alternate segregation mode seems to be compatible, in our case, with life. Analysis showed that 44.3% of the spermatozoa originated from an alternate segregation mode and carried a normal or balanced chromosome constitution. The frequencies of adjacent I, adjacent II, 3:1, 4:0 and aberrant segregation modes were 15.9%, 6.6%, 28.9%, 0.8% and 3.6%, respectively. These patterns are shown in figure 3.

Segregation of chromosomes X and Y

Chromosomes X and Y were simultaneously detected in 7032 and 5702 control and carrier spermatozoa, respectively. Both the sex ratio and the aneuploidy rate were similar in the two groups (Table 2, figure 1g).

Table 2. Sex ratio and sex chromosome aneuploidy in control and translocation carrier sperm. Values are percentages.

	X	Y	XY	XX	YY	XXY	XXY
Control	49.04	50.18	0.40	0.28	0.09	0.0	0.0
Carrier	48.05	51.57	0.23	0.02	0.12	0.02	0.0

Segregation of chromosomes 13 and 21

The segregation of chromosomes 13 and 21 was studied in 5612 control spermatozoa and 5016 carrier spermatozoa to determine any interchromosomal effect. No significant variation in aneuploidy rate was detected for these two chromosomes (Table 3, figure 1f).

Table 3. Chromosome 13 and 21 aneuploidy in control and translocation carrier sperm. Values are percentages.

	13/21	13/-	-/21	13,13/21	13/21,21	13,13/21,21	-	13,13/-	-/21,21
Control	98.66	0.23	0.29	0.07	0.37	0.11	0.25	0.02	0
Carrier	98.33	0.4	0.7	0.0	0.26	0.06	0.24	0.0	0.02

Discussion

Approximately 30% of embryonic and fetal deaths are associated with chromosomal abnormalities, in a ratio of 1 to 9 structural versus numerical defects (Hook 1985). A balanced carrier of a reciprocal translocation can produce a variety of gametes by meiotic

Figure 3. Schematic explanation of the chromosomes involved in the translocation $t(3;11)(q27.3;q24.3)$ and the probes used. The quadrivalent and the various segregational modes are shown. The corresponding resulting gametes are illustrated. For simplification the CEP11 signal, which is detected in red, is here illustrated in yellow. CEP 11= Yellow, TYAC 162 = Green, and D11S-477 = Red. For colour illustration see page 117.

segregation, whereby the reproductive significance of any particular structural rearrangement depends on the proportion of unbalanced gametes produced and the post zygotic lethality arising from them (Goldman and Hulten 1993a; Goldman and Hulten 1993b). Generally, information for genetic counseling is obtained from empirical studies or sperm karyotyping (Estop *et al.*, 1995), however, none of these techniques enables correct knowledge of the chromosome constitution in sperm cells and a reliable risk estimation for the patient (Van Hummelen *et al.*, 1996).

Some studies used FISH on testis biopsies from a translocation carrier (Goldman and Hulten 1993a; Goldman and Hulten 1993b), on epididimal spermatozoa from Robertsonian translocation-bearing mice (Lowe *et al.*, 1996) and on ejaculated spermatozoa from translocation carriers (Spriggs and Martin 1994; Lu *et al.*, 1994; Rousseaux *et al.*, 1995; Mercier and Bresson 1997). However, until now, the analysis of spermatozoa of translocation carriers by FISH using centromeric probes could not discriminate gametes originating from an alternate or adjacent I segregation mode. Simultaneous detection of structural and numerical abnormalities in spermatozoa requires multicolor FISH and an appropriate choice of DNA probes (Van Hummelen *et al.*, 1996). In our approach, we used two locus-specific probes and one centromeric probe to differentiate between segregation modes (figure 3). We found that 44.3% of spermatozoa from a translocation carrier $t(3;11)(q27.3;q24.3)$ carried a normal or balanced genetic constitution, while the remaining spermatozoa showed a chromosomal composition deriving from different segregation modes of the quadrivalent at meiosis. We have shown that although co-hybridization of different DNA probes can influence the hybridization outcome, the present approach can detect structural aberrations in human interphase sperm. These results offer a risk estimation which is reliable enough to be used for genetic counselling of translocation carriers.

Meiotic segregation

The actual risk of producing unbalanced live births differs greatly from one reciprocal translocation to another (Vauhkoen *et al.*, 1985) and unbalanced offspring produced by carriers of a particular reciprocal translocation are nearly always the result of the same mode of translocation.

Factors such as the size of the translocation segments, the presence or absence of heterochromatin, and the centromere index all play a role in defining quadrivalent orientation and first meiotic segregation (Sybenga and Rickards 1987). However, the true mechanisms responsible for determining the segregation of structural rearrangements remains unknown. Alternate segregation yields chromosomally normal gametes and gametes carrying both translocated chromosomes. Information from most of the translocations studies suggests that meiosis produces an equal ratio of chromosomally balanced and normal spermatozoa (Guttenbach *et al.*, 1997). However, with FISH in interphase, and locus specific probes, it is not possible to distinguish between these two forms. Most unbalanced liveborn offspring from reciprocal translocation heterozygotes are the result of adjacent I segregation (Jalbert *et al.*, 1988). In abortion material obtained from the couple described in this paper, this chromosome constitution was found and in the present study we found 15.9% of spermatozoa with this particular genotype.

Exchanges of interstitial chiasmata between the centromere and translocation breakpoint render alternate and adjacent I segregation products cytologically indistinguishable (Sybenga and Rickards 1987). This can be bypassed using a specific probe cocktail which hybridizes to the specific fragments involved in the translocation, as in our tailored approach (figure 3).

Data arising from sperm chromosome studies (see review by Estop *et al.*, 1995) suggest

that in male meiosis, adjacent II segregations are more prevalent than 3:1 segregations. However, this was not observed in our case, where the frequency of 3:1 segregations was 28.9% whereas adjacent II segregation occurred at a frequency of 6.6% only. Jalbert *et al.*, (1980) hypothesized that the features of preferential 3:1 segregation were (i) unequal sizes of the translocated segments and (ii) participation of acrocentric chromosomes. In our study, 28.9% of spermatozoa were the result of a 3:1 segregation. However, this frequency may have been biased by technical artefacts as discussed later. Additionally, 4:0 and aberrant segregation modes were detected at frequencies of 0.8% and 3.57%, respectively (figure 3).

Interchromosomal effects

The possibility of an increased frequency of chromosomal abnormalities unrelated to the specific translocation, an interchromosomal effect, has been postulated (Aurias *et al.*, 1978). This is still controversial, however, and there is little if any evidence to support the concept (Rousseaux *et al.*, 1995). Verily, when analyzing spermatozoa with alphoid or satellite probes for chromosomes X and Y and locus specific probes for chromosomes 13 and 21, we found neither alteration of the sex-ratio nor differences in aneuploidy. The aneuploidy rate found for the sex-chromosomes was in agreement with data from the literature (see Downie *et al.*, 1997 for review; Martini *et al.*, 1996; Bernardini *et al.*, 1997). Few studies have simultaneously assessed chromosomes 13 and 21 in spermatozoa, and all reported higher disomy rates than the present study (see Downie *et al.*, 1997 and Guttenbach *et al.*, 1997; both for review). However, when using a single probe for chromosome 21, Blanco *et al.*, (1996) reported similar frequencies to our study, whereas Rousseaux and Chevret (1995), with the same probe, reported lower aneuploidy rates. In order to completely exclude an interchromosomal effect, the segregation of other autosomes should be studied.

Technical limitations

Like all the techniques, however, there are some limitations to the approach used in this study. The use of FISH and the consequent identification of "spots" in interphase nuclei is not as convincing as the clear identification of chromosomes by cytogenetic analysis. The same analysis performed on lymphocytes and on control spermatozoa revealed hybridization rates of 97.17% and 91.55%, respectively. These data indicate that the efficiency hybridization was reduced in the triple FISH procedure, especially in spermatozoa, where the DNA is compacted by disulfide bonds between protamines which have to be reduced (Martini *et al.*, 1995). Moreover, the individual probes themselves showed different properties.

The fact that we found a high level of loss and/or gain of signals in control spermatozoa can be explained by one or a combination of the following theories. First, the combined use of small locus-specific probes with a large centromeric probe can either mask the signal of the smaller probes by superposition or, second, the smaller probes cannot hybridize as efficiently due to sub-optimal conditions of the protocol. Indeed, excesses of nullisomies for chromosomes 3q29 and 11q25 compared to the corresponding disomies were observed in controls, and technical artefacts are likely to explain these discrepancies. The superposition of green (TYAC162) or red (D11S-477) signals by the large signal for CEP11 (or overlapping signals) could have led to mis-scoring of some cells with a normal constitution (see also efficiency of each probe in triple hybridization). The reduced hybridization efficiency of the probes for 11q25 and 3q29 (probably due to the insert size, the non-optimal co-hybridization situation) could also have contributed to the mis-scoring of some cells. Moreover, an apparent gain of a fluorescence signal might indeed be a split

hybridization domain due to overdecondensation of the sperm DNA (Wyrobek *et al.*, 1994) or to a physical chromosome break within the targeted region of the probe accompanied by a small migration (Van Hummelen *et al.*, 1996).

Finally, the material analyzed has to be considered. It is known from previous studies that analysis of aneuploidy in spermatozoa yields a high variation among different laboratories (Downie *et al.*, 1997). The reason for these variations may be several fold: the quality of the spermatozoa, the decondensation protocol used, single, double or triple FISH protocols, the (im)possibility to identify the sperm tail, the morphological criteria used for the identification of spermatozoa, and the stringency of signal scoring criteria (Martin *et al.*, 1995; Martini *et al.*, 1995; Van Hummelen *et al.*, 1996; Downie *et al.*, 1997).

