European Society of Human Reproduction and Embryology

COURSE 2

Spermatogenetics

Special Interest Group Andrology
Special Interest Group Reproductive Genetics

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Contents

Course 2: Joint Special Interest Group "Andrology" and "Reproductive Genetics"

"Spermatogenetics"

PROGRAM

Course Co-ordinators: J. Geraedts (NL) & H. Tournaye (B)

Course Description: A precongress course proposed by the Special Interest Groups on Reproductive Genetics and Andrology. This course will overview the current knowledge on the genetic basis of spermatogenesis and spermatology.

09.00 - 09.30: Introduction: genetics of male infertility. - *T. Hargreave (UK)*

09.30- 10.00: Y palindromes and male infertility. - *P. Vogt (D)*

10.00 - 10.30: Discussion

10.30 - 11.00: Coffee break

11.00 - 11.30: Aneuploidy in human sperm - *J. Egozcue (E)*

11.30 - 12.00: Spermatozoal RNA-profiles - *D. Miller (UK)*

12.00 - 12.30: Discussion

12.30 - 13.30: Lunch break

13.30 - 14.00: Sperm HALO-FISH - *S. Repping (NL)*

14.00 - 14.30: Sperm chromatin organisation and DNA integrity - *L. Björndahl (S)*

14.30 - 15.00: Discussion

15.00 - 15.30: Coffee break

15.30 - 16.00: Surgical sperm recovery and sperm DNA damage - *L. Ramos (NL)*

16.00 - 16.30: CF and CBAVD - *W. Lissens (B)*

16.30 - 17.00: Discussion and Conclusions

17.00 - 18.30: Business meeting Special Interest Group "Andrology"

Introduction - Genetics of male infertility

T. Hargreave
Department of Oncology
University of Edinburgh
Western General Hospital
Edinburgh EH4 2XU Scotland
United Kingdom
E-mail: tbhargreave@btinternet.com

Learning Objectives

1) Basic understanding of the Human Genetic Code
    a. Chromosome and abnormalities
    b. Balanced and unbalanced translocations
    c. Deletions and microdeletions
    d. Genes and abnormalities
    e. Exons and introns
    f. Non chromosomal inheritance
        i. RNA
        ii. Mitochondria
        iii. cytoskeleton
2) Classic chromosomal and genetic abnormalities relevant to Male Fertility
    a. Klinefelters syndrome
    b. Sex linked gene disorders
        i. Kalmans syndrome
    c. Autosomal gene disorders
        i. CBAVD and the CFTR gene
3) Abnormalities inherited from Parents and de novo abnormalities
    a. Abnormalities passed on from parents
    b. Abnormalities arising de novo in the testis or sperm
        i. DNA fragmentation
        ii. Oxidative damage
4) Who to test and what tests to do in clinical practice
    a. Tests on peripheral blood
    b. Tests on sperm
5) Ethical problems
    a. Conflicts of interest between parents and future child
    b. Germline correction

The intention of this opening presentation is to set the scene for the rest of the days presentations and to give a broad overview of chromosomal and genetic factors and male fertility.

Introduction

The advent of in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) has opened the door to fertility for many couples for whom there had been no reasonable prospect of conception. IVF was introduced to overcome the problem of bilateral Fallopian tube occlusion, however, it

soon became apparent that this technology could enable fatherhood for men with very low sperm counts and severely impaired spermatogenesis. It has been known for a long time that there is a higher rate of chromosomal and genetic abnormalities detected in peripheral blood leucocytes from such men and it is now known that some men with a normal constitutional genetic make-up have genetic abnormalities confined to the germ-cell line and in whom abnormalities can only be detected by the examination of sperm. There is a need for infertility clinicians to have a good understanding of the genetics of male as well as female infertility.

Basic information about the Human Genetic code

The normal human cell has 46 chromosomes arranged as 22 pairs of autosomes and either two X chromosomes in the female or an X and Y in the male. On these chromosomes are arranged approximately 30,000 genes that encode proteins[1]. The chromosomes package 6 to 7 billion base pairs of deoxyribonucleic acid (DNA). The DNA is arranged as sequences of the four DNA nucleotides; Adenosine, Cytosine, Thymine and Guanine (ACTG) and it is this sequence that spells out the sequence of amino acids in proteins. The sequences of the nucleotides are arranged into protein describing areas called EXONS and non-protein describing areas called INTRONS and also special sequences to indicate the beginning and end of genes. Usually the sequence of amino acids in a protein is derived from information from several EXONS joined or "spliced" together in the nucleus before the proteins are made in the cytoplasm. Splicing is under the control of proteins and RNAs in a complex called the spliceosome. Splicing allows mixing and matching of exons to allow the formation of different proteins with shared common sequences and thus maximizes the efficiency of the genetic information. Only 2% of the human genome encodes proteins. The function of the remaining 98% of the genetic material is largely unknown although some of it may be to do with regulating genes. The preservation over 400 million years of some non protein coding sequences between men, mice and fugu fish indicates a likely vital role for some of these non protein coding sequences.

Humans share common genes with all living creatures. We differ from our closest relatives the chimpanzees by approximately 1.6% of our DNA and diverged from a common ancestor about 7 million years ago. Our kinship with primates and mammals allows appropriate animal models to be used to study human genetic mechanisms both generally and with respect to male fertility.

Spermatozoa and oocytes are different from other cells in the body because the DNA content is half that of other cells. This reduction in DNA content occurs when the spermatocytes undergo meiotic division to form haploid round spermatids. The round spermatids elongate in a process called spermiogenesis and during this process the DNA becomes compacted in the sperm head. It is estimated that there are 2000 genes that are involved in the regulation of spermatogenesis and of these 20- 30 genes are located on the long arm of Y chromosome[2]. In general autosomal genes that regulate spermatogenesis are concerned with regulation of metabolic processes in other cells in the body as well as in the cells of spermatogenesis, whereas Y genes are not essential for vital functions other than male reproduction.

Chromosomal abnormalities and male fertility

There are various different types of chromosomal abnormality including the absence of chromosomes or the presence of supernumerary chromosomes or translocation of sections of chromosome or abnormal shape chromosome such as ring forms.

Peripheral blood chromosomal abnormalities are more common in infertile men and in infertile male partners in couples seeking ICSI than in the general population[3]. In a survey of pooled data from 11 publications reporting on 9766 infertile men[4], there was an incidence of chromosomal abnormalities of 5.8%. Of these, sex chromosome abnormalities accounted for 4.2% and autosomal abnormalities for 1.5%. For comparison, the incidence of chromosome abnormalities in pooled data from three series including a total of 94<th>465 newborn male infants was 0.38%, and of these 131 (0.14%) were sex chromosome abnormalities and 232 (0.25%) were autosomal abnormalities[4] .

In a population of 781 male partners in couples undergoing ICSI, 30 men (3.8%) had chromosomal abnormalities. Of these there were 10 (1.2%) sex chromosome aberrations and 20 (2.6%) autosomal aberrations[5].

Chromosomes are relatively sticky and fragile and translocation of genetic material from one chromosome to another occurs in approximately 1 in 500 people. A balanced reciprocal translocation is when there is exchange between two different chromosomes but there is no loss or gain of genetic material. People with balanced translocations are normal but will produce some sperm or eggs with extra or missing genetic material and this will result in a foetus with an imbalanced translocation. One of the more common examples is translocation of genetic material between chromosome 21 and 14 and whilst the carrying parent is normal some gametes contain extra material from chromosome 21 and this can result in a baby with Downs syndrome. This particular scenario accounts for approximately 4 percent of cases of downs syndrome. Another type of translocation is where chromosomes join around their centre to form one long chromosome. This is called a Robertsonian translocation. It occurs among chromosome 13,14,15,21 and 22 (most commonly between chromosomes 13, 14 and 14, 21) and is present in 1 in 1000 in the general population. A person with a Robertsonian translocation only has 45 chromosomes but is normal provided there is no missing material (balanced Robertsonian translocation). However gametes may contain a whole extra chromosome and, for example, this is another cause of trisomy 21 (Downs syndrome).

Sex Chromosomal abnormalities

Klinefelter's syndrome and variants (47XXY, 46XY;47XXY mosaicism)

Klinefelter's syndrome is the most frequent sex chromosome abnormality, which occurred in 66 (0.07%) phenotypically newborn males in pooled data from cytogenetic analysis of 94<th>465 newborn infants[4] . In azoospermic men the prevalence of Klinefelter's syndrome has been found to be approximately 10% in Western countries[6]. Adult males with Klinefelter's have firm small testicles with very few or no germ cells; this is to be distinguished from men with other causes of damage to spermatogenesis who usually have soft small testicles. The phenotype can vary from a normally virilised man to one with stigmata of androgen deficiency including female hair distribution and scanty body hair. Classically a man with Klinefelter's syndrome has long legs and if eunochoid long arms as well, because of late epiphyseal closure. A longitudinal study of children with sex chromosome abnormalities has been reported[7].

Leydig cell function is commonly impaired in men with Klinefelter's syndrome[8] Testosterone levels may be normal or low, oestradiol levels normal or increased and FSH levels increased. Libido is often normal despite low testosterone levels but with aging there is often a need for androgen replacement. Reduced Leydigs cell function is also relevant in the context of sperm retrieval for ICSI as there may be long term post biopsy lower testosterone[9]. Mature spermatozoa can be recovered from testicular tissue from men with apparent complete Klinefelter's syndrome[10] but at present

there are no reliable prebiopsy criteria to indicate the chance of success . There is a risk of offspring with Klinefelters and Preimplantation Genetic Diagnosis (PGD) is now being offered in some centers[12].

Sex chromosome abnormalities in babies conceived through intracytoplasmic sperm injection

There are a number of reports that indicate higher than normal frequency of sex chromosome abnormalities in children conceived through ICSI compared with in the normal population. The underlying mechanism is not yet known, but there are several possibilities discussed in the literature. These include Klinefelter's mosaicism with an aneuploid cell line confined to the germ cells and thus not detectable by peripheral blood karyotyping[13] or the production of 47XY diploid sperm; this has reported in sperm from severely oligozoospermic men with a normal karyotype[14]. It has been suggested[15] that mutations confined to the germ cells may arise when the primordial germ cells are in the extra-embryonic cell mass before these cells migrate back into the embryo. A third possibility is that low levels of mosaicism may be missed during routine karyotype analysis.

Autosomal abnormalities in men with azoospermia and severe oligozoospermia

In addition to an increased in sex chromosome abnormalities there is also and increase in autosomal abnormalities in populations of men with non-obstructive azoospermia or severe oligozoospermia[16,17]. Usually autosomal disorders do not cause infertility in isolation but reduced spermatogenesis is the consequence of a more general disturbance in phenotype and patients with these problems are often known to doctors because of other developmental abnormalities.

Genetic defects and male fertility.

Changes in the sequence of DNA are called mutations and while mutations may occasionally be beneficial and become preserved in evolution often they result in defective genetic function. Mutations may involve relatively large chunks of the gene but there are also many examples of where the alteration of a single base pair on the original DNA (point mutation) can have a profound effect on phenotype. Genetic defects may be single or multiple and may be inherited or the result of new mutation. They may be inherited in a dominant pattern as in Huntington's chorea or a recessive pattern as in cystic fibrosis and may have variable penetrance.

