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Meiotic Chromosome Abnormalities and Spermatic FISH in Infertile Patients with Normal Karyotype

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1. Introduction

It is generally accepted that infertility affects 15% of couples at reproductive ages. The causes of infertility are 38% female, 20% male, 27% mixed and 15% unknown (Ferlin et al., 2007). The male factor is the sole responsible party or a copartner in infertility in 50% of couples. About 7-8% of men have infertility problems or are the cause of miscarriages. Chromosomal causes rank high among the causes of infertility. 50% of first-trimester abortive eggs have aneuploidy (Hassold et al., 1980). In second and third-trimester miscarriages, the aneuploidy rate drops to 15% and 5%, respectively (Simpson, 2007). Among living newborns, 0.5% to 1% shows aneuploidy (Gardner and Sutherland, 2004). Among the infertile population, 15% of infertility is due to chromosomal or genetic reasons (Griffin and Finch, 2005).

The prevalence of chromosomal alterations that are only meiotic, with a normal mitotic karyotype, is unknown. Checking for sperm chromosomal alterations requires testicular biopsy, which is an invasive procedure because meiotic cells, spermatocytes I and II present in ejaculate does not tend to be valid, due to their scarcity and poor condition. Unlike mitosis, which can affect other organs and functions, chromosomal alterations of solely meiosis may have an impact on reproductive capacity. Meiotic chromosomal anomalies can cause alterations to one or more of the basic seminal parameters including sperm count, motility and morphology, even lead to the formation of aneuploid gametes. At a clinical level, men with meiotic anomalies will have primary or secondary infertility or produce gestations with miscarriages. Secondary infertility or difficulty in having another child can be explained by the coexistence of altered cell lines and other normal cell lines (mosaicism).

The meiotic chromosomal anomalies may be studied by directly observing meiotic cells obtained from testicular biopsies. Understanding impact on fertility requires: 1) ascertaining the patient's reproductive history with his current and past partners, if there were any; 2) semen analysis, which can be normal with regard to its three basic parameters, have somewhat severe alterations in some or all sperm parameters, or even azoospermia; 3) studying the testicular histopathology that, at an optical level, can swing between complete blockage at the level of spermatocyte I or II, through apparently normal spermatogenesis and 4) studying aneuploidy present in sperm.

Genetic–not chromosomal– alterations that have an impact on fertility such as Kallmann Syndrome, cystic fibrosis, globozoospermia, 9+0 Syndrome, Y-chromosome microdeletions, etc. will not be the focus of this chapter. Nonetheless, the limits between genetic and chromosomal alterations are more academic than real, as genetic alteration of meiosis tends to be the grounds for chromosomal meiotic alteration.

This chapter will set forth the results of FISH on sperm and of the study on meiotic chromosomes in testicular biopsy for infertile patients. Among the patients on whom both studies were conducted -testicular biopsy and sperm-FISH- we present the findings for 60 with more precise clinical data on semenology for FISH and testicular meiosis.

The aims of this paper are two: a) to know the incidence and types of spermatic aneuploidies and testicular meiotic anomalies in general infertile men, and b) to correlate the results of both studies: spermatic aneuploidies with meiotic chromosomes.

2. Spermatogenesis

The spermatogenesis process lasts some 64 days (Heller and Clermont, 1964) and takes place inside the seminiferous tubules in adult testes. There are three different stages:

2.1 Spermatogonial stage

In this phase, the spermatogonia divide by mitosis. Some remain as cell reserves to divide again later and others enter meiosis. The spermatogonia are located in the basal compartment of the seminiferous tubule between the tubular wall, the Sertoli cells and the inter-Sertolian tight junctions. The Sertoli cells and the tight junctions form the hemato-testicular barrier and create an avascular space (the luminal compartment) in the centre of the seminiferous tubule. The spermatogonia that go into meiosis move from the basal to luminal compartment through the spaces between the Sertoli cells, thanks to the dissolution and reformation of the tight junctions (Byers et al., 1993).

2.2 Spermatocytal or meiotic stage

During this phase, meiosis has two cell divisions that take place in spermatocytes I and II. In the first meiotic division, the spermatocyte I gives rise to two spermatocytes II. The division of the spermocyte II, the second meiotic division or equational divisions, gives rise to two spermatids. This process usually lasts about two weeks (Heller and Clermont, 1964). Finally, each spermatid spawns one spermatozoon.

2.3 Spermiogenesis stage

There is no cell division in this stage, but cell differentiation of spermatid into spermatozoon.

2.4 Spermiation

The sperm detach from the Sertoli cells and are released into seminiferous tubule lumen.The meiotic and spermiogenic stages take place in the luminal compartment of the seminiferous tubule. There, specific hormonal conditions are created, among which the high concentration of testosterone must be pointed out. This chapter will only deal with the meiotic stage.

3. Meiosis

During the meiotic process, two essential and specific events occur: genetic recombination and reductional cell division. Genetic recombination is produced by the exchange of genes between homologous chromosomes that form a pair, with one inherited from the father and the other from the mother. This interchange of genes gives rise to an astronomical genetic variability of spermatozoa (and also the oocytes), on the order of 2^{23} per gamete and $2^{23} \times 2^{23}$ per embryo. Thus, the process facilitates the appearance of gene combinations that are different than the ones the man and woman have. Some of these new gene combinations can be advantageous for the individual and for the species. Others can be more or less pathological. Meiosis is the physical foundation of Mendelian genetic inheritance. The second crucial event that occurs during meiosis is reductional cell division. Meiosis means reduction in Greek. All of the body's nucleated cells contain 46 chromosomes (23 pairs). Only the cells from meiosis I: spermatocytes II, spermatids and spermatozoa, have 23 chromosomes instead of 23 pairs. Spermatocyte II chromosomes contain two chromatids and spermatids and spermatozoa chromosomes contain a single chromatid. The opposite of meiosis is fertilization. In this process, the chromosomes of the haploid spermatozoon (n=23) join with those from the oocyte, also haploid (n=23) and through syngamy form a zygote, which is diploid, with 23 pairs of chromosomes (n=46).

Sexual reproduction is based on meiosis and fertilization. Meiosis assures that the number of chromosomes remains constant from one generation to the next, from parents to children, and that they have different gene combinations than their progenitors. Each chromosome, except for the sexual XY pair, has its homologous chromosome: one of paternal and the other of maternal origin. The X chromosome is always maternal and the Y is always of paternal origin. A chromosome is determined by a centromere and can have one or two chromatids –called sisters- depending on the phase of the cell cycle. There is no genetic recombination between them, as one is a copy of the other. Genetic recombination takes place between homologous chromatids, not sisters.

3.1 First meiotic division

Premeiotic or preleptotene phase: During premeiotic synthesis (phase S), two chromatids are produced in each chromosome via DNA replication with identical genetic content, called sister chromatids, which remain joined, bound, through the G2 phase of the cellular cycle. Premeiotic synthesis is particularly long, lasting some 24 hours. When the S phase ends, the DNA content of each homologous chromosome pair is a tetrad, namely, each pair has four chromatids. The two chromatids from the same chromosome are called sisters. They are termed homologous with respect to the chromatids in the homologous chromosome. The union of the sister chromatids is maintained by a ring-shaped structure that mediates cohesion between them (Gruber et al., 2003) and is formed of proteins from the cohesin complex. At a centromeric level, it is formed by the cohesins shugoshin and sororin. Shugoshin has been located in the pericentromeric region (Lee et al., 2008).

3.1.1 Prophase I

Attachment of chromosomes to the internal nuclear membrane

Dispersed throughout the nucleus, when meiosis begins the chromosomes start to move towards the nuclear membrane, attaching by their telomeres (Fig. 1A). The karyotheca is

denser at the sites where the telomeres attach. The telomeres move along the internal face of the karyotheca and congregate around the centrosome, shaping a bouquet (Fig. 1B) (Zickler and Kleckner, 1998; Scherthan, 2001; Bass, 2003). The formation of the bouquet requires actin (Trelles-Sticken et al., 2005). Each chromosome approaches its homologue, which it recognizes.