Hence, this method still presents some limitations due to the hybridization efficiency of the smaller probes, and these preliminary data should therefore be considered with some caution. Nevertheless, the data give a reasonable estimate of the possible risks for the offspring and should enable more effective genetic counselling.

The couple described in this study will be offered preimplantation genetic diagnosis. From the data obtained it is clear that about one in two pre-embryos is likely to be rejected after the treatment because of imbalanced genomic constitution.

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Chapter 6

Analysis of unfertilized ICSI oocytes using two rounds of FISH and probes to five chromosomes

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Abstract

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 FISH procedure that would enable us to investigate aneuploidy in unfertilized ICSI oocytes. 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: (I) chromosomes 1, 7, 15 (overnight hybridization); (II) 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 (85%) 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, although interpretation of the patterns of DNA fragments and FISH signals requires further clarification.

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 foetal 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 chromosomes. 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 pre-implantation genetic diagnosis (PGD) of X-linked diseases and aneuploidy (Griffin *et al.*, 1992; Harper *et al.*, 1994; Veiga *et al.*, 1994; Munné *et al.*, 1995), as well as to study early embryogenesis (Benkhalifa *et al.*, 1993; Munné *et al.*, 1994b; Muggleton-Harris *et al.*, 1995; Sultan *et al.*, 1995). FISH has also been used to investigate aneuploidy in sperm 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).

Coonen *et al.* (1994) presented an alternative method for preparing and isolating interphase nuclei from pre-implantation 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 couples 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 sperm 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%) fertilised 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 μ l) of 0.01N HCl and 0.1% Tween 20 in bi-distilled water on Super Starfrost Plus slides (Maenzel Glaeser[®], Germany). The 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 (Figure 2a-c). 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 (RT) up to one week or processed immediately for FISH.

DNA probes

Five centromeric probes were used: (i) pUCC 1.77 (satellite III, insert size 1.77 kb), specific for the centromeric region of human chromosome 1 (Cooke and Hindley, 1979); (ii) p7t1 (alphoid, insert size 0.68 kb), recognises a tandem repeat in the centromeric region of human chromosome 7 (Waye *et al.*, 1987); (iii) pBam X5 (alphoid, insert size 2.0 kb), specific for the centromeric region of human chromosome X (Willard *et al.*, 1983); (iv) DYZ1 (satellite II, insert size 2.1 kb), specific for the long arm of human Y chromosome (Cooke *et al.*, 1982); and 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], 2x SSC buffer) and used at a final concentration of 1 ng/ μ l in hybridization solution (60% FA, 2x SSC, 10% dextran sulfate, 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 Y-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 (100 μ g/ml) in 0.01N 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 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. Ten μ l of hybridization mixture containing the first 3 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 2x SSC, 0.05% Tween 20 for 5 min at 42°C, then twice in 0.1x SSC for 5 min at 60°C, twice in 4x SSC, 0.05% Tween 20 for 5 min at RT, then dehydrated through an ethanol series and mounted in Vectashield antifade (Vector laboratories, Burlingame, CA, USA) containing 0.01 μ g/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 4x SSC, 0.05% Tween 20 for 5 min at RT. The slides were dehydrated, 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 (Figure 2d-i).

Evaluation and scoring criteria

The specificity of each FISH probe was tested concurrently on interphase nuclei obtained from ethanol-fixed suspensions of male leucocytes. 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 Munné *et al.* (1995). Minor hybridization spots that had much lower fluorescence 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

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, 2 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 (Figure 2d-i).

FISH results

Oocytes exhibited one or more DNA fragments and were categorised 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 (Munné *et al.*, 1995; 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 2d,e).

FISH on Unfertilized MII Oocytes

Figure 1. Classification of oocytes into groups in relation to DNA patterns and hybridization results. The different pattern(s) of DNA fragments and signals in group I, II and III oocytes are shown. Subgroups are shown individually (group I: I and ii; group II: I-vii) and each spot represents one signal for a given chromatid.

Group II consisted of 23 oocytes (35.4%) which displayed an ambiguous chromosome complement due to an abnormal number of DNA fragments (Figure 1, categories i-vii). These oocytes lysed into one to three DNA fragments (mean 1.9, range 1-3), which probably represents artefactual loss or breakage of the DNA or unidentified sperm DNA. These oocytes displayed either paired signals, indicating normal, univalent metaphase II chro-

mosomes, or only one small spot which was interpreted as a single chromatid signal, or a combination of the two patterns (Figure 1, patterns i-vii, group II). Nonetheless, each DNA fragment exhibited paired or single signals for all the chromosomes analyzed without any aneuploidy. Some of these fragments might have been disrupted PB or metaphase II DNA in which a pre-division of the chromatids had begun (Angell, 1994) (Figure 2f,g).

Group III consisted of 25 oocytes (38.4%) which exhibited aneuploidy for one or more of the chromosomes studied, regardless of the number of DNA fragments present (Figure 1; Figure 2h,i). Interestingly, a heterogenous chromosome constitution was found in this group, and it was possible to relate aneuploidy to the DNA fragment patterns of Groups I and II. We found that 47% of the oocytes which had a normal DNA pattern (two fragments, from the metaphase II and PB, equivalent to Group I) were aneuploid, while 74% of the oocytes with one to three DNA fragments (equivalent to Group II) were aneuploid. The relative frequency of hyperhaploidy and hypohaploidy for chromosomes 1, 7, 15 and X is presented in Table 1. Hypohaploidy for chromosome X and hyperhaploidy for chromosome 1 were common.

Table 1. Number of oocytes with single chromatid hyperhaploidy and hypohaploidy.

Chromosome	Hyperhaploidy	Hypohaploidy	Total chromatids
1	11	-	221
7	3	3	209
15	3	3	193
X	1	16	192

An undecondensed sperm head was seen in 10 oocytes, although a Y signal was present in only 3 of these oocytes. This low frequency might have been due to limited decondensation of sperm DNA (Martini *et al.*, 1995) which hampers efficient hybridization, or to the unrecognized presence of sperm DNA (carrying for example a single set of chromatids and the X-chromosome) which was misclassified into group II.

Discussion

About 60% of oocytes ovulated in women over 40 years of age may be chromosomally unbalanced (Hassold and Jacobs, 1984). Plachot *et al.* (1988) found an increasing rate of aneuploidy in oocytes from women > 35 years, whereas Eichenlaub-Ritter *et al.* (1988) reported similar rates of anomalies in oocytes from young (< 35 years) and older (35-39 years) women. There is evidence that predisposition to non-disjunction (NDJ) is correlated to a shortened cell cycle in oocytes from aged female mice (Eichenlaub-Ritter and Boll, 1989). At a molecular level, these alterations in maturation could result from disturbances in protein phosphorylation and alteration in the phosphorylated status and activity of the catalytic subunit of maturation promoting factor (MPF) (Choi *et al.*, 1991). The high rate of chromosomal abnormalities in oocytes 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.

Figure 2. Spreading of the oocyte (a-c): (a) Oocyte with first polar body (PB) and no pronuclei. At the beginning of spreading, the oolemma is still present. (b) Lysis of the PB, the DNA is visible. (c) Isolated metaphase DNA. FISH results (d-i): 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 ICSI oocytes. First and second FISH rounds respectively on the same DNA (d,e): 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. First and second FISH rounds respectively on the same DNA (f,g): 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. First and second FISH rounds respectively on the same DNA (h,i): Example of aneuploidy for all the chromosomes analyzed. 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. For colour illustration see page 118.

Angell *et al.* (1993) studied human oocytes by karyotyping and reported that the major type of chromosomal abnormality was single chromatids replacing whole chromosomes. This suggests that precocious division (pre-division) of a chromosome univalent during the first meiotic division might be the most common mechanism of trisomy formation, and not NDJ of whole bivalents as generally believed. Paired chromosome signals corresponding to the expected metaphase II pattern were reported in FISH 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 the present 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 5 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 2d,e). 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 pre-division 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.*, 1995; Dyban *et al.*, 1996). In this case, two discrete signals for a chromosome would be observed instead of two conjoined signals (Figure 2f,g). 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.*, 1995). Second, chromatid overlap would produce a dyad with only a single hybridization signal instead of a pair. Munné *et al.* (1995) 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.* (1995) and scored a DNA fragment with single signals as normal, because the probability of having a monosomy for all 5 chromosomes due to pre-division of all the chromatids is remote (Munné *et al.*, 1995).