When mutations are in exons there is an alteration in the protein produced and in many cases a marked resulting alteration in phenotype. Mutations of non coding areas (Introns) are less well understood but can affect the amount of protein produced by making the reading of protein-encoding RNA from the original DNA less efficient; for the Andrologist one of the best known examples of this is the 5T intron abnormality that occurs in the Cystic fibrosis transmembrane gene complex (CFTR) and which is found either alone or with exon mutations in men with congenital absence of the vas deferens (see below).

Most if not all genes on the Y chromosome are to do with the regulation of male differentiation. X-Linked genes may be dominant (e.g. hypophosphatemic rickets) or recessive (e.g.Duchenes muscular dystrophy). X-linked dominant genes will alter the phenotype in both sexes but x-linked recessive genes will only be manifest in the male and the defect will be transmitted through his daughters to his grandsons.

Kallman's syndrome

The commonest X-linked disorder in infertility practice is Kallman's syndrome and the most common form of this is X-linked recessive caused by a mutation in the KALIG-1 gene on Xp22.3[18] . This gene is involved with the regulation of cell adhesion and axonal path finding. Patients with Kallman's syndrome have hypogonadotrophic hypogonadism and may have other clinical features including anosmia, facial asymmetry, cleft palate, colour blindness, deafness, maldescended tests and renal abnormalities. It is important for the Andrologist to note some men with Kallman's syndrome have an isolated gonadotrophin deficiency without any other phenotypic abnormalities and that these men may sometimes present de novo with infertility, which can be treated successfully by hormone replacement therapy

Androgen insensitivity - Reifensteine syndrome, Morris syndrome, Hairless women

The rare disorder of androgen insensitivity may sometimes first present with infertility. The condition has an X-linked recessive inheritance and is caused by one or more defects in the androgen receptor gene. The phenotype may range from complete testicular feminization with an immature female phenotype, to an apparently normal male with infertility.
Excessive amplification of trinucleotide repeats (CAG) in the translated part of the androgen receptor gene is associated with various neurodegenerative diseases such as Huntington's disease. The number of CAG repeats may also be of relevance to prostate cancer. Men with less than 22 repeats may be at more risk of developing prostate cancer than those with more repeats[19].

Y genes and male infertility

Y chromosome genes are subject to different evolutionary pressure compared with all other genes. Spermatogenesis is lifelong and is the result of rapid cell division and in principle there is much lifetime chance for production of sperm with mutant genes compared with oogenesis which although more error prone linvolves much small numbers of cells.

Ymicrodeletions

In 1992 we reported three men with severe damage to spermatogenesis and an apparently normal chromosome analysis but where molecular probes revealed microdeletions on the long arm of the Y chromosome[20,21]. Following our 1992 report there have been a large number of publications of case series and it is clear that while microdeletions may occur in the fertile population they are more common in an infertile population[22]. Involvement of several genes has been described, and these include RBM[23], DAZ[24], DFFRY[25], DBY and CDY. In general Y microdeletions may affect fertility but cause no other phenotypic consequences, they will however be transmitted to sons.

Cystic fibrosis mutations and male infertility

Cystic fibrosis, a fatal-autosomal recessive disorder, is the most common genetic disease of Caucasians; one in 25 are carriers of recessive gene mutations involving the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This gene, located on the short arm of chromosome 7, encodes a membrane protein that functions as an ion channel and also influences the formation of the ejaculatory duct, seminal vesicle, vas deferens and distal two-thirds of the epididymis. Congenital bilateral absence of the vas deferens (CBAVD) is associated with mutations in the CFTR gene, and is found in approximately 2% of men with obstructive azoospermia attending our clinic in Edinburgh, Scotland . However, the incidence in men with obstructive azoospermia

will vary in different countries, depending on the prevalence of cystic fibrosis mutations in the population and the prevalence of other causes of obstruction such as STDs.

Mutations may be found in both copies of the CFTR gene but in most men with CBAVD they are found in only one copy. In some of these supposedly heterozygous cases there may be an unknown second mutation but there is also another interesting mechanism. In up to 63 percent of these a DNA variant called the 5T allele can be detected in one of the introns of the other allele of CFTR gene and we have confirmed these observations in our own patients. The 5T allele causes the CFTR transcript to be inefficiently spliced in the nucleus, decreasing the amount of functional

Advice for couples where the man has congenital bilateral absence of the vas deferens

CFTR mutations have implications for clinical infertility practice. When the male partner has CBAVD it is important to test the female partner for CF mutations as well as the male partner. If she is also found to be a carrier then there must be very careful consideration about whether the couple wish to proceed with ICSI using the husbands' sperm as the chance of a baby with cystic fibrosis will be 25% if he is heterozygous or 50% if he is homozygous. If the female partner is negative for known mutations her chance of being a carrier of unknown mutations is about 0.4% and in these circumstances the chance of her heterozygous partner siring a child with cystic fibrosis is approximately 1:410. These figures are estimates calculated using the known mutation frequency in a Caucasian population. In the context of ICSI preimplantation diagnosis of CFTR status can be used to avoid a CFTR homozygous baby[29]

Autosomal gene defects with severe phenotypic abnormalities as well as infertility

There are a number of inherited disorders with severe or considerable generalized abnormalities as well as infertility. Such patients will be well known to doctors, often from childhood, and any fertility problem should be managed in the context of the care of the man as a whole, and with consideration of his and his partner's ability to care for a child should treatment be successful. Cryptorchidism is associated with both infertility and testicular cancer, and occurs in 3-4% of newborn boys. Mutations in the genes encoding insulin-like 3 (INSL3) and its receptor GREAT/ Lgr8 are found in some patients with undescended testes,

DNA methylation and gene imprinting and ageing changes

Methylation of the DNA base cytosine contributes to silencing and condensation of DNA, so the corresponding genes tend to be inactivated. Cytosine methylation and inactivation of genes occur as part of the ageing process and also as an important part of the mechanism of sex differentiation by silencing genes on the inactive x chromosome. DNA remodelling also has a very important role in spermatogenesis, since the DNA of the haploid round spermatids has to be condensed into the much smaller volume present in sperm. This occurs during spermiogenesis, and may involve the CDY gene which is deleted in some infertile men[30].

Genetic imprinting disorders in male infertility

There is evidence that there is an increase in the human overgrowth condition called the Beckwith Wiedemann syndrome in babies born by ICSI[31] and this condition is associated with failure of imprinting. Beckwith Wiedemann syndrome is a growth disorder characterised by some or all of the following clinical features; macroglossia, omphalocele, umbilical Hernia, diastasis recti, above average birth weight and length, visceromegaly, hemihypertrophy of part or all of the body, and

finally typical facial features including earlobe creases, prominent occiput, and nevus flammeus (strawberry mark on on the forehead and eyelids). The prevalence of Beckwith Wiedemann syndrome is difficult to establish because of underreporting of cases with minimal stigmata.

Ageing and male infertility

Sperm from older men have an increased incidence of XY, YY and XX disomy[32]. Also, children born of older men may have an increased chance of genetic defects; for example, an increased incidence of the rare congenital disorder Apert's syndrome[33] Apert's syndrome is characterized by craniosynostosis, syndactyly and other malformations, and is caused by a paternal mutation in which cytosine is substituted for guanine in the fibroblast receptor-2 gene. Based on this evidence, there are concerns about the genetic integrity of sperm from older men and this has led to recommendations for an upper age limit for men who wish to donate sperm to a sperm bank. In the USA[34] , UK[35] and France[36], there is now a recommended or required upper age limit of 35--45 years. More research is needed to define the exact risk according to age.

Mitochondrial abnormalities

In addition to the nuclear genome, mitochondria contain their own genetic material. Very few if any mitochondria make a paternal contribution to the embryo, and therefore these are unlikely to be a significant factor in inherited male infertility. However, clearly defective mitochondrial function in sperm may, by way of poor sperm motility, be a cause of infertility. There is concern that the ICSI process may allow transmission of paternal mitochondria between generations, but in a study of nine children conceived by ICSI no paternal mitochondria were detected[37].

Human mitochondrial DNA is replicated by a DNA polymerase encoded by the nuclear genome called POLG, which contains a polyglutamine tract of typically 10 amino acids. In men with sperm quality defects, the number of glutamines in this tract has been reported to vary[38]. Sperm from men homozygous for POLG alleles without the common 10 glutamine repeat are less able to achieve fertilisation, but this could be successfully treated by ICSI[39]

Inherited Cytoplasmic disorders and male fertility

In humans the cytoskeleton is a paternal contribution and thus in theory there is the possibility of non-DNA transmitted cytoskeleton abnormalities when very defective sperms are used . Whether this actually occurs is not known.

Chromosomal and DNA abnormalities in sperm

There is less information about the incidence of genetic abnormalities in sperm. In general, tests on peripheral blood leucocytes may indicate that the man has abnormal chromosomal or genetic make-up, and this may be reflected in his sperm. However, another mechanism for the introduction of genetic abnormality in gametes is derangement of mitosis in the testis, and in this situation tests on peripheral blood leucocytes will all be normal and only by studying the sperm will abnormalities become apparent [41]. In particular, men with meiotic arrest have been shown to have increased frequency of mutations in germ cells, suggesting the DNA repair machinery may be compromised in these cells, and this is obviously important regarding the health of any children they might have as a result of assisted reproduction therapy[42]

Chromosomal abnormalities in sperm

Large numbers of sperm can be tested using multicolour FISH analysis, whereas previously sperm karyotyping was by hamster penetration assay, a method that was both laborious and insensitive. Several studies of spermatozoa using FISH[43,44,45] have shown an increased frequency of both autosomal and sex chromosome aneuploidy. Typically, a standard karyotype analysis is performed on 20--30 cells. However, in a study in which 1000 cells were analyzed using FISH[46] the median incidence of sex chromosome aneuploidy was found to be 1.5% in 10 oligozoospermic men compared with 0.3% in 10 fertile men. Also, it was postulated that mitotic instability in infertile men could be the result of failure of the mitotic checkpoint gene MAD2L1.

DNA strand breakages in sperm

Men with infertility have an increase in damage to sperm DNA compared with fertile men and this increased DNA fragmentation is associated with poorer IVF pregnancy rates . One of the mechanisms of damage to DNA is oxidation and this is particular relevant in men with damaged spermatogenesis and leucospermia where free ovgen radical levels are high. Sperm from older men have increased DNA damage compared to those from younger men and this may be one of the mechanisms contributing to the slight increased in risks to children born of older fathers.