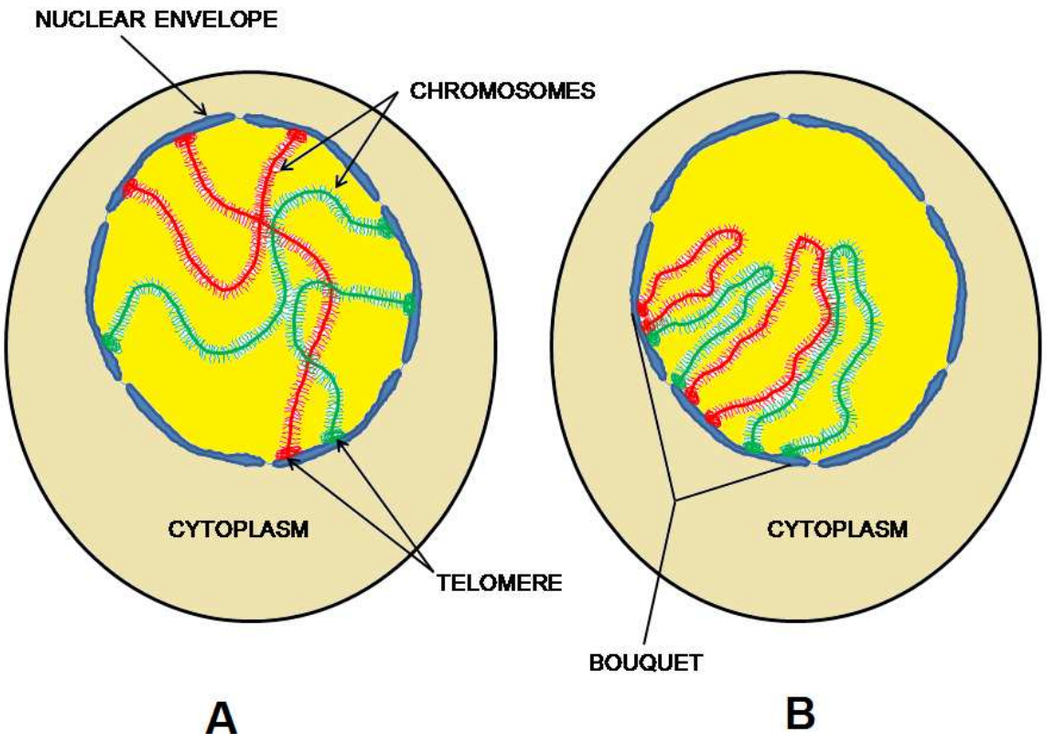


Fig. 1. Fixing the telomere to the nuclear envelope (A). Formation of the bouquet (B)

If the telomeres do not attach to the karyotheca and/or do not form a bouquet, pairing and genetic recombination are altered (Trelles-Sticken et al., 2000). The bouquet shape is seen at the end of the leptotene stage and during the zygotene stage and disappears in the pachytene phase. The chromosomes, already paired and after genetic recombination has taken place between homologous chromatids, disperse over the entire surface of the karyotheca, but are still attached to it. The telomeres detach from the nuclear envelope and the cells proceed to diakinesis (review: Alsheimer, 2009).

Leptotene: The alignment of homologous chromosomes

Almost in parallel to the grouping of the telomeres, the homologous chromosomes align, which is conditioned by the formation of long thin strands along the chromosomes during the first stage of prophase I, or leptotene. These are the lateral elements (LE). Each LE is associated with a pair of sister chromatids. The LEs expand when attaching to the internal nuclear membrane.

Zygotene: The pairing of homologous chromosomes, or synapsis

The leptotene stage is followed by the zygotene, during which the chromosomes thicken and each one pairs up with its homologue. The XY sex pair forms the sex body.

Pairing, or synapsis, requires the formation of the synaptonemal complex (SC) described by Fawcett with the electronic transmission microscope (Fawcett, 1956). The SC (Fig. 2) is a protein structure with three longitudinal elements, two lateral elements (LE), already seen in the previous leptotene stage, and a central element (CE), which provides stability to the SC (Hamer et al., 2006; Bolcun-Filas et al., 2007). The LEs are parallel and equidistant from the CE. The LEs stick to the nuclear envelope. The SC's structure is completed by fine transverse filaments (TFs), which connect the LEs and are perpendicular to the LEs and CE. Each LE is associated with a pair of sister chromatids. The homologous chromosomes are intimately associated at a distance of 100 nm (Zickler, 2006).

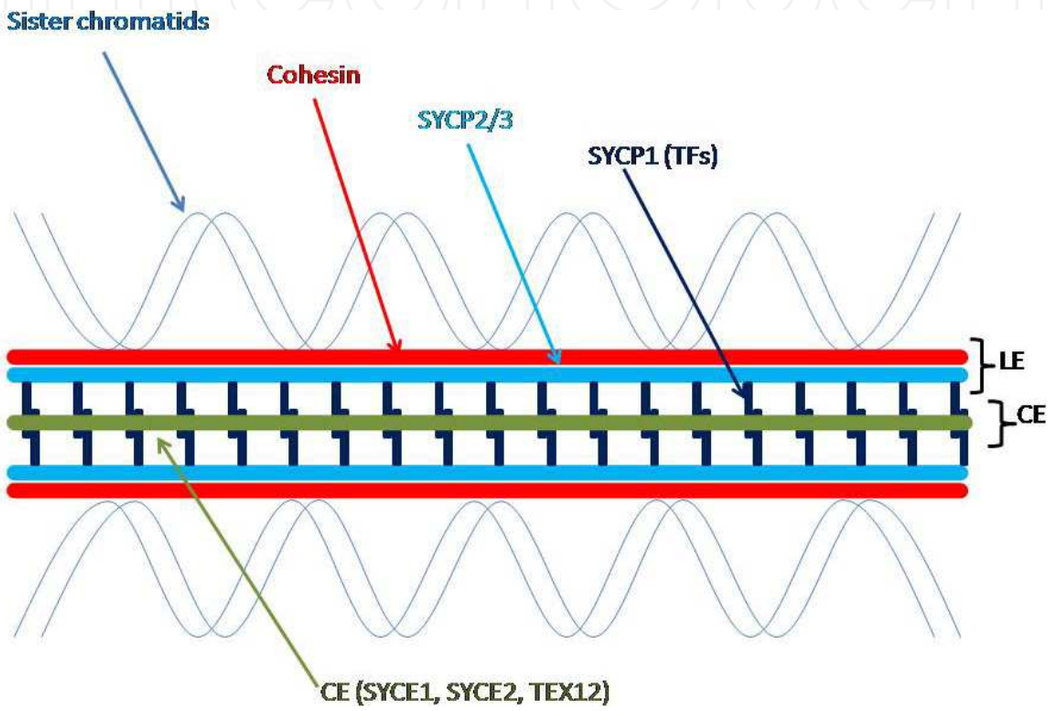


Fig. 2. Diagram of the synaptonemal complex (SC)

LEs are made up of the proteins SYCP2 and SYCP3 and cohesin complexes, which include SMC1 beta, REC8 and STAG3, specific to meiosis (Revenkova and Jessberger, 2006). The specific proteins SYCE1, SYCE2 and TEX12 have been identified in the CE (Costa et al., 2005; Hamer et al., 2006). The protein SYCP1 has been identified in the TFs (Meuwissen et al., 1997). The chromatin is attached to the LEs, forming a series of loops.

Pachytene: Genetic exchange or recombination

Genetic exchange or recombination starts in the DNA double-strand break (DSB) that initiates in the preleptotene and leptotene phases, generated by the enzyme topoisomerase II (Lichten, 2001; Keeney and Neale, 2006). Genetic recombination takes place between homologous chromatids, not sisters. This process is independent in each spermatocyte, which explains the differences between siblings. It takes place predominantly in genomic loci, termed hotspots, close to the telomeres (Lynn et al., 2004). The DSB is an indicator of high genetic exchange activity in the hotspots and is not distributed either randomly or uniformly (Petes, 2001; Nishant and Rao, 2006; Buard and de Massy, 2007).