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 sub-group containing only one DNA fragment which may have been due to artefactual loss of DNA during spreading. However, another sub-group contained three DNA fragments, which might indicate an unrecognised 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 recognised in 10 oocytes; 3 displayed a signal for the Y chromosome, but the sperm head was undecondensed in the other 7 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 recognised 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 (Dozortsev *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 least 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 aneuploidies, whereas karyotyping mostly classify aneuploidy using the broad Denver classification which does not allow classification of chromosome-specific aneuploidies. For example, the high rate of chromosome X hypohaploidy we report might correspond to the hypohaploidy rate reported for group C chromosomes by karyotyping (Macas *et al.*, 1990; Martin *et al.*, 1991; Pellestor *et al.*, 1991b; Roberts and O'Neill, 1995). 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 Dailey *et al.*, (1996). Finally, we cannot exclude loss of chromatids due to technical artifact, 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 conceptuses but they are lost at an early pre-implantation 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 to simultaneously investigate up to six 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 easily be 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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Chapter 7

FISH analysis of six chromosomes in unfertilised human oocytes after polar body removal

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Abstract

We recently reported a two round, multi-probe fluorescence in situ hybridization (FISH) procedure for detecting aneuploidy in human oocytes. In the present study, we have improved the technique by removing the first polar body (PB) before the oocytes were spread so that only the metaphase II chromosomes were evaluated by FISH. We also investigated whether there was an association between aneuploidy and precocious division (pre-division) of chromosomal bivalents. Unfertilised oocytes which were still at metaphase II 18-20 h after insemination or intracytoplasmic sperm injection (ICSI), were treated with pronase to remove the zona pellucida and PB and then spread on slides using HCl and Tween 20. Two rounds of FISH were performed using direct-labelled probes: chromosomes 1, 13, 21 (round 1); chromosomes X, 7, 18 (round 2). Of the 63 oocytes from 18 patients (mean age: 32 years), 48 (76%) had one DNA complement as expected, 9 (14%) had 2 DNA complements, 3 (5%) gave incomplete FISH signals and 3 (5%) were not analysable. Of the 48 oocytes with one set of DNA, 48% were haploid, 44% were aneuploid for one or more chromosomes, and 8% were polyploid. We analysed the position of the chromatids (dyad or pre-division) and found a prevalence of dyads in haploid oocytes and an increased frequency of pre-division in aneuploid oocytes, especially for chromosome 21. This method enables analysis of multiple chromosomes in human oocytes without interference by DNA from the PB.

Introduction

The introduction of assisted reproductive techniques (ART) such as in vitro fertilization (IVF) and ICSI has made it possible to study early events in human reproduction and the contribution of spermatogenesis, oogenesis, fertilization and early embryogenesis to the generation of chromosomal abnormalities.

Unfertilised oocytes from ART cycles are the only readily-available source of human oocytes for research. The oocytes which fail to fertilise after IVF insemination or ICSI can be used for chromosomal analysis. Various lines of evidence imply that oocyte meiosis is sensitive to endogenous or exogenous factors and that the incidence of abnormalities is correlated with female factors. For instance, women with tubal or unexplained infertility have a higher rate of oocyte chromosomal abnormalities than women whose partners are infertile (see Plachot, 1997 for review). The incidence of chromosomal aberrations in uninseminated human oocytes has been reported to vary between 8-50% (Plachot, 1997) and the rate of chromosomally abnormal oocytes appears to be relatively high in cases of fertilization failure after IVF (26.5%). Furthermore, aberrations arising de-novo from meiotic non-disjunction (NDJ), especially paternal NDJ, contribute to chromosomal abnormalities in newborns after ICSI (Plachot, 1997; Liebaers *et al.*, 1995). Studies on metaphase II human oocytes by Angell (Angell, 1994a; Angell, 1994b; Angell, 1997) revealed an unexpected class of chromosome abnormalities which manifest as single chromatids rather than whole chromosomes comprising a dyad of chromatids. These studies also revealed a close correlation, with respect to frequency, distribution and maternal age, between oocyte data and trisomies in aborted fetuses and live newborns (Angell, 1997). Several groups have reported results from large samples of oocyte karyotypes which suggest that a high proportion of human oocytes (25-30%) may be chromosomally abnormal (Plachot *et al.*, 1988; Benkhalifa *et al.*, 1990; Pellestor, 1991a; 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, 1991a; Pellestor, 1991b). The condition of the chromatids

has hampered reliable analysis by karyotyping and necessitated the introduction of a modified protocol based on Tarkowski (1966) to spread the chromosomes. This method however has major drawbacks such as loss of cells and/or loss of morphology, which reduces the number of analysable oocytes and compromises the efficiency and accuracy of the analysis.

We recently developed a reliable method for studying chromosomal anomalies in unfertilised, metaphase II oocytes using the spreading technique of Coonen *et al.* (1994) and a novel FISH procedure involving two triple-probe hybridisations to enable a larger number of chromosomes to be studied (Martini *et al.*, 1997). This new approach allowed us to analyse 85% of the oocytes, compared to the 30-50% routinely obtained by karyotyping (reviewed by Plachot 1997; Angell, 1997). However, it was not always possible to differentiate between DNA from the first PB and the metaphase II chromosomes, which hindered interpretation of the results. The aim of the present study, therefore, was to bypass this problem by removal of the PB prior to spreading the oocytes so that only oocyte chromosomes were studied. Two rounds of FISH and direct-labelled probes for chromosomes 1, 7, 13, 18, 21 and X were used, enabling study of six different chromosomes in each oocyte. Furthermore, since precocious division (pre-division) of bivalents at meiosis I may be responsible for trisomy formation (Angell *et al.*, 1993; Angell, 1997), we also studied the position of chromatids to determine whether or not there was an association with the ploidy status of the oocyte.

Materials and methods

Source of oocytes

Unfertilised oocytes were obtained from 18 couples undergoing ICSI ($n = 8$) or IVF ($n = 10$) in the Reproductive Medicine Unit. The mean female age was 32 (range: 26-39). Our protocols for patient selection and treatment have been described in detail by Payne *et al.* (1991; 1994) and Payne and Matthews (1995). The study was approved by the Ethics of Human Research Committee at The Queen Elizabeth Hospital.

Of the 344 recovered oocytes, 200 (58.1%) fertilised normally and 111 of the unfertilised oocytes were suitable for study. They all failed to show any evidence of fertilization when examined 17-20 h post-injection (ICSI) or post-insemination (IVF) and possessed a first PB, which was either normal or fragmented.

Zona pellucida removal

A 0.5% (w/v) solution of pronase (from *Streptomyces griseus*, specific activity 7000 U/g; Boehringer-Mannheim, Castle Hill, Australia) was prepared in HEPES-buffered HTF culture medium (Quinn *et al.*, 1985) and frozen in aliquots at -20°C . On the day of use, aliquots were thawed, vortexed and then clarified by centrifugation for 20 sec at 14 000g.

Oocytes were processed individually, 20-24 hours post-insemination. Zona removal was performed in 50 μl microdrops, and oocytes were transferred between drops using fine drawn glass pipettes (inner diameter slightly larger than the oocyte). Each oocyte was transferred firstly into a drop of HEPES-HTF containing 0.1% human serum albumin (HSA), then into a drop of pronase solution. It was drawn in and out of the pipette 4-5 times, incubated in pronase for 30-60 sec, then aspirated again until the zona was partially removed. It was then transferred into a fresh drop of HEPES-HTF containing 0.1% HSA after which final removal of the zona and PB was achieved by further aspiration. The oocyte was then rinsed in a fresh drop of HEPES-HTF containing HSA and spread on a slide.

Spreading of oocytes

Oocytes were spread on slides using a modification of the method reported by Coonen *et al.* (1994), as detailed in Martini *et al.* (1997). Slides were subsequently air dried, washed and dehydrated through an ascending ethanol series. They were stored at -20°C in Adelaide before being shipped to Maastricht at room temperature. The slides were then kept at room temperature for up to one month before being processed for FISH.

DNA probes

Six probes were used: (i) pUCC 1.77 (satellite III, insert 1.77 kb), specific for the centromere of human chromosome 1 (Cooke and Hindley, 1979); (ii) p711 (alphoid, insert 0.68 kb), which recognises a tandem repeat in the centromere of human chromosome 7 (Waye *et al.*, 1987); (iii) LSI13, specific for chromosome 13q14 and labelled with Spectrum Green® (Vysis, Downers Grove, IL, USA); (iv) L1.84 (alphoid, insert 0.68 kb), specific for the centromere of human chromosome 18 (Devilee *et al.*, 1986); (v) LSI21, specific for 21q22.13-21q22.2 labelled with Spectrum Orange® (Vysis); (vi) and pBam X5 (alphoid, insert 2.0 kb), specific for the centromere of human chromosome X (Willard *et al.*, 1983). Probes other than LSI13 and LSI21 were labelled by nick translation. The chromosome 18 probe was labelled with FITC-12-dUTP (Boehringer-Mannheim), the chromosome 7 probe was labelled with TRITC-4-dUTP (Amersham, Buckinghamshire, UK), and aliquots of the chromosome 1 and X probes were labelled with FITC-12-dUTP and TRITC-4-dUTP. Probes were dissolved in 60% formamide [FA], 2x SSC and used at a final concentration of 1 ng/μl in hybridization solution (60% FA, 2x SSC, 10% dextran sulfate, tRNA, ssDNA).

FISH protocol

Two rounds of FISH were performed on each oocyte, as described by Martini *et al.* (1997). The first round used a probe cocktail for chromosomes 1, 13 and 21. After evaluation and scoring, the second round was performed using a probe cocktail for chromosomes 7, 18 and X. Briefly, slides were treated with pepsin (100 μg/ml) for 15 min at 37°C to remove any remnants of cytoplasm and make the nuclei accessible for hybridization to the probes, then they were fixed in paraformaldehyde for 5 min at 4°C, rinsed in PBS and then bi-distilled water, and dehydrated through an ethanol series. Ten μl of hybridization mixture containing the first 3 probes (1-FITC and 1-TRITC, 13-Spectrum Green® and 21-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, dehydrated and mounted in Vectashield anti-fade (Vector Laboratories, Burlingame, CA, USA) containing 0.01 μg/ml 4,6-diamidino-2-phenylindole (DAPI) to counterstain the DNA. After analysis and recording of results from the first FISH round, and on the same day, DAPI was removed with two washes, the slides were dehydrated, and the probes for the second round were applied (X-FITC and X-TRITC, 18-FITC and 7-TRITC). A coverslip was sealed on the slide, and denaturation and hybridization were performed as for the first round, except that hybridization proceeded for only 2 h at 37°C. The slides were washed, counterstained with DAPI and re-analysed.