There is increasing evidence that sperm abnormalities may be common, and further studies are needed of sperm from men in well-defined clinical categories (Table 4). Also, when considering such studies, it is relevant to study the population of sperm that would normally be used in an ICSI procedure. In a study of sperm in a swim up preparation the frequency of DNA breaks was reduced by one third in motile sperm <table 4 near here>

Paternal RNA

Paternal RNAs are delivered to the egg at fertilization but it is not known whether these have a role in the early molecular events after fertilitisation but it is hypothesized that defect could account for some cases of unexplained male factor infertility[51]

Risks of intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) is now used to enable men with severe damage to spermatogenesis to father children in situations formerly considered hopeless and where only a very few spermatozoa can be obtained. This has led to worries that children may be born with foetal abnormality because by bypassing the selective processes of female genital tract and coverings of the egg the process could enable defective sperm to fertilize or alternatively eggs may be fertilized that would otherwise not do so. It is reassuring that the collected statistics of foetal abnormality from ICSI centres show similar rates of congenital malformations compared with the general population[52,53] but despite this some concern remains because, the indications for ICSI are constantly being extended to include fertilisation with immature sperm forms and potentially damaged sperm. It will be particularly important to continue to monitor foetal abnormality rates with detailed subgroup analysis according to the clinical and molecular diagnosis of the father (see table 3). ICSI has meant that some men with Klinefelders syndrome can become fathers. One of the most common aneuploidies in humans is trisomy 21 (Downs syndrome) and there is evidence that men with Klinefelders syndrome also produce sperm with high levels of disomy of chromosome 21[54] Hence any embryos from Klinefelters fathers derived by ICSI should be carefully screened.

Ethical considerations, genetic counseling and intracytoplasmic sperm injection

The main difficulties will occur where there is a conflict of interest between the wishes of the couple and the interests of a future child.

The best initial management is to give the couple full information about the risks to the child and then for the couple to decide whether to proceed or not. However, in the situation where both partners are known to carry defects (for example cystic fibrosis mutations), there can be up to a 50% chance of the birth of a child who will develop clinical cystic fibrosis and die young after a number of years of morbidity. In this situation, many clinicians and infertility clinic personnel may feel that their duty of care to the future child and the interests of society as a whole outweigh the wishes of the individual couple, and that it is not ethical to proceed and that ICSI should not be offered to the couple.

In some countries law may govern these matters and then there is no choice, but in the absence of law this type of conflict makes the doctors' role very difficult. Each case has to be judged on its merits and in the context of what is available and affordable in the local health care system. When there is a conflict that cannot be resolved by agreement the interests of a future child probably take precedence over the interests of a couple. If the decision is taken to proceed, it is important for the couple to appreciate fully what may be in store for a future child, and it is often appropriate for arrangements to be made for the couple to visit another family where there is a teenager or older person suffering from the condition. Also, the couple will need to give consideration to preimplantation diagnosis and replacement only of normal embryos or, if this is not available, amniocentesis and genetic diagnosis and the possibility of termination.

ICSI is new technology, but perhaps the greatest potential application is the window of opportunity for germ-line therapy. At present this is considered unethical and sometimes illegal. Nevertheless, the following hypothetical situation may help focus debate. Suppose a couple decide to go ahead with ICSI in a situation in which both partners are known to have cystic fibrosis mutations, but after successful pregnancy would not contemplate amniocentesis and abortion. If in this situation it was possible to correct the mutation before embryo replacement, which would be less harmful -- to repair the defect and enable the birth of a child without cystic fibrosis, or not to repair the defect and for a child to be born with cystic fibrosis? These arguments can be extended to correction of oncogenes and other defects. The risks of germ-line therapy (Table 5) have been summarized by Fiddler and Pergament[55] in a review article, which gives powerful argument in favour of germ-line therapy.

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Y-palindromes and male infertility

P. H. Vogt
Section of Molecular Genetics & Infertility
Department of Gynecological Endocrinology &
Reproductive Medicine
University of Heidelberg
Voßstrasse 9
D-69115 Heidelberg
Germany
E-mail: peter_vogt@med.uni-heidelberg.de

Learning ojectives

- Y-chromosome sequence structure belongs to haplogroup R1*

- Amplicons in palindromes and gene conversion

- Y palindromes and male infertility (AZF deletions)

- Diagnostic of partial AZF deletions needs more than PCR assays

- AZF deletions need intra-chromosomal recombination events

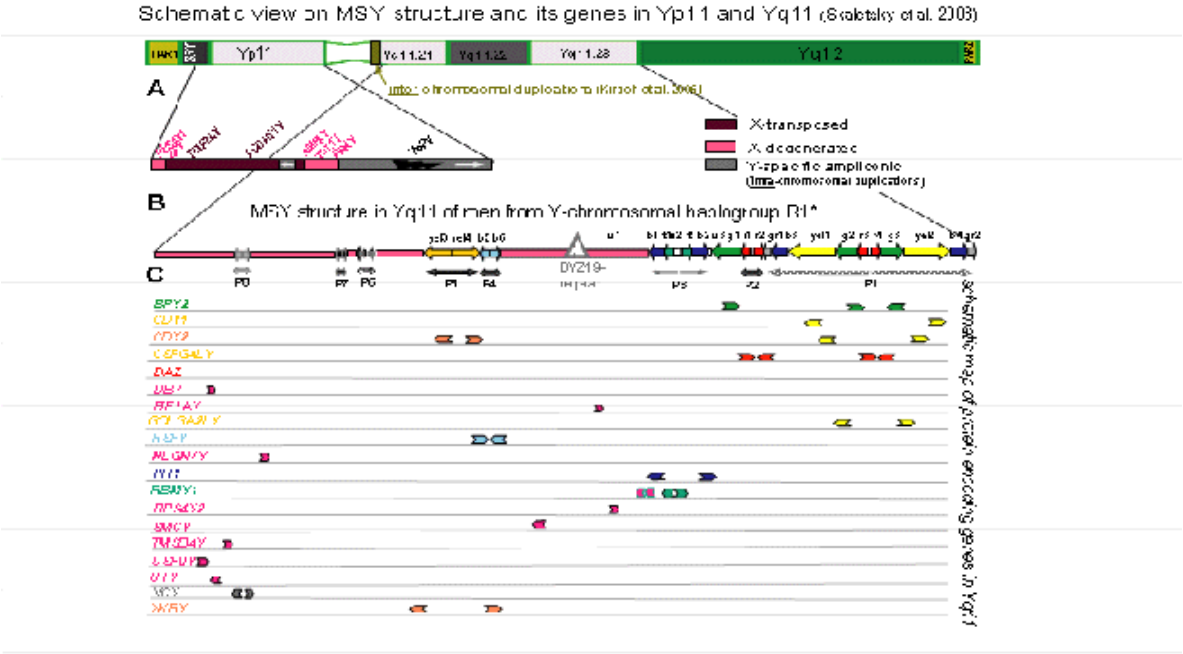
- AZF genes plasticity in Y-chromosomal haplogroups

The published Y-chromosome sequence structure belongs to haplogroup R1*

The male specific region (MSY) of the human Y DNA sequence has now been published (Skaletsky et al., 2003) and deposited in different databases (GenBank: http://www.ncbi.gov; ENSEMBL: http://www.ensembl.org/Homo_sapiens /mapview? chr =Y). It is a mosaic of X-transposed, X-degenerated and repetitive Y-specific sequence blocks, the latter forming large “amplicons” (Fig. 1).

Fig.1: Schematic overview of Y chromosome sequence associated with Y haplogroup R1*. The colour code used for marking the long homologous amplicons in distal Yq11 is given by Kuroda-Kawaguchi et al. (2001). (A): The three domains: X-transposed, X-degenerated, Y-specific ampliconic are distinguished by different colours; (B) Eight palindromes (P1-P8) and a long tandem repeat structure (DYZ19) are mapped in Yq11; (C) Gene map in Yq11. Not sequenced has been the ~3 MB long pseudo-autosomal regions at the tip of the short and long Y-arms (PAR1 and PAR2) and the polymorphic heterochromatic Y region in the distal part of the long Y arm (Yq12) estimated with a length of ~40Mb. The pericentromeric region (~2 Mb) in proximal Yq11 is also not included in the Skaletsky-Y sequence. It was revealed only recently and is composed by inter-chromosomal duplications of sequence blocks located also in the pericentromeric region of different autosomes, namely chromosome 1, 2, 3, 19, 16, and 22 (Kirsch et al., 2005).

[1]*For detailed background information see: Vogt PH (2005b) AZF deletions and Y chromosomal haplogroups: history and update based on sequence. Hum. Reprod. Update, vol.11 (4), in press.*



It has long been predicted that especially the long Y arm is composed of numerous Y-specific repetitive sequence blocks (Foote et al., 1992; Kirsch et al., 1996). Sequence analysis has now confirmed this assumption. However, beyond of that it has revealed a unique pattern of large repetitive sequence blocks (called “amplicons”) ranging in length between 115 kb and 678 kb in distal Yq11. Most interesting, these amplicons were structurally assembled in eight palindrome structures (P1: 2.9Mb; P2: 246kb P3: 736 kb; P4: 419 kb; P5: 996 kb; P6: 266 kb; P7: 30kb; P8: 75 kb). They comprise ~25% of the MSY sequence, that is 5.7 Mb of the genomic Yq11 sequence (Fig. 1B)
Sequence analyses of the homologous amplicons mapped in the different palindrome arms revealed extensive homologies between 99.94-99.997% along the complete amplicon sequence. It was found that this high sequence homology is based on frequent gene conversion events between the homologous palindrome arms (Rozen et al., 2003). It can therefore be assumed that the functional integrity of the Y spermatogenesis genes mapped in the different amplicons (Fig. 1C) is maintained by their palindromic organisation.

Nevertheless, assuming a mutation rate of 1.6 x 10-9 per nucleotide per year and 2.2 x 10-4 conversions per duplicate nucleotide per generation (i.e. per 20 years) it has been calculated that along the 5.4 Mb length of the eight Y palindromes (i.e. 2.7 x 106 duplicated nucleotides) on average 600 duplicated nucleotides undergo arm-to-arm gene conversion per generation, thus distinguishing the Y chromosome of father and son (Rozen et al., 2003). It can therefore be assumed that during human evolution Y-chromosomal sequences have largely diverged.

Worldwide, a rooted pedigree of 18 Y-chromosomal haplogroups forms now the base of divergent Y-sequences evolved during development of the different human populations (The Y chromosome Consortium [YCC], 2002; Jobling and Tyler-Smith, 2003; Fig. 2).