There is proof of epigenetic control in genetic recombination, as hotspot activity is not determined by the local DNA sequence (Neumann and Jeffreys, 2006). The factors and mechanisms that determine the location of hotspots in the genome are unknown. The hotspot is believed to be the minimum functional unit of recombination. To detect hotspots, a resolution power between 100-200 kb is needed. Crossovers are more frequent close to the telomeres (Lynn et al., 2004). They are the basis of chiasmata (crossings) observed with the optical microscope (Nishant and Rao, 2006). Only two homologous chromatids intersect in each chiasma. The other two do not participate. The number of chiasmata observed in the pachytene and diplotene is similar. This number oscillates from 50 to 53, with an inter- and intra-individual variation of 3-10% (Codina-Pascual et al., 2006). During the pachytene stage, homologous chromosomes are attached only by the crossovers or chiasmata. A single chiasma is observed in the XY pair in the pseudoautosomal region. This pair of sexual chromosomes has a condensed chromatin and forms a corpuscle, termed the sex body, glued to the internal face of the spermatocyte's karyotheca (Solari, 1974). The breaking of the DNA chain needed for genetic exchange must be repaired. The broken DNA chain that ends in 3′ remains free. It associates with recombinases and starts searching for complementary base sequences in another DNA molecule corresponding to the homologous chromatid.

Diplotene: Desynapsis

The SC is dismantled in the following diplotene stage. The homologous chromosomes desynapse, separate, but remain connected by chiasmata and the sister chromatids continue to be attached.

3.1.2 Metaphase I

The nuclear membrane disappears in this phase and the homologous chromosome pairs (bivalent) align along the equator of the meiotic spindle. A bivalent chromosome has two centromeres and four chromatids. Each homologous chromosome has at least one chiasma required for correct segregation.

3.1.3 Anaphase I

During anaphase I, the arms of the homologous chromosomes lose cohesion, with the chiasmata disappearing and segregating. The centromeres attach to the spindle's microtubules, which pull each homologue towards an opposite pole. Half of the chromosomes are attracted towards each opposite pole by the spindle fibres. Homologous chromosomes separate completely, without separation of the sister chromatids. Each chromosome has two chromatids. Centromeres do not duplicate or divide. The segregation of homologous chromosomes in anaphase I requires the release of the REC8 cohesin by the separase enzyme (Kudo et al., 2009). Cohesion between the arms of the sister chromatids is lost, although cohesion is maintained at a centromeric level.

3.1.4 Telophase I

Each spermatocyte II has a haploid number of chromosomes (n=23), but each chromosome is formed of two chromatids. All the homologous chromosomes are pulled towards opposite poles and the nuclear membrane is formed.

3.1.5 Interphase or interkinesis

Interkinesis, or interphase, between the first meiotic cell division and the second is very short. The second meiotic division is not preceded by DNA synthesis. It is a quick phase, similar to mitotic cell division. The division of the cytoplasm is incomplete and the two cells remain in communication via intercellular bridges.

3.2 Second meiotic division

During anaphase II, the shugoshin cohesin becomes inactive and the centromeres' cohesion is lost (Marston and Amon, 2004). The two kinetochores, elliptical disks on each side of the centromere, separate. The kinetochores have an anphitelic orientation (biorientation, namely, each one is pulled towards an opposite pole). They attach to the spindle microtubules and the chromatids segregate by action of the separase enzyme, like during anaphase I (Kudo et al., 2009; review: Barbero, 2011).

The resulting cells –spermatids- only contain one set of haploid chromosomes (n=23), where each chromosome has one chromatid. Through cell differentiation, spermatids give rise to spermatozoa.

Of the approximate two weeks that meiosis lasts, prophase I takes some 12 days, and the other phases of meiosis happen in one to two days.

During the two weeks of meiosis, two cell divisions have taken place and four haploid cells have been generated from a cell with tetrad DNA, each one with a different genetic content. Both genetic recombination and haploidization have occurred.

4. Causes of meiotic chromosome abnormalities

Meiotic alterations can be due to different causes and different mechanisms. To summarise, we can group them into the sections below.

4.1 Mitotic alterations of spermatogonia that have an impact on meiosis

In Klinefelter Syndrome with a 47, XXY karyotype, XY pairing is altered. At least part of the spermatogonia in patients with euploid spermatozoa seems to be euploid (Bergère et al., 2002). Robertsonian and reciprocal translocations produce trivalents and tetravalents at a meiotic level, respectively.

4.2 Alterations of genes involved in meiosis

More than 200 genes are expressed in meiosis. If there is gene expression in other organs and tissues as well, man will exhibit other pathologies. These may be revealed in alterations of the cohesins (cohesinopathies) that intervene in meiosis, in DNA repair (Watrin and Peters, 2006), and in gene expression (Dorsett, 2007). Roberts' Syndrome is due to a cohesinopathy (Gerkes et al., 2010). The alteration of SC proteins can lead to infertility. Patients with heterozygosis mutation in the SYCP3 protein gene exhibit azoospermia (Miyamoto et al., 2003). The absence of the REC8 cohesin also causes infertility by altering synapsis (Bannister et al., 2004; Xu et al., 2005). Shugoshin and sororin are proteins from the cohesin group needed to maintain the cohesion of the centromere. The inactivation of shugoshin in meiosis II allows the separation of

the sister chromatids. If there is no inactivation, then there is no disjunction of these chromatids and aneuploid gametes are produced. Infertility in mice has been described owing to the lack of the cohesin SMC1 beta that causes blockage during pachytene (Revenkova et al., 2004). The lack of the SYCP1 protein on TFs lets the chromosomes align, but they do not create pairs (de Vries et al., 2005).

4.3 Epigenetics

Epigenetic entails temporary inheritable changes in gene expression without changes to the DNA base sequence. Gene expression is influenced by the degree of DNA methylation. Methylation can be affected by cadmium chloride, arsenic and nickel compounds. There are claims that incorrect DNA methylation can induce aneuploidy (review: Pacchierotti and Eichenlaub-Ritter, 2011).

4.4 Organophosphate pesticides

Organophosphate pesticides pass through the hemato-testicular barrier, interfere with chromosome segregation and affect fertility (Perry, 2008). This effect depends on exposure time and pesticide concentration (Härkonen, 2005).

4.5 Folate deficiency

This acid provides methyl groups for DNA methylation. Deficiency causes an alteration to chromosome segregation (Pacchierotti and Eichenlaub-Ritter, 2011).

5. Diagnosis of meiotic chromosome alterations

Diagnosing meiotic alterations with normal mitotic karyotype requires a direct study of the testicle or spermatic aneuploidy as a consequence of meiotic anomalies, but testicular biopsy is invasive and has some limitations. The fragment of testicular parenchyma may not contain meiotic cells, may have a reduced number of them, reveal only cells in prophase I but not in metaphase I or particularly, in metaphase II, given the brevity of the second meiotic division (Hultén et al., 1992). (Fig. 3). The most frequent result is to observe cells in prophase I, which is the meiotic stage that lasts longest. Another limitation of the meiotic study is that the specific chromosomes are not identified.

The study of meiotic chromosomes only reveals if the affected chromosomes are large, medium or small sized. It is not uncommon to see two cell lines, one with normal meiosis and another with altered meiosis, and then assess the percentage from each of them.

The study of spermatic aneuploidy using FISH is not possible in cases of azoospermia. The value drops in patients with cryptozoospermia, as a minimum of 500 to 1000 spermatozoa must be studied in order for the results to have statistical value.

FISH with the used probes only provides information on the studied chromosomes, normally five: 13, 18, 21, X and Y. If aneuploidy affects any of the 19 remaining chromosomes, it is not detected. It is possible to study all chromosomes with FISH or array CGH (comparative genomic hibrization) but the cost is very expensive. Despite these limitations, this test is the one most often employed to diagnose meiotic alterations,

as it is non-invasive. If the FISH is altered, a testicular biopsy does not need to be done. Reproductive history can let meiotic anomalies be ruled out. For example, if the patient has had healthy children that he wanted with a past partner.