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

Evaluation and scoring of slides

Slides were examined using a DMRBE fluorescence microscope (Leica, Germany) equipped with a triple band pass filter block containing filters for FITC, TRITC and DAPI. The stringent scoring criteria used have been described by Martini *et al.* (1997). Paired signals reflect the unique nature of oocyte chromosomes at metaphase II, highly contracted, with sister chromatids widely separated and loosely adjoined in the centromere (Dyban *et al.*, 1996). The position of sister chromatids was scored as dyads or pre-division. Images were recorded using a CCD camera, green, red and blue filters, and the Image Pro System (Metasystem, Sandhausen, Germany). Sharpness was enhanced in Photoshop 3.0 (Adobe Systems Inc., San Jose, CA, USA) at 120 pixels per inch (see Figure 1).

Results

A total of 111 oocytes were processed. After the first FISH round, 63 oocytes could be visualised using DAPI, giving a spreading efficiency of 57%. Of these 63 oocytes, 57 were analysed in both FISH rounds, yielding an overall FISH efficiency of 91%.

Oocytes were categorised into 3 groups based on the number of DNA fragments and the number and arrangement of hybridization signals. Group I consisted of 48 oocytes (76.2%) which had one DNA complement and a haploid ($n = 23$, 36.5% of total, 47.9% of Group I), aneuploid ($n = 21$, 33.3% of total, 43.8% of Group I) or polyploid ($n = 4$, 6.3% of total, 8.3% of Group I) chromosomal constitution. The occurrence of hyperhaploidy and hypohaploidy is shown in Table 1.

Table 1. Single chromatid hyperhaploidy and hypohaploidy rates.

Chromosome	Hyperhaploidy > 2 signals	Hypohaploidy < 2 signals	Total chromatids
1	10	-	52
7	17	1	58
13	2	12	32
18	15	5	52
21	3	16	29
X	6	8	40

Group II consisted of 9 oocytes (14.3%) which exhibited two DNA complements: (i) a haploid chromosome constitution in both DNA complements with only one chromatid signal in each complement ($n = 3$); a haploid chromosome constitution in both complements with dyads and single chromatids ($n = 1$); one haploid complement and one aneuploid complement ($n = 3$) and aneuploidy in both DNA complements ($n = 2$). Group III consisted of 6 oocytes (9.5%), which were either unanalysable ($n = 3$) or yielded incomplete FISH results due to failure of one of the two hybridization rounds ($n = 3$).

The results for haploid and aneuploid oocytes from Group I were further analysed to determine whether there was any correlation between aneuploidy and precocious division (pre-division) of chromosomal bivalents (Angell *et al.*, 1994). The findings are presented in Table 2. Haploid oocytes displayed mostly dyads using centromeric probes for chromosomes 1, X, 7 and 18 (82.6-91.3% dyads) as well as using LSI probes for chromosomes 13 and 21 (73.9-78.3% dyads). Aneuploid oocytes, however, showed an increased rate of pre-division of chromatids for all the chromosomes studied, especially chromosome 21 (65.8%).

Table 2. Incidence of dyads and pre-division of chromatids in haploid and aneuploid oocytes.

Chromosome	Haploid Oocytes (n = 23)		Aneuploid Oocytes (n = 21)	
	Dyads (%)	Pre-division (%)	Dyads (%)	Pre-division (%)
1	91.3	8.7	60.3	39.7
7	82.6	17.4	51.7	48.3
13	73.9	26.1	50.0	50.0
18	87.0	13.0	51.7	48.3
21	78.3	21.7	34.2	65.8
X	91.3	8.7	73.3	26.7

Discussion

It is known that oocyte meiosis is sensitive to endogenous and exogenous factors, which could lead to chromosomally abnormal oocytes and hence to abnormal zygotes. However, the signs of chromosomal imbalance in oocytes may be less obvious than in spermatozoa, in which abnormal morphology and reduced sperm number or motility are readily apparent. Hence, reliable techniques are needed to study aneuploidy and other chromosomal anomalies in otherwise morphologically normal human oocytes. We recently reported a method which utilised a modified spreading technique and two rounds of multi-probe FISH (Martini *et al.*, 1997). One of the aims of the present study was to improve the reliability of that method. This was achieved by removing the PB prior to spreading each oocyte, thereby reducing problems caused by an inability to differentiate between PB and oocyte DNA. This improved the overall accuracy of the FISH analyses. However, the spreading efficiency, i.e. the presence of DNA from the oocyte on the slide, was lower (57% versus 85%) than in our previous study (Martini *et al.*, 1997). This may have been caused by the pronase treatment which may have reduced the adhesiveness of oocytes, and/or the storage and shipping conditions which may have caused the loss of some oocyte spreads.

Incidence of aneuploidy in human oocytes

We found a high rate of aneuploidy (48% of oocytes in Group I). This has also been reported in other studies on human oocytes, but there is still debate as to whether these high estimates are accurate (Plachot, 1997). This high frequency of aneuploidy could be explained in several ways. Firstly, these oocytes failed to fertilise but some of them, especially the ICSI oocytes, would have contained sperm (Flaherty *et al.*, 1995). This might have lead to an incorrect conclusion of hyperhaploidy in a haploid oocyte due to extra signals from the sperm DNA, as already discussed in Martini *et al.*, (1997). However, it is more likely that this would elevate the polyploidy rate more than the hyperhaploidy or hypohaploidy rate. Secondly, the 20-24 h period between insemination and fixation, and/or the pronase treatment, might have adversely affected the meiotic spindle in some of these oocytes leading to de novo aberrations during in vitro culture.

However, it may also reflect the true incidence of aneuploidy in these oocytes which came from a heterogeneous population of follicles and may be physiologically compromised in some way because they failed to fertilise. Dyban *et al.* (1996) recorded aneuploidy rates as high as 37% for chromosomes X and 18 after FISH analysis, which is in agreement with earlier karyotyping reports on metaphase II human oocytes. The results of the present study revealed an overall aneuploidy rate of 44%, but there were different frequencies and patterns for each of the chromosomes studied, which is not surprising given that meiotic NDJ is non-random and exhibits chromosome-specific patterns (Plachot, 1997). In our

Figure 1. FISH results on human oocytes. Images from the first and second FISH rounds are shown in **a, c, e** and **b, d, f** respectively. In the first round, the signals are chr. 1 (yellow), chr. 13 (green) and chr. 21 (red). In the second round, the signals are chr. X (yellow), chr. 18 (green), chr. 7 (red). DNA is stained blue. (**a-b**) Haploid, paired signals in round 1 and hypohaploidy for chr. 18 in round 2. (**c-d**) Haploid, paired signals in round 1 and hyperhaploidy for chr. 7 and pre-division of chromatids in round 2. (**e-f**) Haploid oocyte showing pre-division of chromatids for all six chromosomes and hyperhaploidy for chr. 13 in round 1. For colour illustration see page 119.

study, there was an excess of hyperhaploidy for chromosomes 1, 7 and 18, an excess of hypohaploidy for chromosomes 13 and 21, and an equal distribution of hyper- and hypohaploidy for chromosome X. Karyotyping studies on oocytes typically report a higher incidence of hypohaploidy than hyperhaploidy and this is often attributed to artificial loss of chromosomes. Therefore the incidence of aneuploidy is often calculated by doubling the hyperhaploidy rate, resulting in so called "conservative estimates" of aneuploidy. This was not the case in our study because hypo- and hyperhaploidy were represented equally overall.

Pre-division of chromatids in oocytes

Angell *et al.* (1995) karyotyped human oocytes and reported that hyperhaploidy was not due to extra whole chromosomes (bivalents) but rather to an extra single chromatid or two single chromatids. This suggests that precocious division (pre-division) of chromosomal bivalents during the first meiotic division is the most likely mechanism for trisomy forma-

tion, rather than NDJ of whole bivalents as previously thought. Paired signals corresponding to paired chromatids (dyads) have been reported in FISH studies on human oocytes (Benkhalifa *et al.*, 1996; Dyban *et al.*, 1996; Wall *et al.*, 1996), however the detection sensitivity in those studies was low because only single-probe FISH was applied to a small number of oocytes. We reported in our previous study (Martini *et al.*, 1997) that the centromeres of chromatids were sometimes far apart from each other and not paired, but we did not relate this to the chromosomal constitution of the oocyte. In the present study, we found an association between aneuploidy and the position of the chromatids. Haploid oocytes generally showed dyads (frequency 74-91%), with the lowest percentages being associated with the acrocentric chromosomes (chr. 13, 21) and the use of locus specific probes which do not bind to the centromeric region. Aneuploid oocytes displayed a higher frequency of pre-division for all six chromosomes, especially the acrocentric chromosomes (chr. 13, 21). These results support the hypothesis that trisomy formation in humans occurs as a result of a premature division of bivalents at meiosis I (Angell *et al.*, 1993; Angell 1997). Since chiasma frequency declines with increasing maternal age, the reduction in chiasma frequency may be accompanied by the production of univalents, probably at the origin of NDJ (Angell, 1995). Alterations in cell cycle progression might in turn be the principal cause for maternal age-related predisposition to NDJ (Eichenlaub-Ritter and Boll, 1989). Moreover, Angell (1997) studied oocytes which had been in culture for ~ 70 hours prior to fixation. This period is longer than the total culture time the oocytes used in our study were exposed to. Munné *et al.*, (1995) and Dailey *et al.*, (1996) also suggested that some type of abnormalities found by Angell (1991) and Angell *et al.*, (1994) might be artifacts of in vitro culture and specifically related to time in culture. Unfortunately, the patient group in the present study was too small to search for correlations between aneuploidy, pre-division of bivalents and maternal age. This warrants further investigation.