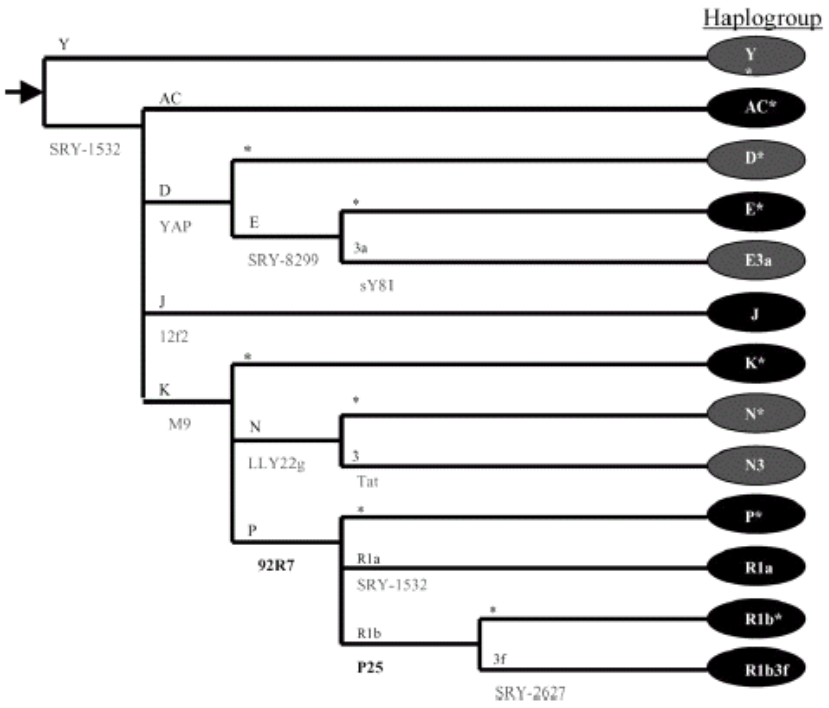


Fig.2. Y-chromosome haplogroup tree including the bi-allelic polymorphisms mainly present in Europe (extracted from Sanchez et al. (2004); in Forensic Science Int. vol 140. 241-250).
Further specification in between a specific haplogroup is revealed by analysis of the pattern of sequence variants of highly polymorphic microsatellites (the so called STR-loci) establishing specific Y-lineages (Kayser et al., 2004). For their detailed descriptions the reader is adviced to study the original papers, respectively, to visit the corresponding websites (http://ycc.biosci.arizona.edu).

Considering the derived state of the marker M207 in position 139.206 in the RPCI-11 BAC clone 386L3 (GenBank accession no. AC006376) the published Y sequence belongs to Y-haplogroup R*. This finding is further specified by a Single Nucleotide Variant (SNV) marker in the yellow amplicon of the AZFc region (AZFc-P1-SNV I: GenBank accession no. G73351) with both alleles (A+B) present only in the Y chromosome of haplogroup R1* but absent in the other Y lineages (Fernandes et al., 2004). Consequently, this Y sequence is not “universal” but associated with this distinct Y-haplogroup.

Based on this now commonly accepted YCC nomenclature system bi-allelic marker sets can be selected for efficient and informative Y-haplotype assays on any given Y DNA sequence (Fig. 2). For reasons discussed below, it is advisable to apply one of these assays in each case when a so-called “partial” AZF deletion has been diagnosed in the clinic, before the identified AZF deletion is considered as to be associated with the occurrence of the patient´s testicular pathology.

Y-palindromes and male (in)fertility (AZF deletions)

“Complete” AZF deletions

Molecular diagnosed AZF (AZoospermia Factor) deletions in Yq11 are defined as microdeletions in the euchromatic part of the long arm of the human Y chromosome (Yq11) causing male infertility (Vogt, 2005a). This does not say, that each microdeletion in Yq11 is an AZF deletion. The molecular extension of the three AZF deletion intervals in Yq11 designated as AZFa, AZFb, and AZFc (Vogt et al., 1996) comprise only ~8.73Mb, that is ~60% and not 100% of the complete Yq11 sequence

(Fig. 3A).

The occurrence of these now called “complete” or “classical” AZF microdeletions seemed not to be dependent on a distinct Y-haplogroup (Quintana-Murci et al, 2001; Krausz et al., 2003). Their high frequency is explained by the occurrence of frequent intra-chromosomal recombination events of some of the large homologous sequence blocks in Yq11 organised in the same polarity. These instabilities in Yq11 are the negative evolutionary aspect of the high density of segmental duplications and palindromes in the Yq11 chromosome region (Fig. 1). All “complete” AZFa, AZFb and AZFc deletions -estimated by appropriate STS deletion assays (Fig. 3A)- cause a distinct testicular pathology.

“Partial” AZF deletions

No distinct testicular pathology is observed for the “partial” AZF deletions now also described repeatedly in the literature. With knowledge of the R1*-Y sequence it is evident that the Y chromosome has two faces, namely, on one site being functional for spermatogenesis, on the other site being polymorphic, i.e., functional AZF genes are structurally linked to Y-specific highly polymorphic DNA regions (Vogt and Fernandes, 2003) with a different arrangement in probably each Y-chromosomal haplogroup (Fig. 1; 3).

Most prominent is the polymorphic DYS1 (49f probe) locus reflecting the different numbers and composition of the DAZ exon 7 variants in the four DAZ gene copies (Fig. 3B). The DAZ genes organised in the red amplicons are part of the P1 and P2 palindromes (Fig. 1). A quick exchange of homologous DAZ exons in these palindromes by gene conversion or intrachromosomal crossing over events can explain their dynamic exon compositions and the polymorphic DYS1 fragments, respectively.

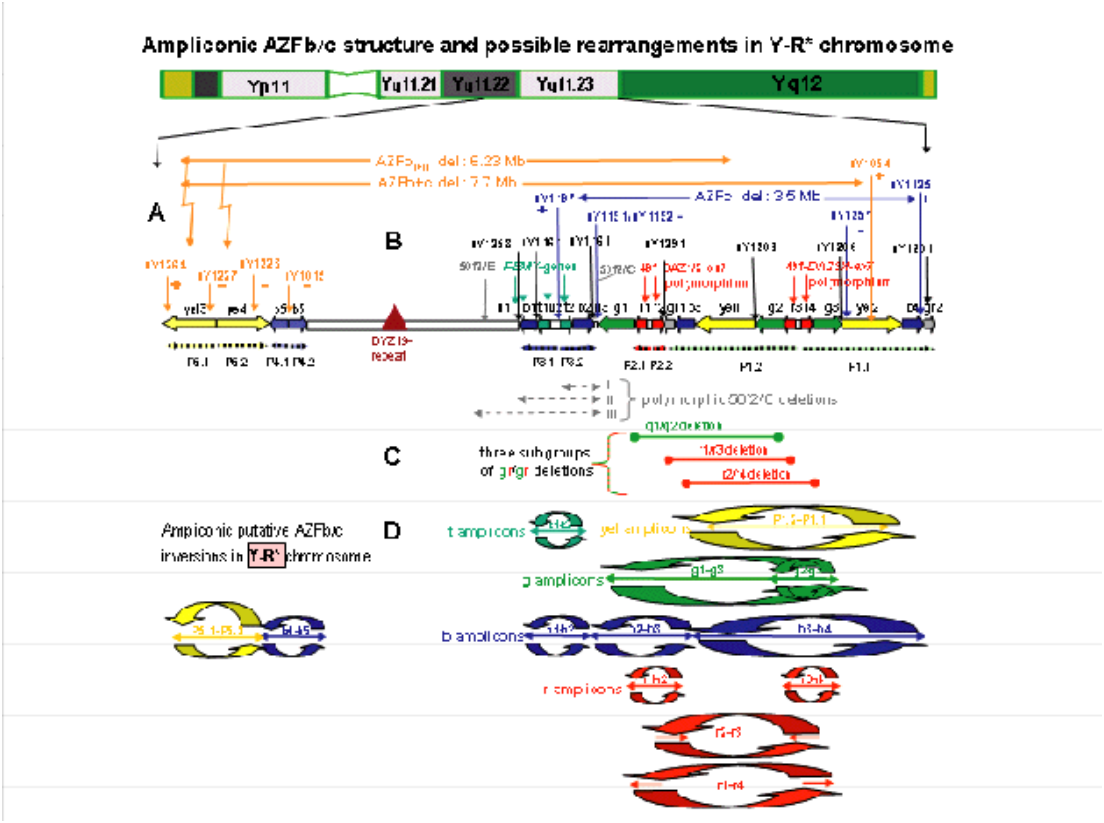


Fig. 3 (A). Schematic view on the molecular extensions of the complete AZFb and AZFc deletions

overlapping in distal Yq11. STS markers used to map their breakpoints (+: present; -, absent) are given in orange (AZFb) and blue (AZFc) colour.(B). The polymorphic 49f-sites in the DAZ exon 7 blocks (DYS1 DNA locus) is located in the red amplicons. The polymorphic 50f2-DNA-locus (DYS7) is located in the AZFc-u3 region and marked with the putative extensions of its deletions in grey colour. STS markers selected for the detection of partial AZFb/c deletion in the Y-R* chromosome (Repping et al., 2003) are given above the AZF amplicon structure. (C) The gr/gr deletions are split into the three subgroups: g1/g2, r1/r3, r2/r4. (D) Schematic overview on putative 11 ampliconic AZFb/c inversion events in the Y-R* chromosome.

The polymorphic DYS7 (50f2 probe) locus is spread in Yp11 (minisatellite MSY1) and Yq11: 50f2/E in AZFb and 50f2/C in AZFc (u3 block) with a distance of ~1.4 Mb (Fig. 3B). Some 50f2/C deletions include the deletion of 50f2/E (extension ~4 MB), others the deletion of neighboured RBMY gene copies. These would have an extension of ~500 kb in the P3 palindrome. An active involvement of this palindrome in any of the 50f2 deletions in distal Yq11 is not yet known. 50f2/C deletions occur with a different frequency in the 12 human populations analysed and taking their different Y chromosomal haplogroups into account six independent deletion events and four duplication events were identified in the proximal AZFc region which overlaps with the AZFb deletion interval (Fig. 3). The highest frequency of 50f2/C deletions (55%) was found in Fins (11 of 20 men had this deletion) making it unlikely that it is associated with spermatogenic failure at least in this population. Consequently, it must be concluded that “partial” AZF deletions are not always a cause for male infertility.

Diagnostic of “partial” AZF deletions needs more than PCR assays

Some STS loci diagnostic for different partial AZF deletions are given in Figure 3. Because gene conversions are expected with a high frequency in the palindromic Yq11 sequences (see above), any partial AZF deletion diagnosed by a distinct set of STS markers has to be confirmed as a real genomic deletion by FIBER-FISH or Southern blot or some other quantitative gene copy assay.
The partial AZFc deletions summarized under the pseudonym “gr/gr” deletions display the same deletion pattern of green-red FIBER-FISH signals in the men´s lymphocyte nuclei (Repping et al., 2003). However, assuming that these are Non-Allelic-Homologous-Recombination (NAHR) events needing for recombination an identical sequence site as observed in similar autosomal NAHR events, the gr/gr coined recombination sites need to be split in recombination sites between the g1/g2 amplicons, the r1/r3 amplicons, and the r2/r4 amplicons (Fig. 3C).

Recently, a g1/g2 based recombination leading to a partial AZF deletion including the DAZ1/DAZ2 gene doublet was reported as “de novo” mutation event in five individuals with severe oligozoospermia. The DAZ1/DAZ2 deletion was identified with a specific DNA blot assay. Since the same DAZ1/DAZ2 blot deletion pattern could not be identified in 107 fertile control samples, it was concluded that the g1/g2 deleted Y chromosome is probably associated with the occurrence of the men spermatogenic failure. However, this study did not yet report the Y haplogroups of the different g1/g2 deleted men nor the exact recombination sites. We expect a large variability of these sites in the g1/g2, and also r1/r3 and r2/r4 amplicons in the 17 different Y lineages where the gr/gr deleted Y chromosome were identified, since all these recombination events must have occurred independently.