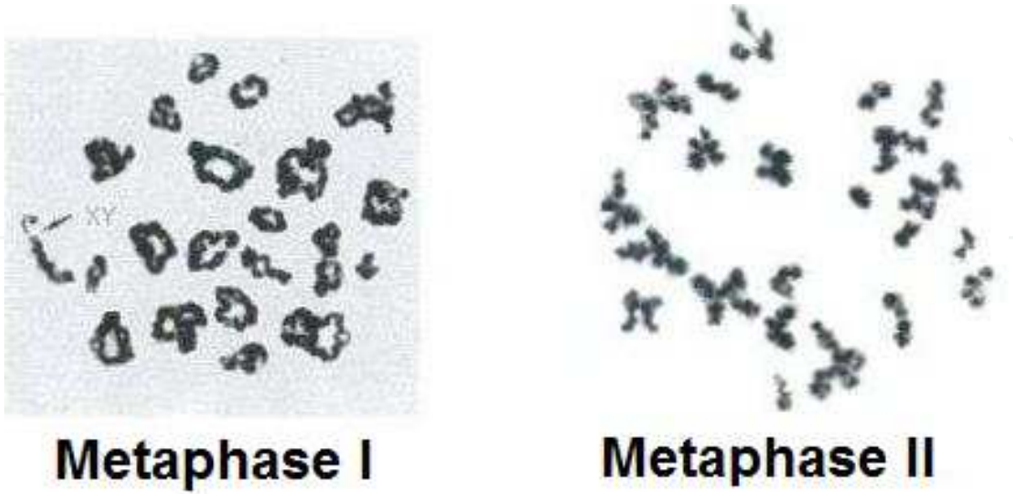


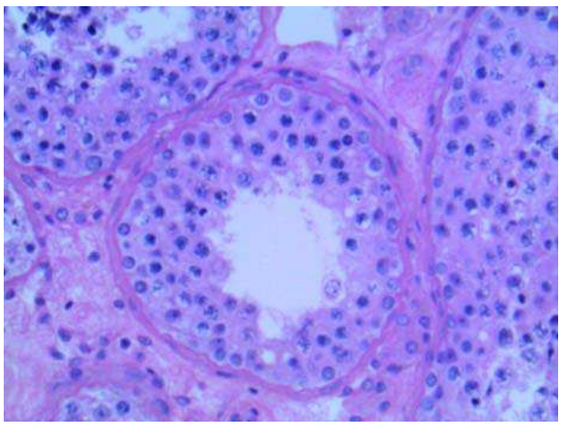
Fig. 3. Normal spermatocytes I and II

Physical examination does not provide representative data on meiotic alterations. Standard semen analysis does not reveal specific alterations of meiotic anomalies, although their frequency is higher with low total spermatic counts (TSC) and in patients with severe teratozoospermia.

6. Repercussions of chromosome alterations limited to meisois

The complex meiotic process requires the precise function and coordination of a large number of genes and their proteins. Some genes are expressed only in the testicles and are specific to meiosis and these gene alterations could impact reproductive ability. Another some genes are expressed not only in testis, but also in other tissues and organs, which is the case of the genes in the cohesin complex. These gene alterations may affect reproduction and also exhibit other pathologies. All men, including fertile ones, have meiotic anomalies. Depending on the type of anomaly and the quantity of affected meiotic cells, the impact on reproductive capacity will range from insignificant to different degrees of severity. Meiotic chromosome alterations can have an impact on testicular histology, blocking meiosis in different phases of the process and preventing it from finishing. Using an optical microscope, maturation blocks can be identified at a spermatocyte I and spermatocyte II level, albeit much less frequently. In parallel, the blocking of meiotic maturation can be complete or incomplete (Fig. 4). In this case, meiosis progresses but is quantitatively reduced.

There may be meiotic alterations without meiotic blockage. In these cases, the testicular histology at an optical level is normal. At a semen level, the repercussions of meiotic alterations may be: 1) azoospermia if meiotic blocking is complete, 2) greater or lesser reduction of total spermatic count (TSC) related to the severity of the maturation block, 3) normal or even high TSC (polyzoospermia) or 4) more or less severe teratozoospermia.



- 32-year-old patient seeking treatment for infertility.
- Anamnesis without pathological data of note.
- Physical examination: Testicles - 25 ml. Rest of the andrological examination normal.
- Ejaculate: 4.6ml. Azoospermia.
- Normal mitotic karyotype.
- Normal micro-deletion of Y.
- FSH: normal (5.2mUI/ml).
- TESE: No sperm were observed in the right or left testicle.
- Biopsy of the testicle. Pathological anatomy: Complete blockage at spermatocyte I. General desynapsis

Fig. 4. Seminiferous tubule H&E stain (X200).

In meiotic chromosome studies during testicular biopsies, the alterations observed are primarily in prophase I (pairing anomalies); in metaphase I (desynapsis) (Fig. 5). In metaphase II (diploidy, hyperploidy), they are less frequent. The absence of cells in metaphase II is not considered a meiotic alteration. The meiotic chromosomal anomalies can produce gametes with aneuploidies, chromosomal translocation, deletion, inversion, etc.... We will focus on aneupliodies.

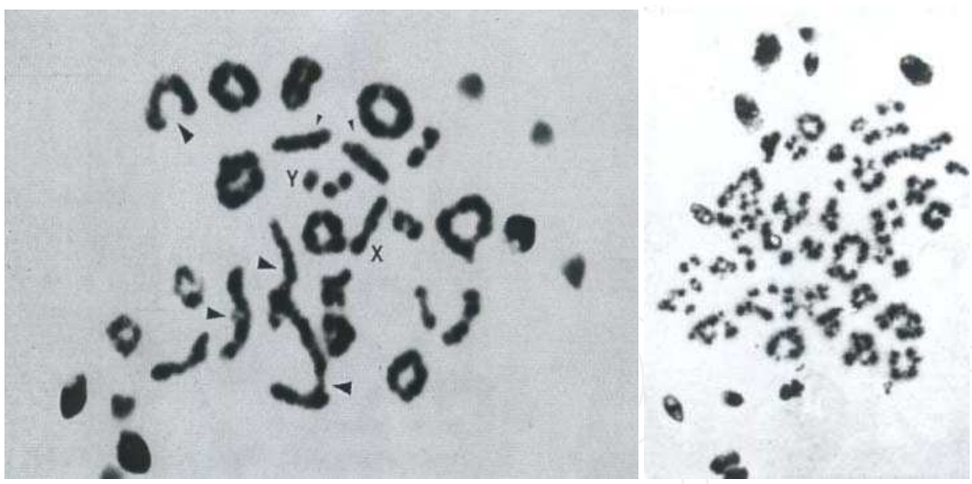


Fig. 5. Desynapsis of bivalent (left) and general desynapsis (right)

At a clinical level, men with complete meiotic blockage and azoospermia are sterile. Patients with incomplete meiotic blocks and reduced TSCs will have reduced reproductive capacity depending on the TSC and the number of aneuploid gametes. They can lead to a lack of gestation; gestation and subsequent miscarriage; or having a healthy child and difficulty having another (including having a sick child).

The quantification of these situations is complex, given the multitude of factors that intervene in attaining gestation, as well as female fertility.

IVF-ICSI treatments are useful in patients with low TSC if they have euploid spermatozoa. IVF-ICSIs do not resolve the problem if gametes are aneuploid. This is why it is crucial to know if the patients' spermatozoa that we treat are aneuploid or not, and what percentage of them.

7. Our experience

The data given here correspond to studies carried out on patients treated in the Barcelona CEFER Reproduction Institute. The sperm FISH study and/or biopsy of testicles with meiotic chromosome were not indicated for all patients. Not all the patients in whom the FISH technique and/or biopsy was indicated actually went through with it.

The sperm FISH and/or testicular biopsy indication criteria were not the same for the entire trial. At the beginning, the FISH study was not indicated if the patient had a healthy son or if the seminogram was normal. It was indicated more frequently in patients with oligoasthenoteratozoospermia (OAT). The patients were divided into three groups.