In conclusion, the problem of differentiating PB DNA and metaphase II DNA was overcome by removing the zona pellucida and PB with pronase before spreading oocytes, and two rounds of FISH and six different chromosome probes gave a good insight into the chromosomal constitution of these oocytes and yielded more data than standard multi-probe FISH approaches using 3 probes. Furthermore, we found an association between pre-division of the chromatids and aneuploidy, supporting the hypothesis of Angell (1997) (Angell, 1997). This method is reliable and enables collection of data on multiple chromosomes in large numbers of oocytes.

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Chapter 8

General Discussion

Introduction

Increases in the success and efficiency of IVF and ICSI have been paralleled by questions about the chromosomal content of the manipulated gametes. During ICSI, motile spermatozoa showing normal morphology are selected for injection into the oocyte. An important presumption is that the selected sperm cells are expected to have a normal chromosomal constitution. It is however known that men involved in ICSI programs have an increased risk of carrying a chromosome abnormality compared with fertile men (Mau *et al.*, 1997; Stuppia *et al.*, 1998; Van der Ven *et al.*, 1998). There is also a risk that the eventual progeny might have infertility problems. The absence of natural selection of sperm prior to injection may increase the risk of injecting an abnormal spermatozoon. Furthermore, the injection procedure itself might induce aneuploidy by disturbing the meiotic spindle of the oocyte (Plachot 1995; Plachot 1997).

The in situ hybridization (ISH) procedure has in recent years been applied with varying success for the detection of chromosomal aberrations in gametes and embryos.

As outlined in the introduction to this thesis, the specific aims of this study were:

- ★ To improve the efficiency, sensitivity and reproducibility of ISH on human gametes.
- ★ To assess whether or not ISH is a valid alternative to karyotyping for estimating the incidence of aneuploidy in human gametes.
- ★ To analyze the incidence of aneuploidy in apparently normal spermatozoa and unfertilized oocytes.
- ★ To estimate the risk of transmitting chromosomal aberrations using gametes from men with karyotypic abnormalities.

The outcome of these studies will be discussed in a broader perspective using these questions as guide lines.

Efficiency, sensitivity and reproducibility of ISH on human gametes

In situ hybridization is a technique used to detect numerical and structural chromosomal aberrations in metaphase and interphase cells (Hopman *et al.*, 1988). Many different centres have used ISH to study aneuploidy in human spermatozoa, however, large discrepancies can be noticed in the published results (see Downie *et al.*, 1997 and Guttenbach *et al.*, 1997a for reviews). These discrepancies are due to the different protocols used for the ISH procedure and the methods used for sample preparation. Also, scoring criteria and interpretation of the results influences the outcome of such studies. As outlined in foregoing chapters of this thesis, partial decondensation of the sperm head prior to ISH is a crucial step for an efficient in situ hybridization. Overdecondensation can cause loss of morphology of the sperm cells and splitting of ISH signals, while underdecondensation reduces the hybridization efficiency (Martini *et al.*, 1995). Wyrobek *et al.*, (1993) showed that in human spermatozoa, fluorescent signals from repetitive probes for chromosomes Y and 8 can split into several signals if the sperm head DNA is overdecondensed. Martin and Spriggs (1995b) and Martin *et al.*, (1995a) who reiterated the importance of optimized sperm pretreatment procedures and strict scoring criteria, validated this. A standard decondensation and hybridization procedure, as well as a correct identification of spermatozoa is therefore essential. In chapter 2 of this thesis it is shown that an exposure of sperm cells for 5 minutes at room temperature to DTT and TRIS (pH 9.5) induces sufficient decondensation of the DNA in the majority of sperm heads to guarantee optimal ISH results (Martini *et al.*, 1995). After counterstaining with Diff-Quik, one can distinguish spermatozoa with aberrant tails (two tails, coiled tails) or deformed heads (two heads, microcephalic, macrocephalic) (Martini *et al.*, 1995). Brightfield microscopy enables better dis-

tion of morphologically abnormal spermatozoa than fluorescence microscopy, especially when analyzing sperm in neat semen (without further preparation by swim-up or Percoll gradient) because of the presence of somatic cells and immature germ cells (IGC). This in turn can influence the aneuploidy rate since somatic cells or IGC might erroneously be classified as aneuploid sperm cells. Moreover, the method we pioneered has the advantage to produce permanent preparations, since the precipitation products and the counterstaining will not fade, as fluorescent preparations do. However, this technique also has some shortcomings. The ISH signals sometimes overlap, do not localize properly and therefore affect aneuploidy estimates. The basic technical factors that may influence the result include the method of nuclear decondensation, the number of spermatozoa scored, the use of internal controls, scoring criteria, the specificity of the probes and the possible influence of DNA polymorphisms. The latter problem is more likely to be encountered when centromeric probes with a large insert size are used, and can be overcome by using locus specific probes with a smaller insert size. However, in this case, a more sensitive amplification detection system is needed and the combined use of fluorescent molecules (FISH) can help to overcome this limitation (Martini *et al.*, 1998a).

Fewer studies have addressed the chromosomal content of human oocytes, which is largely due to the limited amount of material available for research. Most investigation have used methanol and acetic acid (3:1) for fixation or a modification of Tarkowski (1966), and then performed karyotyping or ISH analysis (see Plachot 1997 for review). The cell spreading technique using HCl and Tween developed by Coonen *et al.*, (1994b) which is less aggressive than procedures based on methanol and acetic acid has considerably advanced this area of research. The application of chromosome-specific probes can help to analyse the chromosomal content of unfertilized oocytes and provides more precise identification of chromosomal abnormalities than karyotyping (in oocytes), however, FISH analysis is limited to only a small number of chromosomes. This can partly be overcome by simultaneous detection of multiple chromosomes (Dauwerse *et al.*, 1992; Smith *et al.*, 1998) or by multiple hybridizations, each to a different set of chromosomes (Martini *et al.*, 1998b; Martini *et al.*, 1997). For instance, we have analysed 9 chromosomes in the same oocytes using 3 hybridization rounds. The drawbacks are that the quality of the DNA decreases with each hybridization step and metaphase II chromosomes tend to be lost during the repeated washing and detection steps. Some of the limitations of our approach also need to be discussed. First, spreading inefficiencies remain and oocytes are sometimes lost due to mechanical or external factors during spreading. For instance, this might occur by dissolution of the oolemma in the pipette or loss of the oocyte during transfer from culture medium to the slide. Second, the metaphase II DNA of the oocyte is sometimes very difficult to detect, during spreading as well as during ISH analysis. Third, any ISH procedure engenders a certain risk of damaging the DNA, especially during denaturation steps, and this is even more pronounced when studying single cells.

Perhaps a combination of both techniques, FISH on karyotyped gametes, will in future help to obtain the most reliable and accurate results. It is unrealistic to assume that ISH procedures will ever provide error-free estimations of aneuploidy in gametes. However, it is much quicker and easier to perform than karyotyping, and it enables rapid and reliable accumulation of information with respect to the chromosome constitution of large numbers of human gametes.

ISH as a valid alternative to karyotyping for estimating the incidence of aneuploidy in human gametes

In recent years, molecular cytogenetic techniques such as ISH have been at the forefront

of research into the genetics of gametes. Human gametes were originally studied using more traditional cytogenetic techniques (Angell 1997; Guttenbach *et al.*, 1997a; Pellestor 1991a; Plachot 1995 for reviews) and sperm karyotyping using the hamster oocyte technique is still considered by many to be the "gold standard" against which new methods such as ISH must be evaluated (Robbins *et al.*, 1995). The direct visualization of human sperm chromosomes in the ooplasm of zona-free hamster oocytes was introduced by Yanagimachi *et al.*, (1976), and many studies have since been conducted (see Downie *et al.*, 1997 for review). One of the major drawbacks of this technique is the high incidence of loss of chromosomes during fixation (Jacobs 1992; Martin *et al.*, 1993). This problem led to a conservative estimation of aneuploidy based on doubling the disomy rate, yielding total aneuploidy frequencies of 0.9 to 4.0% (Kamiguchi and Mikamo 1986; Benet *et al.*, 1992; Estop *et al.*, 1991; Guttenbach *et al.*, 1997a; Downie *et al.*, 1997). This method provides valuable data because the entire chromosome complement of each spermatozoon can be analysed, and numerical as well as structural abnormalities can be studied. However, it is labour intensive and time consuming, and the results are potentially biased in that only those spermatozoa which can fertilize zona-free hamster oocytes are karyotyped. This may eliminate sperm cells with chromosomal abnormalities or morphological disadvantages that preclude them from fusing with oocytes. Nevertheless, this method provides useful data to compare with ISH results (Martin *et al.*, 1993; Spriggs *et al.*, 1996; Van Hummelen *et al.*, 1996). It is difficult to reconcile the results obtained by karyotyping and ISH analysis. ISH results show higher aneuploidy rates than determined by chromosomal analysis, which may be due to scoring biases resulting from non-specific hybridization, diploid sperm cells or diploid somatic cells (Martin *et al.*, 1993; Martini *et al.*, 1995 and Downie *et al.*, 1997 for review), the tendency of some chromosomes to undergo non-disjunction, especially chromosomes 21, X and Y, and the effect of alpha satellite DNA polymorphisms on detection of chromosomes using centromeric probes (Egozcue *et al.*, 1997 for review). Moreover, studies have shown considerable intra- and inter-individual variability in the frequency of aneuploidies (Downie *et al.*, 1997).