Thus the gr/gr coined partial AZFc deletions are summarizing multiple different recombination events of which the specific breakpoint sites can spread along ~600 kb of the green/red amplicon pair. Their frequency is not evenly distributed between these amplicons but recombinational hot-spots are reported (Machev et al., 2004). In summary, their artificial combination under the

pseudonym “gr/gr” oversimplifies the complexity of these molecular events and inhibits true statistically based interpretations, which are only valid when comparing the same molecular mutation event. Statistical evaluations whether a distinct gr/gr based AZF deletion is indeed contributing to the man´s infertility is therefore only valid when comparing the data of men from the same Y-chromosomal haplogroup.

Unfortunately, not all STS/SNV based studies on gr/gr deleted Y chromosomes did include further assays to confirm the proposed genomic AZF deletion, or analysed the associated Y-chromosomal haplogroups. This may explain the opposite conclusions drawn by some of these studies with regard to the association of the identified AZF deletion with male infertility (Hucklenbroich et al., 2004; Llanos et al., 2004). Both studies diagnosed the partial AZF deletion as gr/gr deletion only by marker sY1291. The sY1291 marker is a polymorphic sequence site deleted in men with Y haplogroup J independent of their fertility status (Machev et al., 2004).

AZF deletions need intrachromosomal recombination events

Intrachromosomal recombination events are based on the same polarity of the homologous amplicons (Yen, 2001). A high frequency of g1/g2, r1/r3 and r2/r4 deletions is therefore expected only in the Y sequence structures which have a similar polarity of their amplicons as found in Y haplogroup R1* (Vogt, 2004). Accordingly, no gr/gr deleted Y chromosomes were found in 29 Y-branches whereas in 13 non-R1* branches only single gr/gr deletions were identified. Instead of this, 12 gr/gr deletions were reported in the 94 men from the R1*- haplogroup (Repping et al., 2003).
In one study on partial AZF deletions, two inversions (b2_b3 and b3_b4) in the R1*-AZFc amplicon structure leading to different polarities of some AZFc amplicons (see Fig. 3) were proposed in order to align the observed marker deletion- and FIBER-FISH-patterns along a continuous DNA segment (Machev et al., 2004). The 32 “gr/gr” –typed deletions were identified in 17 Y-chromosomal haplogroups, that means representing at least 17 independent recombination events. In each Y haplogroup two gr/gr deleted Y chromosomes were identified with an associated b2_b3 or b3_b4 inversion. It can therefoe be predicted that these and other inversion events of amplicons will generally occur frequently in the Y chromosome (Fig. 3).

The b2_b3 inversion with a subsequent g1/g3 recombination was first proposed to be present in the AZFb/c amplicon structure of men from a pre-N-haplogroup (Fernandes et al., 2004). The g1/g3 recombination leading to deletion of the DAZ3/DAZ4 gene doublet and confirmed by Southern blot was found in all individuals from the Y haplogroups N3* and N*(xN3). However, the same rearranged AZF amplicon structure would be also created when a g1_g3 inversion in the R1*-AZF amplicon structure with subsequent b2/b3 recombination would have happened in the Y-N* lineage. If derived from the polarity pattern of the R*-AZF amplicon blocks (Fig. 3), a b2/b3 inversion followed by a g1/g3 recombination in the pre-N* lineage is most likely because the sister clade of the N* haplogroup in the Y phylogeny, haplogroup O, has displayed a b2_b3 inversion and not a g1_g3 inversion in its AZF amplicon structure (Repping et al., 2004).

However, it must be admitted that in many cases, nor STS/SNV deletion analysis, nor FIBER-FISH or blot analyses are suitable to identify and to distinguish all alternative recombinatorial routes which would lead to the same AZFb/c amplicon structure. Additional analyses of sequences and structural maps from the Y chromosome of other lineages –perhaps choosing first the most divergent A haplogroup (YCC, 2002)– are therefore highly desirable.

In summary, although several partial AZFc deletions summarized under the pseudonym “gr/gr” might have some influence on the man´s reproductive fitness this influence is probably variable in

different Y lineages and its penetrance with respect to spermatogenic failure is certainly lower than that for complete AZFc deletions induced by the b2/b4 recombinations. Whereas complete AZFc deletions were almost always found as “de novo” deletions, i.e., only present in the patient´s Y chromosome, the gr/gr deleted Y chromosomes were found also in the probands´ fathers in all instances where a father of the infertile gr/gr deleted man was available.

AZF genes plasticity in Y chromosomal haplogroups

Considering the variable copy number of the AZFb and AZFc genes in Y haplogroup D2b, F(xH,K), I and N (Fernandes et al., 2004; Machev et al., 2004; Repping et al., 2004) and probably more Y chromosomal haplogroups not yet identified, the question is raised which the Y genes in the polymorphic AZFb/c subintervals are really all essential for spermatogenesis? If we assume that the basic mechanisms leading to the different rearrangements in the AZF deletion intervals are the same, we can expect that they occur frequently in the different Y-lineages currently present in the global population of over three billion men. Molecular methods which compare the age of the Y-lineage with a distinct ampliconic AZFb and AZFc deletion structure and its gene content would then help to determine whether the structure identified is compatible with neutrality (i.e. fertility) or associated with some spermatogenic failure effects and whether the preference of multi-copy genes in the ampliconic Y-sequence domains reflects some functional constraints from the germ line or genetic redundancy need as a counterbalance for their unstable structure to reduce the risk of male infertility. Knowledge of the haplogroup of the Y chromosome of men diagnosed with a distinct partial AZF deletion or only single AZF gene deletion will obviously help to decide whether the observed AZF (genes) deletion contributes to the individuals´ specific testicular pathology or can be neglected.

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Aneuploidy in human sperm

J. Egozcue
J. Blanco,
E. Anton, Z. Sarrate,
F. Vidal
Department of Cell Biology
Universitat Autònoma de Barcelona
Edifici CS, 08193 Bellaterra
Spain
E-mail: josep.egozcue@uab.es

Learning objectives

1)The proportion of aneuploid sperm is increased in several circumstances: normal individuals with a tendency to meiotic errors, carriers of sex chromosome anomalies (XXY, XYY), carriers of balanced chromosomal rearrangements (inversions, translocations), and individuals with synaptic anomalies; 2) The presence of a rearrangement in meiotic cells can induce the abnormal segregation of other chromosome pairs in some cases (interchromosomal effect; ICE); 3) The analysis of aneuploidy in sperm may be helpful for reproductive counseling.

Introduction

In human males, the tendency to meiotic errors (non-disjunction, anaphase lag, predivision) is scarce. For this reason, the proportion of abnormal sperm in fertile individuals is low. However, the use of intracytoplasmic sperm injection has allowed males with abnormal semenograms to reproduce. Often, the presence of an abnormal semenogram is related to constitutional chromosome anomalies, such as sex chromosome aberrations (XXY, XYY), structural chromosome rearrangements or synaptic errors that give raise to the production of chromosomally abnormal spermatozoa which, in turn, are responsible for sterility or recurrent pregnancy losses (Egozcue et al., 2003)1.

Human spermatozoa can be analyzed by fluorescence in situ hybridization (FISH) on decondensed sperm heads. FISH analysis is based on the use of a limited number of probes, and thus can only detect a variable proportion of chromosomally abnormal sperm. However, at present it is widely used to indirectly study the chromosomal constitution of human sperm.

Chromosome anomalies in controls

FISH studies have been performed in several series of fertile individuals, showing a considerable interindividual but also intersample variability1. Based on an estimated mean incidence of disomy of 0.13 % per autosomal pair, of 0.37 % for the sex chromosomes and on an incidence of diploidy of 0.20 %, the mean anomaly rate has been estimated at about 7 % (Figure 1), about 4 times lower than the incidence estimated for oocytes.

Some fertile individuals may have a greater tendency to undergo meiotic errors. This has been demonstrated for some fathers of children with Down syndrome or Turner syndrome of paternal origin (Templado et al., 2005)2(Figure 2).

The incidence of numerical errors and of structural anomalies derived from unrepaired or abnormally repaired chromosome breaks increases with age2, but this increase probably has no clinical significance.

Numerical sex chromosome anomalies

Carriers of numerical sex chromosome anomalies usually show moderate increases in the proportion of sperm with sex chromosome disomies1 (Figure 3). These disomies are mainly of the XY type, both in XXY and XYY males. It has been suggested that XXY cells may be meiotically incompetent, and that XYY cells would have an extreme difficulty to complete meiosis (Blanco et al., 2001)3.

Thus, abnormal sperm in these patients would derive from a normal XY line present in a testis with an abnormal environment (high FSH, compromised blood supply, etc.), suggesting that at least XXY males who produce sperm should necessarily be mosaics.

Considering the low frequency of abnormal sperm in these individuals, and the very limited or absent clinical severity of the condition in most cases, it has been suggested that screening for sex chromosome disomies in these cases would be useless.

Structural chromosome rearrangements

Carriers of balanced Robertsonian translocations (chromosome fusions), reciprocal translocations and inversions produce increased numbers of abnormal sperm (Figure 4). In inversion carriers, the production of chromosomally abnormal sperm is related to the length of the inversion and its possibility of producing an inversion loop during meiosis I (Anton et al., 2005)4.

In Robertsonian and reciprocal translocation carriers, the proportion of abnormal sperm is high, but the risk should be estimated individually, because it depends on the chromosomes and chromosome segments involved, the situation of the breakpoints and the characteristics of the affected segments. These factors have been analyzed by Roux et al. (2005)5 for Robertsonian translocations, and by Benet et al. (2005)6 for reciprocal translocations. Unfortunately, in these patients the frequency of abnormal embryos produced is still higher, probably as a result of interchromosomal effects (see below).

Examples of abnormal spermatozoa in these conditions are shown in Figures 5-7. In each case, the probes used must allow to identify the different types of segregation of the chromosomes involved in the rearrangement.

The presence of a structural rearrangement in a meiotic cell may affect the segregation of other chromosome pairs not involved in the rearrangement, and induce novel chromosome aberrations in that cell. This is known as an interchromosomal effect (ICE), which can be detected in some cases, but not in others4 (Figure 8).

Infertile males with a normal karyotype

Most infertile males have a normal karyotype, but abnormal semenograms. These patients have extremely variable proportions of chromosomally abnormal sperm (Figures 9 and 10). In many cases, the production of chromosomally abnormal sperm is related to the presence of synaptic errors, which may affect up to18 % of patients with a severe oligoasthenozoospermia (<1x106

motile sperm/ml), and although the data are still preliminary, may reach as many as 27 % of normozoospermic patients with previous IVF/ICSI failures (Egozcue et al., 2005)7. Although in these cases the use of sperm FISH studies often does not detect the anomalies present in sperm, because the probes used do not correspond to the chromosomes affected8, sperm FISH studies should also be performed in these cases.

References

Reference 1 is the more recent review of the subject. It is a mini-review but provides all important data. References 2, 4, 5, 6 and 7 are included in a special issue of Cytogenetic and Genome Research, edited by Renée H. Martin and entitled "Cytogenetics of human germ cells" which will have been published by the time the Pre-congress course takes place, and will be the most up-to-date at length reviews of sperm aneuploidy in each particular case. Reference 3 is a study of the meiotic behavior of XXY and XYY cells.

Reference 8 is the only male meiotic study using Multiplex-FISH published so far.