7.1 Groups of patients

Group 1. Patients with FISH (n= 1813; 100%)

All infertile patients had a normal mitotic karyotype and sufficient sperm in the semen for FISH analysis.

Group 2. Patients with testicular biopsy (n= 216; 100%)

This group included all patients with the meiotic biopsy study in the last three years. All of these sought consultation due to infertility or miscarriage and had normal mitotic karyotype. The seminogram varied from azoospermia to normal semen.

Group 3. Patients with FISH and testicular biopsy (n=60; 100%)

This group includes sterile patients or patients prone to miscarriage with the sperm FISH and meiotic study on the testicular biopsy.

However, the following patients were excluded: i) patients with healthy children from their current partner or a previous one; ii) patients whose karyotype showed morphological variations not considered to be of pathological significance, such as pericentric inversion of chromosome 9. All the patients included in our study had normal mitotic karyotypes; iii) patients with a total sperm count below 0.10×10^6; these patients were not included because of the difficulty of evaluating the FISH results, given the reduced number of sperm available; and iv) patients whose semen contained many non-gamete cells; these patients were rejected due to the difficulty of interpreting the FISH results.

7.2 Semen analysis

Semen analysis was carried out following WHO recommendations (WHO, 1999). In some cases, analysis was done using the sample provided closest to the date of the sample used for FISH; in the majority of patients, semen analysis and FISH were performed on the same semen sample.

7.3 The FISH technique

The semen samples were fixed in a methanol/acetic acid solution (3:1). Sperm nuclei were decondensed by incubating the slides in dithiothreitol (DTT) (5 mM) and Triton X-100 (1%). The details of semen fixation, nuclear decondensation and FISH have been described (Vidal et al.,1993). Three-colour FISH was performed using centromeric probes for all patients (Vysis Inc., Downers Grove, IL, USA) for chromosomes 18 (spectrum aqua), X (spectrum green) and Y (spectrum orange), and two-colour FISH was performed with locus-specific probes for chromosomes 13 (spectrum green) and 21 (spectrum orange). The incubation and detection protocol suggested by the manufacturer (Vysis) was followed. Evaluation was carried out using an Olympus BX51 microscope fitted with specific FITC, TRITC, Aqua and DAPI/FITC/PI filters. Only nuclei identified as decondensed sperm nuclei (either by their oval shape and/or the presence of a tail) were evaluated. The following criteria were used to avoid subjective observation: 1) overlapping sperm or those without a well-defined contour were not evaluated; 2) in the case of disomy and diploidy, the signals had to have the same intensity and be separated by a distance equivalent to the diameter of one of them (Blanco et al., 1996). The hybridization efficiency had to be greater than or equal to 98% and was calculated as a percentage of the haploid sperm plus twice the percentage of disomic sperm plus the percentage of diploid sperm (Blanco et al., 1996).

A minimum of 1000 sperm were analysed per patient (500 sperm for each of the two kinds of probes studied: centromeric and locus-specific probes) The chi-square test was used for statistical analysis. Results were considered to be statistically significant when $P<0.05$. The frequency of chromosomal abnormalities was expressed as a percentage with a 95% confidence interval. The control group has been published (Blanco et al., 1997).

7.4 Study of meiotic cells in testicular biopsies

Open testicular biopsies, only on one testicle, were carried out under local anaesthesia on an outpatient basis. The testicular tissue was placed in a hypotonic solution of potassium chloride (0.075 M). Fixation was carried out in accordance with the described technique (Egozcue et al., 1983).Treating meiotic cells with hypotonic solution produces swelling, breaks down the cells' nuclear membrane in prophase, causes the spindle to disappear, and the normal topography of bivalent chromosomes is lost. At diplotene, it is possible to see the cross-over sites, called chiasmata, where genetic exchange takes place. Differentiating between the chromosome figures at diakinesis (the last phase of prophase I) and metaphase I is not easy. We shall use the term metaphase I for both phases. The technique used does not allow for the identification of individual chromosomes, except for the pair of sex chromosomes and chromosome 9, due to its secondary constriction.

7.5 Results

7.5.1 Group 1. patients with FISH (n=1813)

From the 1813 (100%) patients that underwent sperm FISH, 1576 (86.9%) showed normal results; these results were altered in 237 patients (13%). Figures 6, 7 and 8 are box plots which showed a correlation between the FISH results and the semen parameters, count, motility and morphology.

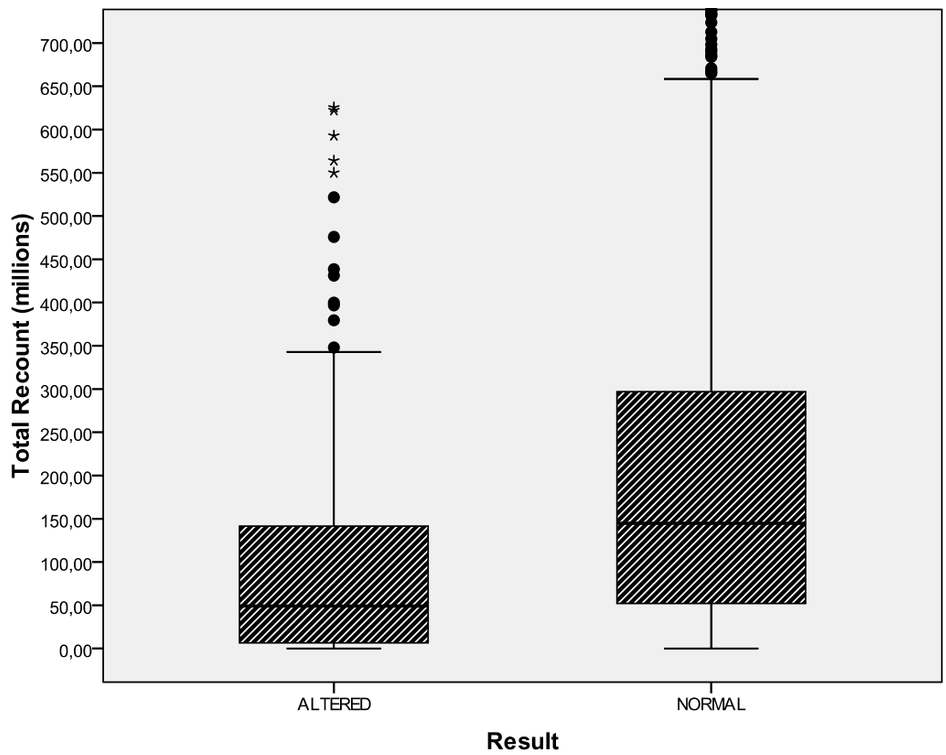


Fig. 6. Relationship between the FISH results and the overall spermatic count.

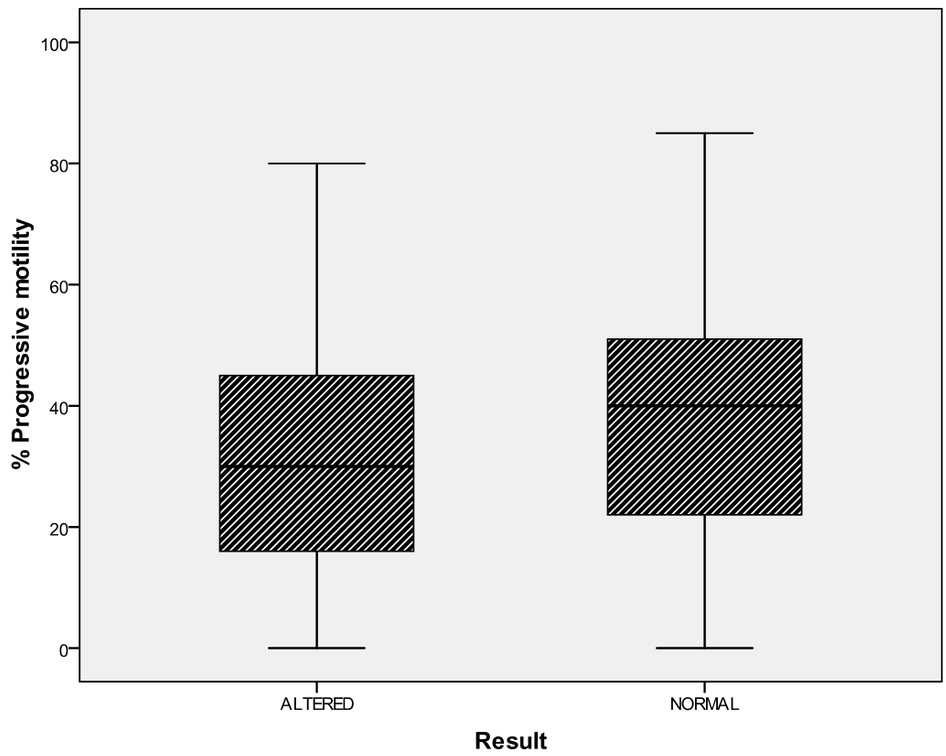


Fig. 7. Relationship between the FISH results and spermatic motility.