Many authors have published results of cytogenetic analysis of oocytes that failed to fertilize after IVF (Plachot 1995; Plachot 1997 for reviews). To date, karyotypic data on 3000 oocytes indicate that the overall aneuploidy rates varies from 8.1 to 54.2%, with an average of 26.5% (Plachot 1995; Plachot 1997). Aneuploid metaphase II plates can be attributed to loss of chromosomes during fixation (especially in the case of hypohaploidy), but also to non-disjunction, anaphase lag, anomalies in the structure or distribution of microtubules, and alterations in the cytoskeleton. The two main difficulties encountered while studying chromosomes in human oocytes are first, the scarcity of material, and second, the unusual and condensed nature of the chromatids on the metaphase II spindle (Pellestor 1991b). The condition of the chromatids hampers reliable analysis by karyotyping and necessitated the introduction of a modified protocol based on Tarkowski (1966) to effectively spread the chromosomes (Munné *et al.*, 1995c; Pellestor 1991a). However, this method has several drawbacks, such as loss of cells and loss of morphology, which reduces its overall efficiency and reliability.

Another major drawback of karyotyping is that the arrangement and morphology of the chromosomes is critical for obtaining an analysis, but many oocytes do not meet these criteria and therefore cannot be analyzed. Some studies report exclusion of almost 50% of the oocytes for this reason (Angell 1997; Pellestor 1991a). In contrast, the protocol used in our ISH studies on unfertilized human oocytes yielded spreading efficiencies of 57 to 85% and ISH efficiencies of 91 to 97%. Moreover, we were able to simultaneously investigate up to six different chromosomes in each oocyte (Martini *et al.*, 1998b; Martini *et al.*,

1997). All in all it can be concluded that the ISH procedure is a valid alternative to karyotyping for the detection of chromosomal abnormalities in human gametes.

Incidence of aneuploidy in apparently normal spermatozoa and unfertilized oocytes

Aneuploidy rates for specific chromosomes in spermatozoa vary greatly between published studies. For example, the lowest reported disomy rate for chromosome 1 was 0.02% (Van Hummelen *et al.*, 1996), while the highest was 0.96% (Pang *et al.*, 1994). Similarly, disomy rates for chromosomes X and Y vary widely (0.02 to 0.7% and 0 to 0.6%, respectively) (see Guttenbach *et al.*, 1997a for review). It is extremely unlikely that these differences arise solely as a result of inter-individual variations in the disomy rate. It is more likely that, as with the hamster technique for karyotyping sperm, different approaches in the experimental design are responsible for this wide variation (see Guttenbach *et al.*, 1997a and Downie *et al.*, 1997 for reviews).

Similarly, estimates of aneuploidy rates in human oocytes should be viewed with caution. Women undergoing IVF are not representative for the total population. The hormonal status leading to superovulation is very different from natural cycles and it is plausible that variations in the hormonal regimen may account for some of the reported differences in ploidy levels of oocytes. Whether or not superovulation induces an increase in the aneuploidy rate has not been determined yet (Gras *et al.*, 1992; Plachot 1997). It is important to remember that most studies have used sub-optimal material in that the oocytes used were generally those which failed to fertilize in vitro. Multiple factors associated with this failure are usually not taken into account and the oocytes may therefore not be representative of the entire cohort of oocytes. Procedural differences between studies and the choice of which oocytes are studied may influence such comparisons as well. Furthermore, there are marked differences between studies in the age of oocytes at the time of analysis. For example, Angell (Angell *et al.*, 1993; Angell *et al.*, 1994a and Angell 1994c; 1997) analyzed oocytes at 70 hours after collection, while Munné *et al.*, (1995c) fixed oocytes within 6 hours of retrieval. The oocytes analyzed in our studies were in culture for approximately 18-20 hours. The influence of oocyte age and other factors on the aneuploidy rate estimates warrants further investigation.

In the past years, only a few studies have been published in which ISH was used to analyse unfertilized human oocytes (Benkhalifa *et al.*, 1996; Dyban *et al.*, 1996; Wall *et al.*, 1996). Dailey *et al.*, (1996) reported 20% of oocytes to be aneuploid, despite the fact that they examined only four chromosomes, namely 13, 18, 21 and X. Since these chromosomes are responsible for approximately one fifth of all trisomies in spontaneous abortions, an implausibly high proportion of oocytes should have been aneuploid to account for the observed distribution of trisomies. A similar problem occurs in ISH studies on cleavage stage human embryos produced in vitro, in which unexpectedly high proportions of aneuploidy were scored (Munné *et al.*, 1995b). Our ISH studies on unfertilized oocytes (Chapter 6 and 7) also suggested a high aneuploidy rate. The fact that we found a higher percentage of aneuploid cells in ISH (38.4 to 44%) as compared to karyotype studies (25 to 30%) (Benkhalifa *et al.*, 1990; Pellestor 1991b; Plachot 1995; Plachot *et al.*, 1988), might be due to the small number of oocytes analyzed, however, it may more accurately reflect the true situation, since the oocytes were obtained from subfertile women who underwent fertility treatment. It should also be kept in mind that oocyte meiosis is very sensitive to endogeneous and exogeneous factors, which could lead to chromosomal abnormalities in oocytes (Plachot 1997).

The risk of transmitting chromosomal aberrations using gametes from men with karyotypic abnormalities

Useful information can be gleaned from studying the chromosomal constitution of spermatozoa in cases of known chromosomal abnormalities (e.g. XXY, XYY, translocations), unexplained infertility or a history of recurrent abortions (Bernardini *et al.*, 1997; Martini *et al.*, 1996; Martini *et al.*, 1998a).

In the study described in chapter 2, we used ISH to examine sperm from three men with 47,XYY and 47,XXY numerical chromosomal abnormalities (Martini *et al.*, 1996). The results revealed that a 46,XY germ cell line with a proliferative advantage over 47,XYY and 47,XXY cells must be present in these patients, which was recently confirmed by others (Mercier *et al.*, 1996; Blanco *et al.*, 1997; Chevret *et al.*, 1997; Guttenbach *et al.*, 1997b; Mercier and Bresson 1997).

However, the important point to bear in mind is that these patients have an increased risk of spermatozoa carrying sex chromosomal abnormalities (15% in XYY, 3% in XXY). Recently studies were published where the same findings are supported.

Meiotic segregation, recombination and aneuploidy have been assessed for spermatozoa from translocation carriers in recent studies by sperm chromosome complements (Colls *et al.*, 1998; Estop *et al.*, 1997) or by ISH (Guttenbach *et al.*, 1997a; Van Hummelen *et al.*, 1997). Chapter 4 presents the results of a study on sperm from a translocation carrier in which a combination of three probes, with different insert sizes (centromeric, cosmid and YAC) was used to evaluate the potential risk to offspring (Martini *et al.*, 1998a). Although this approach furnished results about the sperm cells and provided useful information, it has some deficiencies which are discussed extensively in chapter 4. In particular, the correct choice of DNA probes appeared to be essential and co-hybridization of the probes requires adaptation of the hybridization mixture.

Conclusions

Unquestionably, gamete genetics still has many challenging areas waiting to be explored. With the continuous evolution of medically assisted reproduction such as ICSI, the study of human gametes will become of greater importance in the clinical management of these patients. Knowing the chromosomal constitution of gametes can assist the geneticist to provide better counselling and risk estimations, and help patients to choose appropriate forms of treatment.

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Summary

Aim of the study:

The study of the chromosomal content of single gametes can provide valuable diagnostic information and contribute to basic knowledge in the field of reproductive biology. Patients carrying chromosomal defects or requiring medically assisted reproduction (MAR) techniques may benefit from gamete analysis.

Numerical and structural chromosomal aberrations are correlated with embryonic and fetal wastage, perinatal and infant mortality, mental retardation and congenital anomalies. Some studies suggest that approximately half of all human conceptuses bear chromosomal abnormalities. Analysis of gametes provides information on the origin and frequency of these chromosomal aberrations.

Methodology:

For this purpose a new protocol was developed to screen a relatively large number of gametes avoiding labour intensive and time-consuming pre-treatment steps. The non-radioactive in situ hybridization (ISH) technique allows the detection of chromosomal abnormalities in human interphase nuclei, and can thus be used for detection of numerical and structural chromosomal abnormalities in sperm cells and oocytes.

The combination of ISH, brightfield microscopy and Diff-Quik staining enables visualisation of single sperm cells and makes it possible to discriminate between nullisomic, disomic, diploid and morphologically abnormal spermatozoa, overlapping cells, somatic cells and immature germ cells (Chapter 2).

Spermatozoa:

In Chapter 3 semen from two 47,XYY males and one 46,XY/47,XXY male with fertility problems were analysed with the brightfield ISH procedure. The ratio of X- to Y-bearing spermatozoa did not differ from the expected 1:1 ratio, but the sex chromosome aneuploidy rate was much higher than in normal 46,XY males. In both 47,XYY males, approximately 15% of the sperm cells were aneuploid, while in the 46,XY/47,XXY male, the aneuploidy rate was 3%. The most striking finding was the relatively low percentage of spermatozoa in these patients, with an average of 65% in the XYY males and 84% in the XY/XXY male. The other cells were immature germ cells (IGC), including spermatogonia and spermatocytes. However, the finding that the majority of spermatozoa in semen of 47,XYY and 47,XXY males were not aneuploid strengthens the hypothesis that a 46,XY germ cell line must be present, apparently with a proliferative advantage over the 47,XYY and 47,XXY cells.