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Acknowledgements

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- **Basal incidence of chromosome abnormalities**
  - * Mean frequency of disomy for the autosomes: 0.13%
  - * Mean frequency of disomy for the sex chromosomes: 0.37
  - * Diploid sperm: 0.20%
  - * Important interindividual and interchromosomal differences have been reported

Estimated percentage of chromosomally abnormal spermatozoa: 7%

Figure 1

- **Fathers of aneuploid offspring**

	Disomy									Diploidy
	4	6	13	18	21	22	XX	YY	XY	
DS fathers			*		*	*			*	*
TS fathers			€		€	€			*	
DS and TS Fathers			*		*	*			*	

* statistically increased frequency with respect to the control group
€ increased frequency with respect to the control group, with tendency to significance

Figure 2

- **Carriers of sex chromosome anomalies**
  - * 47,XXY; 47,XXY/46,XY; 47,XYY; 47,XYY/47XY
  - * Incidence of sperm with sex chromosome abnormalities

Klinefelter syndrome and variants
- **Sex chromosome disomies:** mean 5.7% (range 0%-20%)
- **Diploid sperm:** mean 0.6% (range 0%-4.2%)

47,XYY and variants
- **Sex chromosome disomies:** mean 3.7% (range 0.1%-14.4%)
- **Diploid sperm:** mean 0.5% (range 0%-3.3%)

Controversial opinions about the meiotic competence of the abnormal cell line and the origin of the anomalies observed

Figure 3

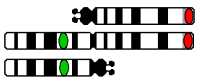
- **Carriers of structural chromosome anomalies**
  - * Translocations, inversions
  - * Classical meiotic studies: estimates of the theoretical risk of production of unbalanced spermatozoa
    Meiotic behaviour depends on each particular reorganization
  - * Sperm studies

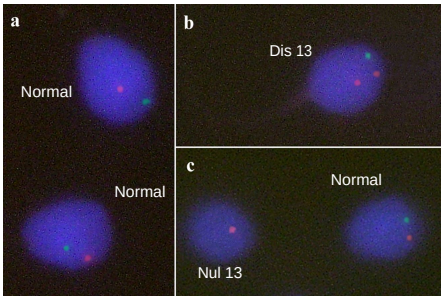
RB: mean 14% (range 7%-36%)
RC: mean 53.2% (range 29.4%-70.2%
inv: mean 8.8% (range 0%-37.8%)

* **Depends on each particular reorganization, chromosomes and breakpoints involved**

Figure 4

- **Carriers of structural chromosome anomalies**



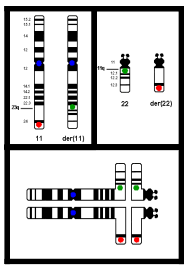


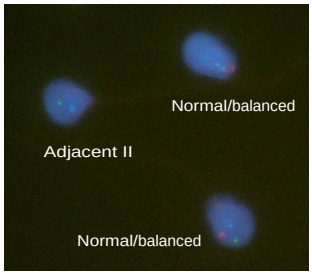
Anton E, Blanco J, Egozcue J, Vidal F (2004)
Sperm FISH studies in seven male carriers of Robertsonian translocation t(13;14)(q10;q10).
Hum Reprod 19:1345-1351

Probes:
LSI 13, RB1, Spectrum Green (Vysis, Inc.)
TelVysion 14q, Spectrum Orange (Vysis, Inc.)

Figure 5

- **Carriers of structural chromosome anomalies**

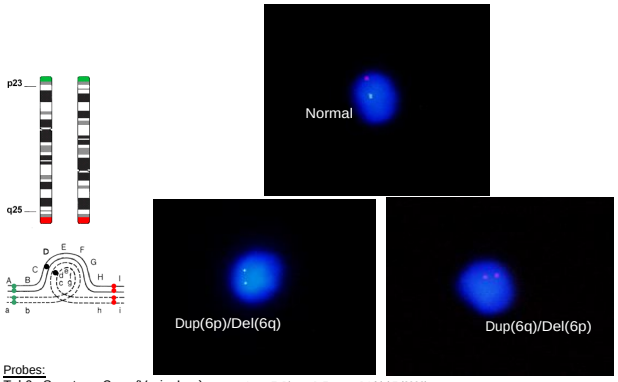




Probes:
LSI 22 (bcr, 22q13.1), Spectrum Green (Vysis, Inc.)
TelVysion 11q, Spectrum Orange (Vysis, Inc.)
CEP 11, Spectrum Aqua (Vysis, Inc.)

Figure 6

- **Carriers of structural chromosome anomalies**

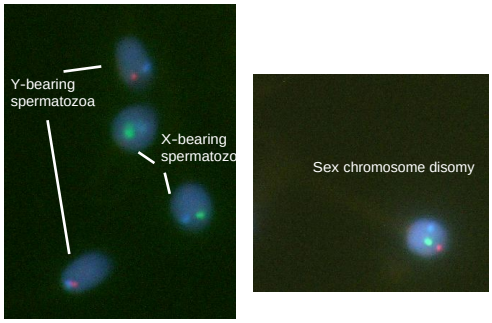


Probes:
Tel 6p Spectrum Geen (Vysis, Inc.)
Tel 6q Spectrum Orange (Vysis, Inc.)

Anton E, Blanco J, Egozcue J, Vidal F (2002)
Risk assessment and segregation analysis in a pericentric inversion inv(6)(p23q25) carrier using FISH on decondensed sperm nuclei. Cytogenet Genome Res 97:149-154

Figure 7

- **Interchromosomal effects in carriers of structural chromosome anomalies**



Positive ICE : 5/13 RB analysed
11/32 RC analysed

Figure 8

- **Infertile males with normal karyotype**

Very heterogeneous population

AS | **OAT** | **severe OAT** | **AZ**

Ejaculated, epididymal, testicular

Meiotic anomalies

Heterogeneous results:
Aneuploidy ranges: normal - 20% (x130 vs. controls)
Diploidy ranges: normal-10% (x50 vs. controls)

Figure 9

- **Infertile males with normal karyotype**

Authors	Infertility	Increase incidence in:
Bernardini *et al.* (1997)	OA (n=9)	Sex chromosome disomy Diploidy
Arán *et al.* (1999)	OA (n=14)	Sex chromosome disomy Diploidy
	A (n=5)	Diploidy
Pang *et al.* (1999)	OA (n=9)	Autosomal and gonosomal disomy Diploidy
Bernardini *et al.* (2001)	OAT (n=22)	Autosomal and gonosomal disomy Diploidy
	TESE (n=3)	Autosomal and gonosomal disomy Diploidy
Rodrigo et al. 2004	Epididym. (n=2)	-------
	TESE (n=20)	Sex chromosome disomy Diploidy

Figure 10

Spermatozoal RNA profiles: isolation and analysis of human spermatozoal RNA

D. Miller
Reproduction and Early Development Research Group
Faculty of Health and Medicine
University of Leeds
U.K.
E-mail: d.miller@leeds.ac.uk
G.Ch. Ostermeier,
Wayne State School of Medicin
Detroit, MI, 48201
USA
D. Dix
Reproductive Toxicology Division (MD-72)
Research Triangle Park, NC, 2711,
USA
Stephen A. Krawetz
Wayne State School of Medicine
Detroit, MI, 48201
USA

Learning Objectives

Following the lecture, participants should be aware of the following:

- The compelling evidence for the presence of RNAs in ejaculate spermatozoa.
- Possible reasons for selective retention of mRNA.
- Minimising somatic cell contamination.
- Development and choice of methods aimed at isolating pure spermatzoal RNA
- Specific markers of spermatzoal RNA.
- Methods for analysing spermatozoal RNA populations.
- Prospects for infertility (and other) research.

Introduction

The presence of spermatozoal RNA has been the subject of some controversy since the work of Maclauchlin and Terner showed that the terminally differentiated spermatozoa have lost any intrinsic mechanism for synthesising nuclear RNA [1]. This finding is in full agreement with the accepted shutdown of the spermatozoal nucleus during spermiogenesis and the concomitant uncoupling of transcription from translation [2]. Until very recently, the general assumption has been that the terminally differentiated spermatozoon has no mRNA and that any detection thereof is likely to be artefactual. This workshop will provide an overview of the direct evidence that established that ejaculate spermatozoa indeed retain a complex entourage of mRNAs. This complex population may be informative with respect to the diagnosis of male infertility and aberrations in the gene expression pathways that underpin spermatogenesis.

The presence of RNAs in ejaculate spermatozoa.

There have been many reports of mRNAs in human spermatozoa and the spermatozoa of other species. These have mostly relied on the use of RT-PCR based amplification strategies using target-specific primers on cDNA generated from total spermatozoal RNA or affinity purified mRNA. The number of reports using this strategy has grown over recent years and now includes the detection of mRNAs encoding HLA class molecules, L-type calcium channels, N-cadherins, oestrogen receptors, cyclic nucleotide phosphodiesterases (PDE), various integrins, aromatase and Nitric Oxide Synthase (NOS) among others [3]. The interrogation of cDNA arrays covering thousands of gene targets using spermatozoal probes has increased the number of detected mRNAs to at least 5,000 based on comparisons between individual sample ejaculates. These studies have also revealed the likely presence of two populations of mRNAs in the spermatozoa of individual ejaculates reflecting a core invariant subset of mRNAs that may be essential for fertility and a variable subset that may not be essential (but possibly belies inter and intra-sample heterogeneity). At its crudest, sperm RNA can be used to test the effectiveness of vasectomy using the detective power of RT-PCR; no signal for sperm-specific protamine 2 (PRM2) should be detectable after one round of PCR compared with a standard housekeeping mRNA such as GAPDH (derived from somatic cells contained within the ejaculate that are not removed following vasectomy).

Minimising somatic cell contamination

Human semen normally contains somatic cells including some leucocytes and epithelial cells (almost exclusively squamous in origin). Depending on the type of analysis required, these must be either removed or minimised. We have found that sedimentation at 1000g through one or two (sequential) discontinuous gradients of Percoll or Suprasperm (90%:45% in Earle's HEPES buffered salt or P.B.S. containing 0.1%v/v H.S.A.) will remove almost all of these cells. Clearly, however, there will be a significant loss of spermatozoa in the upper layers of these media. The final pellet can be washed to remove the gradient medium and then re-suspended in DEPC-treated distilled water containing 0.5% (w/v) SDS and 0.5% (v/v) Triton X-100 for 10 m at room temperature to lyse any remaining somatic cells (Figure 1). As a single ejaculate spermatozoon contains approximately 10-15 fg of RNA, the yield of total RNA by this method is wholly dependent on the efficiency of centrifugation and hence the number of spermatozoa that sediment into the final pellet. Less than 100ng of spermatozoal RNA will suffice for several target-directed RT-PCR experiments corresponding to approximately 6-10 x 106 cells. This number should be easily harvested from an average normozoospermic ejaculate even after two sequential rounds of centrifugation. However, samples with low sperm counts and/or high levels of somatic cell carriage can be problematic. We have found that on fresh samples or samples stored in frozen storage buffer (FSB; 50 mM HEPES buffer-pH 7.5, 10 mM NaCl, 5 mM Mg-acetate, and 25% v/v glycerol), density gradient centrifugation can be omitted from the procedure and substituted by either one or preferably two rounds of hypotonic lysis alone. After removing the seminal plasma (and FSB) by mild centrifugation (two washes in HEPES buffered saline), this method alone has been successfully employed to destroy contaminating somatic cells. Increasing the concentration of SDS and Triton X-100 to 0.5% w/v and 0.75% v/v, respectively can help treat semen samples containing high numbers of round cells, but their use at higher concentrations is not recommended. While spermatozoa are resistant to detergent treatment, the effect of SDS and Triton X-100 on RNA carriage and stability remains unknown. Moreover, we have found that many commercial freezing preservatives including SpermFreeze™ reduce the ability of hypotonic solutions to lyse somatic cells (as does preservation in RNA stabilising solutions including Ambion's RNA Later™). Hence, if FSB cannot be used, then samples should ideally be processed to remove soma prior to either freezing or stabilisation in RNA preservatives if long-term storage is required. It should also be noted that even some fresh

samples are resistant to hypotonic, detergent-assisted cell lysis and it is therefore wise to check processed samples under a microscope to ensure that round cells have been destroyed prior to further processing.