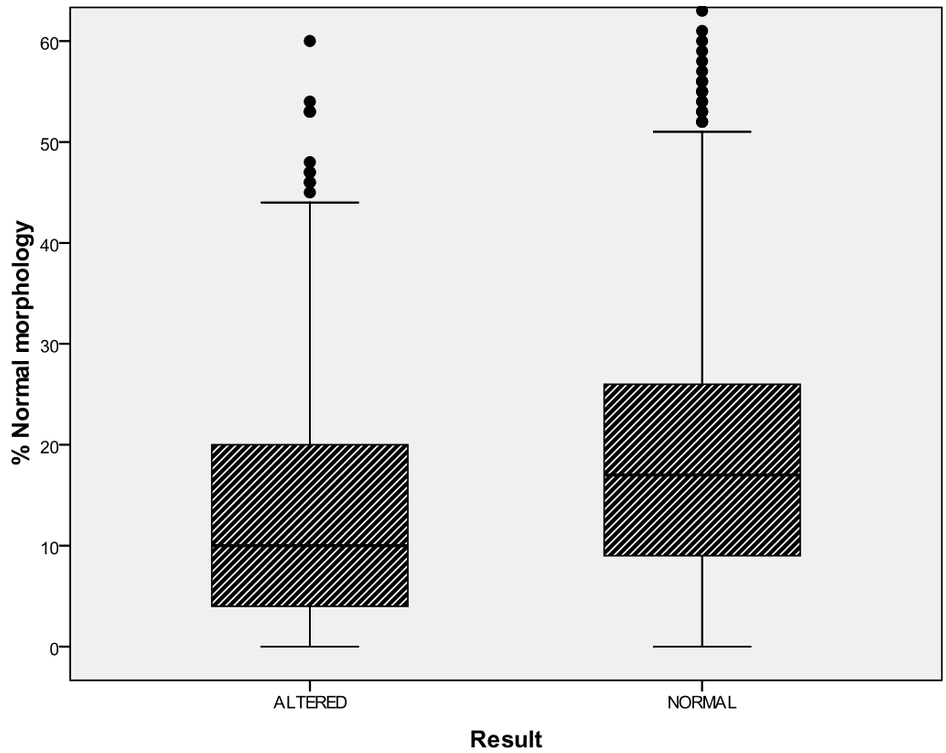


Fig. 8. Relationship between the FISH results and spermatic morphology.

7.5.2 Group 2. patients with testicular biopsy (n=216)

All patients sought consultation due to infertility or miscarriage and had normal mitotic karyotype. The seminogram varied from azoospermia to normal semen in the basic sperm count, motility and morphology parameters. All cases of spermatocytes in prophase I and metaphase I without alterations were considered normal. The absence of spermatocytes II was not considered pathological. The coexistence of normal and altered spermatocytes were diagnosed as mosaicism. Any cases with a variety of abnormalities were included in the group with the most frequent abnormalities. Table I gives the results for this group of patients.

DIAGNOSIS	No.	%
Normal meiosis	67	31
Only Sertoli cells	16	7.4
Only cells in prophase I	31	14.3
Altered meiosis	42	19.4
Mosaicism	60	27.7
Total	216	100

Table I. Results of the meiotic study on the testicular biopsy.

The types of meiotic abnormality observed in 42 patients (100%) are given in table II.

ABNORMALITIES	No	%
Abnormal mating in PI	6	14.2
Desynapsis of the bivalents	14[a]	33.3
Reduction in the number of chiasmata	3	7.1
Presence of univalents in MI	11[b]	26.2
XY separation	2	4.7
MI hyperploids	5	11.9
MII diploids	1	2.3
Total	42	100

a)we observed spermatocyte II diploids in 4 cases and 2 cases with general desynapsis, b) there were also 2 cases with separation of the XY pair.

Table II. Meiotic abnormalities. PI, MI and MII: Cells in prophase I, metaphase I and II.

7.5.3 Group 3. patients with FISH and testicular biopsy (n=60; 100%)

The study of FISH and meiosis in the testicular biopsies did not reveal any abnormalities in 18 of the 60 cases studied (30%) (table III). The partners of 13 patients in this group had experienced miscarriages (21.6%): six of 18 in the group with normal meiosis (33.3%); and seven of 42 in the two groups with altered meiosis (16.6%)(tables IV and V). These figures were low, but significant differences in the miscarriage rate were observed in both groups, with normal meiosis and with altered meiosis. An absence of figures in metaphase II in the fragment of testicular tissue studied was observed in 13 of the 18 cases with normal meiosis (72.2%). Of those 13 cases, nine had a normal total sperm count, which would seem to be incompatible with the total absence of figures in metaphase II. The shortness of this phase explains the frequent absence of figures observed in metaphase II in testicular-biopsy samples (Hultén et al., 1992).

The sperm FISH in 25 patients was normal and the testicular meiosis showed alterations. The seminological data and testicular meiosis are presented in table IV.

The data collected from the 17 patients with sperm FISH and testicular meiosis altered are contained in table V.

The abnormalities found in the FISH for this group of patients are given in table VI.

Of the 17 patients with altered FISH results and altered meiosis, the partner of only one had a history of miscarriages. Total sperm count was normal in nine cases (52.9%) Sperm motility was normal or moderately low (≥30%) in nine cases (52.9%); and sperm morphology was normal or moderately low (≥20%) in 11 patients (64.7%). Individual data with meiotic results are shown in table V.

The meiotic abnormalities observed (n=42; 70%) and the individual data for each case are summarized in tables IV and V. All these patients (n=42; 100%) had meiotic figures at prophase I and all of them had sex body. Pairing of homologous chromosomes at pachytene was normal in 24 cases (57.1%). At metaphase I the most common abnormality was incomplete desynapsis observed as bivalents with some but not all of their chiasmata, small univalents or an association of both abnormalities. It affected some metaphase figures in 29 out of 38 cases in which figures at metaphase I were observed (76.3%). Complete desynapsis was observed in four cases (10,5%).