In the study described in Chapter 4, semen samples from 15 infertile males were analysed with sex chromosome probes in order to study whether a correlation exists between infertility and increased aneuploidy levels. Semen from fertile volunteers, unexplained infertility patients and severe male factor patients were analysed, and morphologically normal spermatozoa carrying an abnormal chromosome constitution for the sex chromosomes were detected at frequencies of 0.86%, 0.75% and 1.35%, respectively. The value of aneuploidy in the severe male factor group of patients was significantly higher than in the other two groups.

A fluorescent ISH approach was used to detect structural and numerical chromosomal aberrations in spermatozoa from a reciprocal translocation t(3;11)(q27.3;q24.3) carrier

(Chapter 5). The presence of chromosomal aberrations in spermatozoa is a direct consequence of the meiotic behaviour of the translocation chromosomes. To analyse the meiotic segregation modes, a tailored FISH approach was developed in order to study both numerical and structural aberrations in spermatozoa. A combination of three different probes, hybridising to the centromeric region of chromosome 11 and to two specific DNA loci (a cosmid probe for chromosome 11q and a YAC probe for chromosome 3q) were used. Spermatozoa from the patient and from a normal donor were analysed and the abnormal chromosome constitutions were established. In the translocation carrier, 44.3% of the analysable sperm cells carried a normal or balanced karyotype, while the remaining cells were abnormal resulting from adjacent I segregation mode (15.9%), adjacent II segregation (6.5%), 3:1 segregation (28.9%), 4:0 segregation (0.8%) and aberrant segregation (3.6%). In the control sperm cells, 92.0% exhibited a normal chromosome pattern.

Oocytes:

Although far from being ideal, unfertilised oocytes from IVF or ICSI cycles are the only source of human oocytes available for research. Overall, 30-50% of oocytes recovered for IVF fail to fertilise and can therefore be used for chromosome analysis. Various lines of evidence imply that oocyte meiosis is very sensitive to endogenous or exogenous factors and that the incidence of abnormalities is correlated to female age.

In Chapter 6 a method for studying chromosomal anomalies in unfertilised, metaphase II oocytes is presented. This new technical approach allowed us to analyse 85% of the oocytes, compared to the 30-50% of analysable cells obtained by karyotyping. Two rounds of fluorescent ISH were performed using directly labelled probes: chromosomes 1, 7, 15 (round 1); chromosomes 1, X, Y (round 2). The chromosome 1 probe was included in both rounds as an internal control for the specificity, sensitivity and efficiency of the fluorescent ISH procedure.

In the initial study it was not possible to differentiate between polar body (PB) DNA and metaphase II DNA. Therefore, in the following study (Chapter 7), we modified the technique by removing the first PB with pronase so that only the metaphase II chromosomes were evaluated by FISH. Six different probes were used: chromosomes 1, 13, 21 (round 1) and chromosomes X, 7, 18 (round 2). Furthermore, since precocious division (pre-division) of univalents at meiosis I is thought to be responsible for trisomy formation, the position of chromatids was investigated and an association was found with the chromosomal status of the cell. A prevalence of dyads was found in haploid oocytes while there was an increased frequency of pre-division of chromatids in the aneuploid oocytes, especially for chromosome 21.

Conclusions:

The techniques described in this study were successfully applied to study chromosomal abnormalities, both numerical and structural, in human gametes. ISH analysis of spermatozoa from carriers of translocations, numerical sex-chromosomal abnormalities, or on spermatozoa of patients with unexplained fertility problems is realistic. However, it is unrealistic to think that ISH will ever provide error-free estimations of aneuploidy rates in human spermatozoa and oocytes. Nonetheless, it is much quicker and easier to perform than karyotyping. Therefore it enables the accumulation of information about the chromosomal constitution of large numbers of gametes.

Samenvatting

Doel van het onderzoek:

De bestudering van de chromosomale inhoud van individuele gameten kan waardevolle diagnostische informatie bieden en bijdragen aan de basale kennis op het gebied van de reproductieve biologie. Het onderzoek van de gameten kan van nut zijn voor patiënten die drager zijn van chromosomale afwijkingen of gebruik maken van medisch geassisteerde voortplantingsmethoden.

Numerieke en structurele chromosoomafwijkingen veroorzaken embryonale, foetale en perinatale sterfte en mortaliteit op de kinderleeftijd, mentale retardatie en aangeboren afwijkingen. Door sommige auteurs wordt gesuggereerd dat ongeveer de helft van alle humane zygoten chromosomaal afwijkend is. Het onderzoek van de gameten geeft inzicht in het ontstaan en de frequentie van deze chromosomale afwijkingen.

Methodologie:

Er werd een nieuw protocol ontwikkeld dat het mogelijk maakt een relatief groot aantal gameten te onderzoeken zonder bewerkelijke en tijdrovende voorbehandeling. Met behulp van de niet-radioactieve in situ hybridizatie (ISH) techniek kunnen chromosoomafwijkingen in humane interfase kernen aangetoond worden. Deze methode kan derhalve ook gebruikt worden voor het onderzoek van numerieke en structurele chromosoomafwijkingen van zaadcellen en eicellen. Door ISH te combineren met de Diff-Quik kleuring kunnen individuele zaadcellen in een doorvallend lichtmicroscop beoordeeld worden op afwijkingen als nullisomie, disomie, diploidie, morfologische afwijkingen, overlap en kunnen zaadcellen onderscheiden worden van somatische cellen en onrijpe geslachtscellen (Hoofdstuk 2).

Spermatozoa:

In hoofdstuk 3 is de nieuwe methode toegepast bij twee 47,XYY mannen en een 46,XY/47,XXY man met vruchtbaarheidsproblemen. De verhouding X-: Y-dragende zaadcellen week niet af van de verwachte 1:1 verhouding, maar het percentage numerieke geslachtschromosomale afwijkingen was veel hoger dan bij normale 46,XY mannen. Bij beide 47,XYY mannen was ongeveer 15 % van de zaadcellen aneuploid, terwijl het aneuploidie percentage bij de 46,XY/47,XXY man ongeveer 3 % bedroeg. De meest opvallende bevinding was het relatief lage percentage zaadcellen dat werd waargenomen bij deze patiënten. Dit bedroeg gemiddeld 65 % bij de XYY mannen en 84 % bij de XY/XXY man. De andere cellen waren voornamelijk onrijpe geslachtscellen, zoals spermatogonia en spermatocyten. De bevinding dat de meerderheid van de zaadcellen in het semen van 47,XYY en 47,XXY mannen niet aneuploid was is een sterke aanwijzing voor de hypothese dat er een 46,XY geslachtscellijn aanwezig moet zijn, die waarschijnlijk in proliferatief opzicht in het voordeel is ten opzichte van de geslachtschromosomaal afwijkende cellen. Het onderzoek dat beschreven wordt in Hoofdstuk 4, heeft betrekking op de analyse van zaadmonsters van 15 onvruchtbare mannen. Dit werd gedaan om na te gaan of er een verband bestaat tussen onvruchtbaarheid en toename van aneuploidie. Semen van vruchtbare vrijwilligers werd vergeleken met dat van patiënten met onverklaarde infertiliteit en patiënten met een ernstige infertiliteit op basis van een mannelijke factor. Bij deze drie groepen werden in morfologisch normale zaadcellen percentages afwijkingen van de geslachtschromosomen gevonden van resp. 0,86 %, 0,75 % en 1,35 %. Het aneuploidie percentage in de laatste groep was significant hoger dan dat van de andere twee groepen.

Deze bevindingen werden bevestigd door gebruik te maken van probes voor de autosomen 1 en 17.

Fluorescente in situ hybridizatie (FISH) werd toegepast om structurele en numerieke chromosomale afwijkingen te onderzoeken in zaadcellen van een drager van een reciproke translocatie $t(3;11)(q27.3;q24.3)$ (Hoofdstuk 5). De aanwezigheid van chromosomale afwijkingen in de zaadcellen van een dergelijke translocatiedrager is een direct gevolg van het gedrag van de translocatiechromosomen in de meiose. De verdeling van de bij de translocatie betrokken chromosomen over de meiotische dochtercellen werd onderzocht met behulp van een speciaal ontwikkelde FISH methode waarmee zowel numerieke als structurele afwijkingen van de zaadcellen konden worden aangetoond. Er werd gebruik gemaakt van een combinatie van drie verschillende probes, die hybridiseerden met respectievelijk het centromeer gebied van chromosoom 11 en twee specifieke DNA loci (een cosmide probe op chromosoom 11q en een YAC probe op chromosoom 3q). De zaadcellen van de translocatiedrager werden vergeleken met die van een normale donor en de afwijkende chromosomale constituties werden vastgesteld. Bij de translocatiedrager had 44,3 % van de analyseerbare zaadcellen een normaal of gebalanceerd karyotype, terwijl de overige cellen afwijkend waren tengevolge van adjacent I segregatie (15,9 %), adjacent II segregatie (6,5 %), 3:1 segregatie (28,9 %), 4:0 segregatie (0,8 %) en andere afwijkende segregaties (3,6 %). In het controle monster liet 92,0 % een normaal segregatiepatroon zien.

Oöcyten:

Ofschoon ze zeker geen ideaal onderzoeksobject vormen, zijn na IVF en ICSI onbevruucht gebleven oöcyten het enige beschikbare materiaal voor onderzoek van de vrouwelijke humane gameet. In het algemeen kan gesteld worden dat 30-50 % van de oöcyten onbevruucht blijft en derhalve beschikbaar is voor chromosomaal onderzoek. Er zijn allerlei aanwijzingen dat de meiose bij de vrouw erg gevoelig is voor endogene en exogene factoren en dat de incidentie van chromosoomafwijkingen gecorreleerd is met de leeftijd van de vrouw.