Methods aimed at isolating pure spermatzoal RNA (and yields)

Compared with the average somatic cell and probably spermatid with 1-3pg of RNA, the low RNA content of ejaculate spermatozoa (~0.015pg) requires that efficient isolation procedures be chosen. On the whole, methods based on guanidinium isothiocyanate (GHCN) disruption of between 107 and 108 cells meet these requirements. In general, 4M solutions of GHCN in neutral or slightly acid citrate buffers containing sodium sarcosinate are optimal (4-6ml depending on cell concentration). We have found that the addition of 2-mercaptoethanol improves isolation yields, presumably by disrupting S-S bonds and hence facilitating access by GHCN to the highly compact sperm nucleus. Dithiothreatol (DTT) is not a good substitute under these conditions. The addition of carriers such as yeast tRNA or glycogen or polyacrylamide carriers can also improve recovery from samples containing fewer gametes although tRNA interferes with the calculation of RNA yields. Incubating the suspension for 30 – 60 min at 68°C prior to further processing is also helpful as is shearing the DNA (carefully) using a 25 guage needle. Total RNA (0.5µg - 3µg) is recovered from these solutions using standard phenol/chloroform based procedures and as there is little or no ribosomal RNA in the preparation, much of this RNA is message (Figure 2). Indeed, the absence of 28S and 18S species is a good, albeit crude marker of spermatozoal RNA purity (see below). Unless adjustments to the volume of GHCN are made, RNA yield does not necessarily increase linearly with cell concentration due to competition with sperm DNA (particularly where columns are used). Hence, It is almost always the case that the RNA will require treatment with RNAse-free DNAse as residual DNA is frequently encountered. The alternative route of re-processing the RNA through an additional phenol/chloroform extraction step is not recommended, as RNA losses are inevitable.

Of the commercially available RNA isolation kits, the Qiagen RNEasy™ system works well (http://www1.qiagen.com/Products/RnaStabilizationPurification/OtherSamples.aspx. To counteract the much higher DNA/RNA ratio of these cells, we recommend doubling the number of columns suggested for a given concentration of somatic cells or halving the quantity of sample for processing and passing both halves through the same column concurrently. 2-mercaptoethanol should also be added to the GHCN solution. This should then be followed with vigorous homogenisation using either a polytron or teckmar homogenizer prior to incubation at 68°C for 30 m. We recommend that the manufacturer's instructions are followed from this point onward, including treatment with DNAse 1 either on or off the column (or both) See the 'animal cell' protocol from the Qiagen handbook. However, each lot of DNase 1 and RNase block will require careful quality control measures to ensure the absence of contaminating RNases as it has been our experience that RNAse-free is not necessarily RNase free. The fully detailed protocol as used in the Miller and Krawetz labs, including somatic cell lysis will be available at the Workshop.

RNA can also be amplified if yields are too low for downstream processing. Collaborating laboratories have successfully applied Ambion's MessageAmp II system (http://www.ambion.com/techlib/tn/115/2.html) for both target-directed and array-based analyses of spermatozoal RNA. This approach can extend RNA analysis to oligozoospermic men with < 20 x 106 spermatozoa /ml and should also facilitate the examination of sub-populations of ejaculate spermatozoa fractionated on density gradients or by FACS.

Markers of spermatozoal RNA and assessing DNA contamination

Although spermatozoa contain little or no 28S and 18S rRNA, these are unsuitable (indeed misleading) markers of spermatozoal RNA fidelity because of their likely detection in any sensitive RT-PCR assays. There are a number of more appropriate markers that can be used to assess the specificity and purity of sperm RNA. Intron-spanning primers that amplify a 150bp product from protamine 2 mRNA and a 310bp product from genomic DNA controls for both sperm-specificity and residual DNA contamination (Table 1). Primers amplifying 844bp (CD45) and 780bp (cKIT) sequences have been used to control for RNA derived from leucocytes and immature germ cells, respectively (Table 1;[4]). In addition to target-specific controls, we have used the power of OntoExpress™ (http://vortex.cs.wayne.edu/projects.htm) to assess the level of somatic cell contribution to our RNA preparations as analysed on genomic arrays and have found it to be negligible.

Methods for analysing spermatozoal RNA populations

Currently, the most popular method of analysis of spermatozoal RNA is target (gene-specific) RT-PCR. A full list of papers reporting data from a wide variety of RNA targets will be available at the workshop. At least one group has reported changes in the levels of one or more transcripts in relation to sedimentation rates in density gradient media and to motility [4], indicating that a careful selection of targets can yield informative data. In situ RT-PCR offers an alternative strategy for analysing specific gene targets in histological sections [5]. More recently, microarrays representing thousands of gene sequences have become available from a number of sources. These allow the simultaneous analysis of a large number of spermatozoal RNA targets that for the first time, opens up large-scale molecular analysis of spermatozoal RNA. Such an approach has successfully delineated the fertile 'transcriptome' of spermatozoa and enabled the identification of some key genes that are affected in sub-motile populations [6]. As array-based approaches are now becoming more reasonably priced their deployment in male factor infertility investigations should become more widespread and promises to revolutionise our understanding of the molecular background.

Prospects for infertility (and other) research

The fact that ejaculated spermatozoa harbour thousands of mRNA species is interesting in itself. Many of them are well characterised (see above) and can offer new insights into ion trafficking, sperm-egg interaction, chromatin repackaging, structural anomalies and other aspects of the spermatozoan. We have also noted that the sperm appears to retain a record of gene expression that occurred throughout spermatogenesis or at the very least, spermiogenesis and our own studies suggest that many RNAs are relatively uncharacterised or encode hypothetical proteins. One study [7] has already shown that spermatozoal RNA can delineate an infertile phenotype caused by poor progressive motility. Perhaps the most important feature offered by semen sampling is that it is wholly non-invasive. Since the great majority of male factor infertility does not eliminate sperm from the ejaculate, there is every confidence that studying the ejaculate spermatozoa of men with idiopathic infertility will, in conjunction with microarrays (commercial or bespoke), illuminate the molecular mechanisms underpinning their low fecundity. This could be done at lower financial cost and at higher power than is possible using testicular biopsy since we can obtain many more sample ejaculates than testicular samples. We know that spermatozoal RNA is a good proxy for the testis because it comprises mRNAs that define a subset of those found in testis [6]. This subset most likely reflects the germ line. Finally, spermatozoal RNA offers considerable potential in toxicological studies aimed at analysing the molecular mechanisms underlying the suspected decline in male

fertility in relation to environmental factors. The impact on spermatogenesis of chemotherapies employed in cancer prophylaxis could also be conveniently estimated in the ejaculate rather than the testis. This would provide an excellent route to examining the recovery of spermatogenesis following treatment and assessing the quality of the gametes produced.

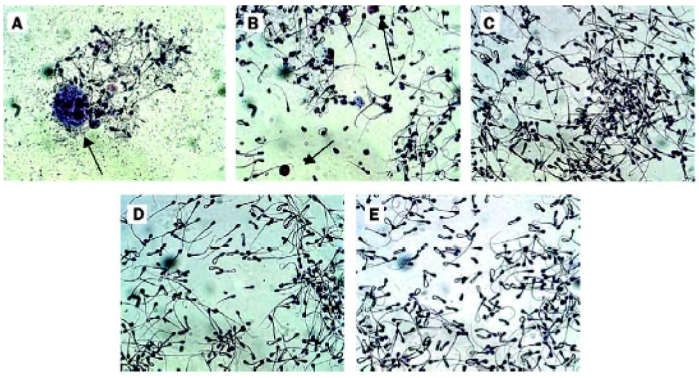


Figure 1 Purification of ejaculate spermatozoa (**A**) through a 90:45% solution of Peroll showing the 90:45 interface (**B**), pellet (**C**), pellet from second round density gradient (**D**) and after detergent assisted hypotonic lysis (**E**). Unwanted cells (arrows) are eliminated by this process. The centrifugation steps can be omitted to the same effect (see text).

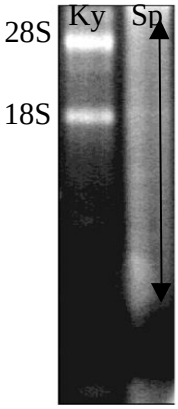


Figure 2
AGE of total Kidney (Ky) and sperm (Sp) RNA. Note absence of rRNA in sperm and presence of streak (arrow)

Table 1 **Control primer sequences**

PRIMER	Sequence (5' to 3')	T_A°C	Amplicon (bp)
PRM2-fwd PRM2-rev	tat agg cgc aga cac tgc gcc ttc tgc atg ttc tct	64 (gDNA control)	310*; 149°[8] * gDNA; °cDNA
CD45-fwd CD45-rev	tgc aga tgc cta cct taa tgc cac att gca gca ctt cca tt	60 (WBC control)	844 [4]
c-KIT-fwd c-KIT-rev	agt aca tgg aca tga aac ctg g gat tct gct cag aca tcg tcg	52 (RC control)	780[4]

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The use of FISH to determine Y-chromosome structure

S. Repping
Center for Reproductive Medicine
Academic Medical Center (A1-229)
Meibergdreef 9
1105 AZ Amsterdam
The Netherlands
E-mail: S.Repping@amc.uva.nl

Learning objectives

- to be aware of various FISH techniques that are available
- to understand the applications of each FISH technique
- to broadly understand the structure of the human Y chromosome
- to list the common deletions on the Y chromosome
- to understand the common variation in Y-chromosome structure

Introduction

The human Y chromosome has been studied extensively by reproductive scientists for its role in male infertility. Originally, in 1976, light microscopy and karyotype analysis indicated a deletion of the entire long arm of the chromosome in a small percentage of men suffering from azoospermia. With the advent of modern day molecular techniques such as PCR and Southern blotting, the long arm of the chromosome was shown to harbor three distinct intervals termed azoospermia factor a, b and c (AZFa, AZFb, AZFc).