	MISCARRIAGES	SEMEN ANALYSIS			STUDY OF MEIOSIS		
Case	n	TSC (x10^6)	Motility (%)	Morphology (%)	Prophase I	Metaphase I	Metaphase II
1	No	738	20	8	Normal	Normal	Normal
2	No	205	10	3	Normal	Normal	Normal
3	No	203	25	6	Normal	Normal	Normal
4	No	157	50	17	Normal	Normal	Normal
5[a]	No	121	15	3	Normal	Normal	No
6	No	116	30	40	Normal	Normal	No
7[b]	No	104	5	18	Normal	Normal	No
8	No	85	55	17	Normal	Normal	No
9	No	57	25	18	Normal	Normal	No
10	No	1.1	40	38	Normal	Normal	No
11[c]	No	0.7	0	4	Normal	Normal	No
12	No	0.13	0	8	Normal	Normal	No
13	6	564	60	47	Normal	Normal	No
14	2	495	50	16	Normal	Normal	Normal
15	4[d]	161	50	18	Normal	Normal	No
16	2[e]	154	40	23	Normal	Normal	No
17	2	90	30	16	Normal	Normal	No
18	3[f]	0.5	30	9	Normal	Normal	No

[a]left varicocele; [b]unilateral microorchidism; [c]unilateral cryptorchidism,[d]one with trisomy 13 and one with 46, XX; [e]one with trisomy 16; [f]one with 46, XX

TSC = total sperm count

Table III. Patients with normal FISH results and normal meiosis (18 out of 60; 30%):miscarriages, semen analysis and study of meiosis

	MISCARRIAGES	SEMEN ANALYSIS			STUDY OF MEIOSIS		
Case	Nº	TSC (x10⁶)	Motility (%)	Morphology (%)	Prophase I	Metaphase I	Metaphase II
1	No	1721	45	19	PA (some)	2-3 desynaptic bivalents[bc]	No
2	No	670	45	17	Normal	2-4 univalents[a]	No
3	No	363	40	4	PA (some)	2-3 desynaptic bivalents[b]	No
4	No	330	40	9	Normal	2-4 desynaptic bivalents[b]	No
5	No	287	11	33	Normal	2-6 univalents[a] and 2-3 desynaptic bivalents[b]	No
6	No	265	3	3	Normal (70%); PA (30%)	Normal (70%), and complete desynapsis (30%)[d]	No
7	No	192	35	27	Normal	2 desynaptic bivalents[b]	No
8	No	159	40	7	Normal	23 bivalents[d]	No
9	No	88	15	5	PA (some)	1 desynaptic bivalent[be]	No
10	No	77	50	21	Normal	Normal (75%), and complete desynapsis (25%)[d]	No
11	No	42	30	5	Normal	1 extra bivalent: hyperploidy	No
12	No	28	35	4	PA	2 desynaptic bivalents[ace]	No
13	No	17	15	11	PA	No	No
14	No	10	25	34	Normal	No	No
15	No	9.6	60	15	Normal	2-3 desynaptic bivalents[bcd]	No
16	No	9	65	23	PA (some)	2-4 univalents [a]	No
17	No	4	16	2	Normal	2-3 desynaptic bivalents[ce]	No
18	No	0.2	8	10	Normal	23 bivalents[d]	No
19	No	0.18	50	8	PA and tetraploids[f]	Complete desynapsis	Diploids
20	3	1716	40	54	Normal	2-3 desynaptic bivalents[b]	No
21	5	489	40	25	Normal	2-4 univalents[a]	No
22	3	218	30	11	Normal	23 bivalents[d] and 23 normal bivalents	No
23	4	208	50	35	Tetraploids (12%)	Tetraploids (some)	1 diploid
24	5	198	15	19	PA (some)	2-4 univalents[a] and 2 desynaptic bivalents[abe]	No
25	4	73	55	20	PA (some)	2-3 desynaptic bivalents[c]	No

[a]small; [b]medium-sized; [c]big; [d]degenerative in appearance; [e]early separation of XY pair; [f]some tetraploid figures with two sex vesicles

TSC = total sperm count; PA = pairing anomalies

Table IV. Patients with normal FISH results and altered meiosis (25 out of 60; 41.6%): miscarriages, semen analysis and study of meiosis

	MISCARRIAGES	SEMEN ANALYSIS			FISH RESULTS	STUDY OF MEIOSIS		
Case	Nº	TSC (x10⁶)	Motility (%)	Morphology (%)	Alteration	Prophase I	Metaphase I	Metaphase II
1	No	260	45	23	Gonosomal disomies	Normal	2 univalents[ad]	No
2	No	250	15	39	Diploidies and disomies 21	Normal	2-6 univalents; 1 desynaptic bivalent[b]	No
3	No	202	35	22	Diploidies	Some tetraploids	2 univalents; 1-2 bivalents[abc]	Some diploids or hypoploids
4	No	176	45	45	Diploidies	Normal	Normal (25%); 2 desynaptic bivalents (75%)[c]; 2-4 univalents[a] (some)	No
5	No	80	3	9	Disomies 13	Normal	2 univalents[a]; 2-3 desynaptic bivalents[bcd]	No
6	No	74	30	20	Gonosomal disomies	PA	2-3 desynaptic bivalents[bc]	No
7	No	66	15	4	Diploidies and disomies 13	Normal	2-4 univalents[a]; desynaptic bivalent[b]	No
8	No	64	45	54	Diploidies	Normal	4 desynaptic bivalents[bc]	No
9	No	38	55	20	Diploidies	PA in 3 bivalents [bc]	No	No
10	No	15	35	33	Diploidies	Normal	4-6 univalents[a]; 2-3 desynaptic bivalents[bc]	No
11	No	9	20	46	Diploidies and disomies 13	Normal	2-3 desynaptic bivalents [b]	No
12	No	7	30	8	Diploidies and gonosomal disomies	Normal	2-4 univalents[a]; 1 desynaptic bivalent[b]	No
13	No	3	10	15	Diploidies and gonosomal disomies	Normal	univalents[a]; desynaptic bivalents[d]	No
14	No	1.2	16	10	Diploidies and gonosomal disomies	PA	Complete desynapsis	No
15	No	0.9	5	1	Diploidies	PA	No	No
16	No	0.46	15	22	Disomies 13	PA	2-4 univalents[a]; 1-2 desynaptic bivalents[bd]	Some hyperploids
17	2[e]	396	55	53	Diploidies	PA	10 univalents[a]; 4 desynaptic bivalents[bd]	Some diploids

TSC: total sperm count; PA: pairing anomalies; [a]small; [b]medium-sized; [c]large; [d]early separation of XY pair; [e]one with 92,XX,YY

Table V. Patients with altered FISH results and altered meiosis (17 out of 60; 28.3%):

FISH ABNORMALITIES	No.	(%)
Diploidies	7	41,1
Diploidies and gonosomic disomies	3	17,6
Diploidies and autosomic disomies [a]	3	17,6
Gonosomic disomies	2	11,7
Autosomic disomies [b]	2	11,7
TOTAL	17	100,0

[a] Chromosome 13 and 21 were affected
[b] Chromosome 13 was affected in both cases

Table VI. The anomalies observed with the FISH technique (n=17; 100%)

Incomplete desynapsis and/or the presence of small univalents was observed in patients with polyzoospermia and those with normal, low or very low total sperm counts: normal, low or very low sperm motility; and normal, low or very low sperm morphology (tables IV and V). No relationship was observed between incomplete desynapsis and more or less severe alterations of basic semen parameters. No relationship was detected between the type of meiotic abnormalities observed in the testes and the kind of gamete aneuploidy (diploidies, disomies). The synaptic process is controlled individually for each chromosome and not at cell level (Templado et al., 1981). The cases of complete desynapsis of all bivalents and in all cells had very low total sperm counts: 0.18 and 1.2 x 10^6 (Case 19 in table IV and Case 14 in table V). Cases six and ten in table IV, which had complete desynapsis but not in all cells (only in 30% and 25%, respectively) presented with normal total sperm counts: 265 and 77 million, respectively. No figures at metaphase II were observed in 37 of 42 cases (88%).

The meiotic report from the testicular biopsy cannot simply be extrapolated to the entire testicular parenchyma.

8. Remarks

When the first successful birth was achieved using the IVF-ICSI technique (Palermo et al., 1992) and one year later when this technique was used successfully on sperm extracted from the testicle (TESE) (Schoysman et al., 1993), andrology entered a new era. It simply became a matter of obtaining a dozen or so sperm from the semen or the testicle in order to have a child. These 20 years of experience in IVF-ICSI have come to show that the reality is not quite as straightforward as this. The few sperm used have to be euploids. IVF-ICSI cannot resolve the problem of infertility if the sperm introduced into the ooplasm is an aneuploid.