In hoofdstuk 6 is een methode beschreven voor de chromosomale bestudering van onbevruucht gebleven, metafase II oöcyten. Deze nieuwe benadering maakte het mogelijk 85 % van de eicellen te analyseren. Na klassieke karyotypering kan een resultaat verkregen worden bij 30 tot 50 % van de cellen. Hiertoe werd FISH met direct gemerkte probes toegepast in twee opeenvolgende ronden: chromosoom 1, 7 en 15 probes in ronde 1 en chromosoom 1, X en Y probes in ronde 2. De chromosoom 1 probe werd in beide ronden toegepast om een interne controle te hebben voor de specificiteit, sensitiviteit en efficiëntie van de FISH procedure.

In het eerste onderzoek was het niet mogelijk het DNA van het poollichaampje te onderscheiden van dat van de metafase II. Daarom werd de methode in het vervolgonderzoek aangepast door eerst het poollichaampje te verwijderen met pronase zodat slechts de metafase II chromosomen overbleven voor FISH onderzoek (hoofdstuk 7). Zes verschillende probes werden gebruikt: chromosoom 1, 13 en 21 probes in ronde 1 en chromosoom X, 7 en 18 probes in ronde 2. Bovendien werd de positie van de chromatiden onderzocht, aangezien vroegtijdige deling (pre-divisie) van univalenten tijdens meiose I verantwoordelijk wordt gehouden voor trisomie vorming. Er werd een verband gevonden met de chromosomale status van de cel. Bij haploide oöcyten overheerste de aanwezigheid van normale univalenten terwijl er sprake was van een verhoogde frequentie van pre-divisie van de chromatiden bij de aneuploide oöcyten en speciaal bij aneuploidie voor chromosoom 21.

Conclusies:

De methoden die beschreven zijn in dit proefschrift zijn met succes toegepast bij de bestudering van numerieke en structurele chromosoomafwijkingen van menselijke geslachtscellen. ISH analyse van zaadcellen van translocatiedragers, patiënten met geslachtschromosomale afwijkingen of patiënten met onverklaarde infertiliteit is haalbaar. Het is echter niet realistisch te veronderstellen dat het ooit mogelijk zal zijn met behulp van ISH tot foutloze bepalingen van het percentage aneuploidie van humane spermatozoa en oöcyten te komen. Daar staat tegenover dat ISH veel sneller en gemakkelijker uitgevoerd kan worden dan karyotypering. Daardoor is het mogelijk informatie te verzamelen over de chromosomale inhoud van grote aantallen gameten.

Curriculum Vitae

Elena Martini was born on the 29th of February, 1968 in Livorno, Italy. She graduated in 1987 at the Deutsche Schule Genua and in the same year started her Biology study at the University of Genoa, where she obtained her Masters degree in 1993. In September 1993 she came to the Stichting Klinische Genetica in Maastricht financed by a Commett Grant from the EC. This initial stage of 6 months developed into a Ph.D. project, to be performed at the University of Limburg, Department of Molecular Cell Biology & Genetics (Prof. Dr. FCS Ramaekers and Prof. Dr. JPM Geraedts, under supervision of Dr. AHN Hopman) in collaboration with the Department of Obstetrics & Gynaecology, University of Genoa, Italy (Prof. Dr. GL Capitano). In 1996 she obtained a 2-years Training and Mobility Research Fellowship from the EC and continued her project as researcher at the University of Maastricht. In September 1996 she visited on basis of a joint grant from the Netherlands Organisation for Scientific Research and Australian Research Council (NWO-ARC), The Queen Elizabeth Hospital in Adelaide, Australia (Prof. Dr. CD Matthews and Dr. SP Flaherty) where she stayed 3 months and performed part of the work published in this thesis.

She is married to Maurice Jansen and they have a one-year old son, Martino.

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Chapter 2, page 30 - Figure 1. (magnification for Figures a,b,d,e,f,g,h,i: 1000X; Figure.c: 2000X) (a.) Single-target ISH with a biotinylated probe for chromosome 1, detected with the DAB reaction. The arrow indicates a spermatozoa with 2 ISH signals for chromosome 1. (b.) Double-target ISH with a biotinylated probe for chromosome X and a FITC-labelled probe for chromosome Y. Detected with the Fast-Red and the TMB reactions, respectively. (c-d.) Triple-target ISH with a biotinylated probe for chromosome 1, a digoxigenin-labelled probe for chromosome 7 and a FITC-labelled probe for chromosome 17. Detected with DAB, Fast-Red and TMB, respectively. In Figure 1c the arrow indicates the tail. (a-d.) Counterstaining with haematoxylin. (e-g.) Single-target ISH with a 1-bio probe, detected with DAB. The arrow in (f.) indicates a spermatozoa with 2 ISH signals for chromosome 1. The arrow in (g.) indicates a morphologically abnormal sperm cell, with two heads, two signals, two midpieces, but one tail. (h.) Single-target ISH with 1-bio probe, detected with TMB. The arrow indicates a morphologically abnormal sperm cell with one head but with two ISH signals and two tails. (i.) Somatic cell and double-target ISH with a X-bio probe and FITC-labelled probe for chromosome Y, detected with DAB and TMB, respectively. (e-i.) Counterstaining with Diff-Quik.

Chapter 3, page 41 - Figure 1. Diff-Quik and Haematoxylin stained semen preparation to which double-target ISH was applied with an X-probe (brown) and a Y-probe (green). (a) Control sample showing an XX-bearing spermatozoon. (b, c) Immature germ cells from an XYY male (b) and an XXY male (c) showing a XYY- and XY- constitution respectively. Morphologically normal spermatozoa from an XXY male (d, e) and an XYY male (f, g) showing a normal sex-chromosome constitution and YY constitution respectively. (h, i) Morphologically abnormal spermatozoa from the XXY patient. (Magnification x 1000).

Chapter 4, page 50 - Figure 1. Double target hybridization of semen samples with biotinylated X probe (brown) and fluorescent isothiocyanate (FITC)-labelled Y probe (green). **(a-d)** Morphologically normal spermatozoa. **(a)** Control sample showing either X or Y signal; **(b)** spermatozoa showing an XY sex chromosome constitution; **(c)** Sperm cell with an XX sex chromosome constitution; **(d)** Sperm cell with a YY sex chromosome constitution. **(e)** Morphologically abnormal sperm cells, top left with two heads and two chromosome X-signals; bottom right spermatozoon with two tails and an XY chromosome constitution. **(f)** Immature germ cell with hybridization signal for chromosomes X and Y.

Chapter 5, page 64 - Figure 1. FISH results on peripheral blood cells (a-c) and spermatozoa (d-g) of the patient. (a) Triple target hybridization to metaphase spreads with the probes CEP11, TYAC 162 and D11S-477, respectively in orange, green and red. (b) Particular of the chromosomes involved in the translocation and the probes applied. (c) Lymphocyte, hybridized with triple FISH, showing the difference in signal size for the centromere and the locus specific probe used. (d) Spermatozoa of the patient hybridized with triple FISH (for explanation see figure 3). (i) Adjacent II segregation mode, signals for CEP11 and TYAC162; (ii) 3:1 segregation mode, signals for CEP11 and D11S-477; (iii) Alternate segregation, normal or balanced sperm with signals for CEP11, TYAC 162, D11S-477; (iv) Aberrant segregation mode, two signals for CEP11. (e) Spermatozoa of the patient hybridized with triple FISH (for explanation see figure 3). (i) Adjacent I segregation, signals for CEP11 and twice for TYAC 162; (ii) and (iii) Alternate segregation mode, normal or balanced spermatozoa; (iv) 3:1 segregation mode, signals for CEP11, TYAC 162 and twice for D11S-477. (f): double FISH with chromosomal probes for chromosomes 13 (green) and 21 (red). All spermatozoa show a normal, haploid, chromosomal constitution. (g): double FISH with the sex-chromosomal probes. In green the X-probe, in red the Y-probe. At the bottom, right corner, a sperm cell carrying an XY constitution is visible.

Chapter 5, page 67 - Figure 3. Schematic explanation of the chromosomes involved in the translocation t(3;11)(q27.3;q24.3) and the probes used. The quadrivalent and the various segregational modes are shown. The corresponding resulting gametes are illustrated. For simplification the CEP11 signal, which is detected in red, is here illustrated in yellow. CEP 11 = Yellow, TYAC 162 = Green, and D11S-477 = Red.

Chapter 6, page 80 - Figure 2. Spreading of the oocyte (a-c): (a) Oocyte with first polar body (PB) and no pronuclei. At the beginning of spreading, the oolemma is still present. (b) Lysis of the PB, the DNA is visible. (c) Isolated metaphase DNA. FISH results (d-i): 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 ICSI oocytes. First and second FISH rounds respectively on the same DNA (d,e): 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. First and second FISH rounds respectively on the same DNA (f,g): 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. First and second FISH rounds respectively on the same DNA (h,i): Example of aneuploidy for all the chromosomes analyzed. 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.

Chapter 7, page 93 - Figure 1. FISH results on human oocytes. Images from the first and second FISH rounds are shown in **a, c, e** and **b, d, f** respectively. In the first round, the signals are chr. 1 (yellow), chr. 13 (green) and chr. 21 (red). In the second round, the signals are chr. X (yellow), chr. 18 (green), chr. 7 (red). DNA is stained blue. **(a-b)** Haploid, paired signals in round 1 and hypohaploidy for chr. 18 in round 2. **(c-d)** Haploid, paired signals in round 1 and hyperhaploidy for chr. 7 and pre-division of chromatids in round 2. **(e-f)** Haploid oocyte showing pre-division of chromatids for all six chromosomes and hyperhaploidy for chr. 13 in round 1.