If the sperm selected for microinjection into the mature ovocyte is aneuploid, the oocyte may not be fertilized (Lee et al., 2002) or an aneuploid embryo may be produced but stops developing in the first few days after fertilization. Such embryos have higher levels of chromosomal abnormalities (Almeida and Bolton, 1998); another possibility is that the

embryo does not implant; they can produce abortions (Giorlandino et al., 1998) or the child is born with a pathology.

In fertile men, around 0.1% of disomies were detected per chromosome; lower in chromosome 8 (0.03%) and higher in the gonosomes (0.27%) (Templado et al.,2011), including a very high level (0.43%) of disomies XY (de Massy, 2003). The total disomies detected via the FISH technique in fertile men is around 4.5% (Templado et al., 2011). It is worth noting then that all fertile men present meiotic abnormalities. It is simply a question of percentages. In infertile patients with normal mitotic karyotype, the level of spermatic aneuploidies is significantly higher in comparison to fertile men: 13% according to the data obtained and 14% according to Sarrate et al., (2010). The high number of patients included in both studies, in excess of 2000, gives added solidity to the data. The incidence of spermatic aneuploidies in infertile men is sufficiently high to recommend investigation in all patients with fertility problems.

Infertility is a symptom that can be brought on by a variety of causes, including causes that are not directly attributed to the man (female issues). The symptom of infertility (or a history of miscarriage) does not enable us to confirm or disregard the possibility that meiotic alterations form part of its etiology.

An increase in spermatic aneuploidies has been observed in patients with oligoasthenoteratozoospermia (OAT) (Devillard et al., 2002; Lee et al., 2002). Also in semen with isolated alterations of each of the basic semen parameters: low spermatic count (Templado et al., 2011); or altered morphology (Calogero et al., 2002). Not all the authors found a correlation between altered spermatic motility (Calogero et al., 2001) or morphology (Sbracia et al., 2002) and an increase in the level of aneuploidies.

The variability of the semen parameter results from one laboratory to another, one patient to another and one day to another only enables us to identify trends as opposed to establishing concrete percentages.

Expressing the spermatic count in concentration levels (per ml) instead of the total spermatic count is another variable that causes difficulties in comparing the results published.

The normality of the basic semen, count, motility and morphology parameters including polyzoospermia, does not enable us to rule out the presence of spermatic aneuploidies or testicular meiotic alterations (tables IV and V). The semen parameter that best correlates with gametic aneuploidies is TSC (Fig. 6); followed by spermatic morphology (Figure 8). The data obtained from group 3 refer to a small highly selective group of patients and cannot be applied to the general infertile population or people prone to miscarriage. There is high percentages of abnormalities detected with FISH (17 out of 60; 28.3%) in group 3 (table V) in comparison to the 13% in the more numerous group 2 (237 out of 1813; 13%). This is explained by the rigorous selection process for the cases.

Depending on the reproductive history (infertility, miscarriage, low fertilisation rate in IVF-ICSI cycles, including donated oocytes, embryonic blockages and embryonic implant failure) and the quality of the semen parameters, the incidence of spermatic aneuploidies and meiotic abnormalities will be different. The percentage published by Sarrate et al.,(2010) is very similar (26.5%) to ours (28.3%).

The FISH study of five chromosomes enabled us to detect a higher percentage of aneuploidies, specifically 11.7% of chromosome 13 in the data provided (table VI), than if only three probes were used, typically chromosomes 18, X and Y in this case.

Patients with altered FISH (100%) in this study presented meiotic abnormalities in the testicular biopsy. Sarrate et al., (2010) encountered the situation in 91.7% of patients. If the sperm FISH is altered, it is not necessary to carry out a meiotic study on the testicular biopsy as any gametic aneuploidy is considered to be caused by meiotic alteration. In a small percentage of patients, between 5 and 8%, according to both our not-published data and that published by Sarrate et al., (2010), the FISH is altered and testicular meiosis is normal. We did not observe any cells in metaphase II in the biopsy in our patients. Meiotic alteration causing gametic and aneuploidy could occur in the second meiotic division. Another possible explanation is the presence of mosaicism.

In patients with normal FISH, the meiosis is altered in 41.6% of cases (25 out of 60) in this study and reaches 73.6% in the Sarrate et al., (2010) publication. The data indicate that a normal FISH does not rule out the existence of meiotic abnormalities. This situation may be explained by the existence of altered meiotic cells that do not produce spermatozoa, along with normal meiotic cells that produce euploid gametes. Another possible explanation is that the spermatozoa are aneuploids but for other chromosomes than those studied with the probes used. If the FISH is normal then it will be necessary to consider whether to indicate the meiotic study or not on the testicular biopsy depending on the reproductive history and the semen parameters.

We did not find a correlation between the meiotic anomalies observed in the testicle and the type of FISH alteration: diploidies or disomies of one or other chromosome. We also did not observe an increase in gonosomic disomies in comparison to autosomic disomies. The number of cases presented is small (n=25) and not conclusive.

The detection of meiotic chromosomal abnormalities both with the FISH and by means of testicular biopsy, together with the reproductive history of the patients, has led the andrologist to indicate a study of the chromosomes in the embryos in order to avoid transferring aneuploids. Another option is to propose the use of a sperm bank. Repeating and re-repeating IVF cycles without studying possible meiotic alteration is not the most reliable option.

The meiotic study on the testicle biopsy was normal in 31% of cases and was not informative in 7.4%. The blockage in prophase I (14.3% of cases) may be due to genetic alterations with no translation of mating abnormalities at an optic level. Out of the alterations observed, the most frequent is desynapsis 33,3% (table II).

It is worth noting that 27.7% (60 out of 216) of patients presented mosaicism. This is the group that may benefit from IVF-ICSI. The use of the IMSI technique (Intracytoplasmic Morphologically Selected Spermatozoon Injection) will enable a better selection of spermatozoon for micro-injection. The selection of spermatozoon for introduction into the oocyte can be 16000x instead of 400x the standard ICSI. It has been correlated the presence of vacuoles in the spermatic nucleus with aneuploid spermatozoon. (Garolla et al., 2008)

The PGD technique, particularly the array comparative genomic hibridization (aCGH) technique that studies all chromosomes enables us to select euploid embryos that are suitable for transferring to the uterus.

9. Conclusions

We can draw the following conclusions from the data presented and quoted in the bibliography.

1. The incidence of spermatic aneuploidies is three times greater in the infertile population (13% - 14%) than the fertile population (4.5%).
2. None of the clinical or seminological data enable us to confirm or rule out the possibility that an infertile patient with normal mitotic karyotype may or may not produce aneuploid gametes. We have observed an inverted relationship in particular between the sperm count and the aneuploidy level.
3. The concordance of the results of the altered FISH and testicular meiosis is almost 100% and in this case there is no need to carry out a testicular biopsy.
4. In case of normal FSIH may be necessary to do testicular biopsy because in more than 40% of this patients we observed testicular meiotic abnormalities. It depends on reproductive history.
5. The FISH study must be carried out together with the seminogram for all infertile patients. The information is significant and cannot be obtained in a more straightforward fashion.

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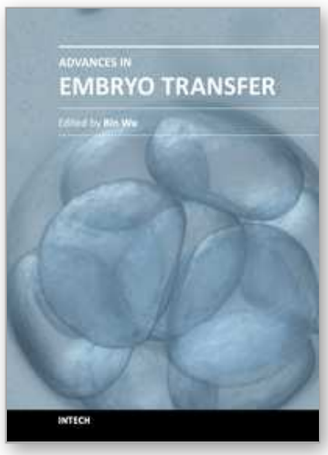

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Embryo transfer has become one of the prominent high businesses worldwide. This book updates and reviews some new developed theories and technologies in the human embryo transfer and mainly focus on discussing some encountered problems during embryo transfer, which gives some examples how to improve pregnancy rate by innovated techniques so that readers, especially embryologists and physicians for human IVF programs, may acquire some new and usable information as well as some key practice techniques. Major contents include the optimal stimulation scheme for ovaries, advance in insemination technology, improved embryo transfer technology and endometrial receptivity and embryo implantation mechanism. Thus, this book will greatly add new information for readers to improve human embryo transfer pregnancy rate.

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