Due to the high proportion of repetitive sequences on the human Y chromosome however, these molecular methods are not always suitable for studying the structure of the chromosome and its aberrations that underlie spermatogenic failure. In stead, our laboratory has used various fluorescence in situ hybridization (FISH) techniques to study the Y chromosome of normal and infertile men. This lecture will discuss these FISH techniques and their use in Y-chromosome research. Furthermore, previously published and novel data will be shown to illustrate the usefulness of FISH.

FISH methods

Fluorescence in situ hybridization is a commonly used technique that is based on the hybridization of directly or indirectly labeled probes to complementary DNA targets. Probes can be labeled with various distinct fluorophores and can be hybridized separately or in combination with other probes labeled with a different fluorophore. FISH probes will generally hybridize to any sequence that has >85% homology to the sequence of the probe. Each FISH technique relies on the same principle but varies in the nature of the DNA target giving it a specific resolution and range (see table 1).

Metaphase FISH

By using metaphase spreads as target for FISH one can easily determine the location of a specific probe in the context of all chromosomes. It is for instance easy to determine if a probe is Y-chromosome specific and if it hybridizes to the short or long arm of the chromosome. For instance, figure one shows the use of metaphase FISH to detect the precise location of Y-chromosome specific heterochromatic sequences. Because of the extreme condensation of DNA during metaphase, the resolution of metaphase FISH is low.

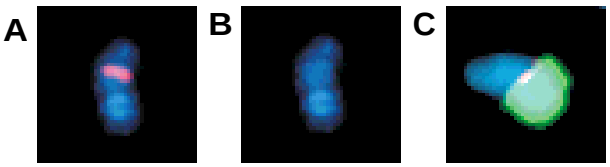


Figure 1. Metaphase FISH. A, Hybridization of a DYZ19 BAC (red) to a DAPI-stained (blue) metaphase Y chromosome. B, DAPI staining alone, revealing slight diminution of DAPI staining at site of DYZ19 hybridization. C, Co-hybridization of two BACs to a DAPI-stained metaphase Y chromosome. The first BAC (red) spans the Yq11 euchromatin/Yq12 heterochromatin boundary; its heterochromatic portion is composed exclusively of DYZ18 sequences. The second BAC (green) is composed exclusively of DYZ1 sequences. The observed yellow signal at the proximal edge of the heterochromatic region results from the superposition of red and green signals. This result illustrates that the Y-chromosome long arm is constructed mostly of DYZ1 repeats1.

Interphase FISH

Interphase FISH is probably the most commonly used FISH method. By starving growing lymphoblastoid cell lines, cells can be arrested in G0 (a phase in which no DNA replication occurs) and used for FISH. During interphase the full genome is compacted into a single nucleus and the chromosomes can not be seen separately. However, as compared to metaphase FISH the resolution is far better and it is possible to see apart two signals that are separated by only 50 kb. Interphase FISH can be used to determine the number of spots for a specific probe (and hence the copy number of a specific sequence) or it can be used to determine the order of two or more probes (see figure 2).

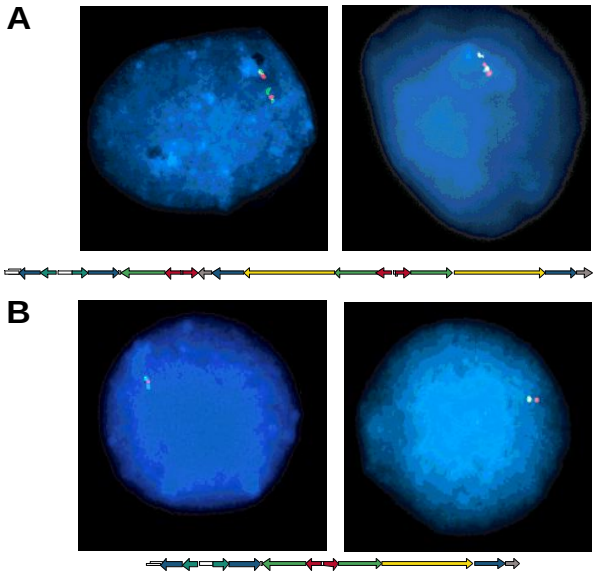


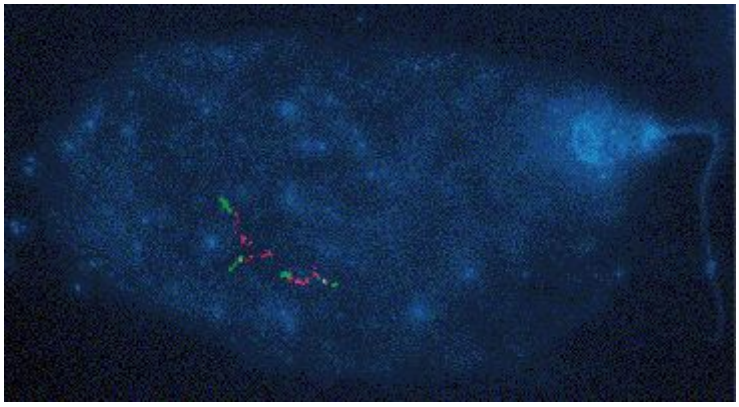
Figure 2. Interphase FISH. A, nuclei from a man with an intact AZFc region. The left panel shows two-color FISH with probes against the green and red amplicons (order G-R-G-R-G); the right panel is a two-color FISH with probes against

red and yellow (Y-R-Y-R). Below the nuclei is a schematic diagram of the AZFc region. B, nuclei from a man with a gr/gr deletion. The order of the signals corresponds to the diagram (below) of a gr/gr deleted Y chromosome2.

Sperm HALO FISH

In the past, we have developed spermHALO FISH as an intermediate technique (in terms of resolution and range) between interphase and fiber FISH3. SpermHALO FISH can achieve the same resolution as fiber FISH (down to only a few kb) but has a far higher range. We have used spermHALO FISH primarily to illustrate that deletions based merely on results from sequence family variants (SFVs) are often false findings, i.e. spermHALO FISH indicates the presence of a complete AZFc region in many men with negative results for one or more DAZ-SFVs. Although useful, spermHALO FISH is nowadays used in our laboratory only in rare cases since it is both laborious and requires a sufficient amount of spermatozoa (which is often not available).

Figure 3. SpermHALO FISH. SpermHALO from a normospermic donor probed with DAZ-probes 18E8 (red; detecting the center of each DAZ cluster) and 46A6 (green; detecting the 3'-end of each DAZ gene) showing two DAZ gene-clusters with a total of four DAZ genes3.



Fiber FISH

By mechanically extracting DNA from interphase nuclei, it is possible to create linear DNA threads on glass slides that can extend up to a few 100 kb in length. Similar to spermHALO FISH, fluorescent probe signals appear as beads-on-a-string. This FISH technique is commonly used to study the sequence order of two probes with a resolution of down to a few kb. Furthermore, it can also be used to accurately estimate the distance between two sequences1. A drawback of fiber FISH is however, that DNA threads break easily. Furthermore, it is impossible to determine whether two signal threads that appear close to each other originate from the same cell or from two separate cells.

Figure 4. Fiber FISH. DNA fiber probed with cosmids 46A6 (red; see figure 3) and 63C9 (green; detecting the complete DAZ gene). The order of signals shows that each DAZ cluster contains two DAZ genes in head-to-head orientation (3' 5';5'➡3')[4].

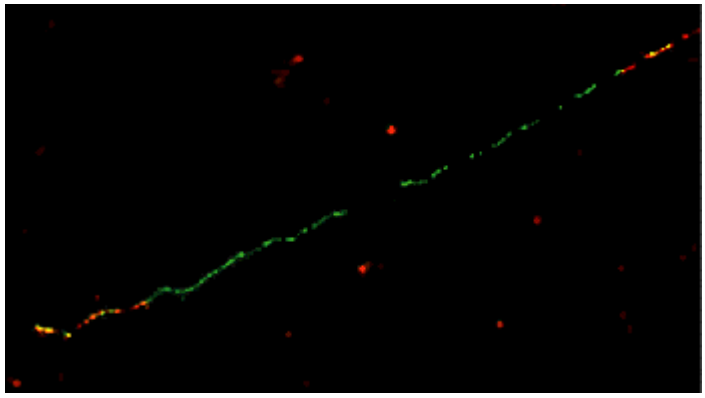


Table 1. Summary of FISH techniques

Method	Range	Resolution
Metaphase FISH	> 1 Mb	Low
Interphase FISH	> 50 kb	Medium
Sperm-HALO FISH	10 kb - ~5 Mb	High
Fiber FISH	5 – ~300 kb	Very high

Results

Deletions

FISH has been used mostly to study Y-chromosome deletions. Originally we successfully used FISH to determine the number and organization of the DAZ genes before the complete AZFc region was sequenced4. By using DAZ specific probes we subsequently showed that some infertile men miss one DAZ-cluster consisting of two DAZ genes (deletions that we now know as gr/gr, b1/b3 and b2/b3 deletions) 5. FISH was also used to show that P5/proximal-P1 deletions (also known as AZFb deletions) overlap with the AZFc region6. More recently, FISH was used to confirm the deletion origin of gr/gr, b1/b3 and b2/b3 deletions. Furthermore, FISH indicated that in some instances gr/gr or b2/b3 deleted chromosomes have undergone subsequent duplications that restore AZFc gene copy number2,7.

Common variation

We have recently used FISH to study the degree of common variation among human Y chromosomes by determining the structure of representative chromosomes from each branch of the Y-chromosome genealogical tree, a tree that represents worldwide diversity. In contrast to previous reports, this study focused on presumably selectively neutral common variation. FISH assays were designed to detect variation based on previously published data as well as variation predicted to occur through homologous recombination. Our study revealed that the structure of the human Y chromosome varies considerably (including the AZFc structure) but the copy number of Y-chromosomal genes seems to be constrained (Repping et al., unpublished data).

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Sperm chromatin organization and DNA integrity

L. Björndahl
Director of Andrology
Assisted Conception Unit.
Birmingham Women's Hospital
United Kingdom
Andrology Centre
Karolinska Univeristy Hospital and Institute
Stockholm
Sweden
E-mail: L.Bjorndahl@bham.ac.uk

Introduction

The spermatozoon represents a unique cell type in the body. In the evolution it has developed into a cell adapted for a safe transfer of the paternal half of the genome from the site of production through the reproductive tracts of both the male and the female to the oocyte in order to create a new individual with an undamaged genome. There are organelles to allow cell mobility, passage between cells and other material surrounding the maternal gamete and to finally fuse the cell membranes to achieve fertilization. The DNA in the sperm nucleus is densely packed and therefore not available for DNA transcription and replication as in somatic cells. This condensed state of the sperm nucleus is believed to be essential for the protection of the genome during storage and transport to the oocytes. However, it is obvious that it is not only the integrity of the DNA itself that is essential for the proper development after fertilization. The sperm DNA is likely to have a unique coding – epigenetic control - for which genes that should be available for activation at different stages of the embryonic and foetal development. Sperm specific epigenetic control should include both a general pattern required for the embryonic development, and also a specifically paternal pattern resulting in activation or inactivation of certain genes in the paternal genome. Errors in the sperm epigenetic control are known as paternal imprinting disorders.

Nuclear changes during spermatogenesis

After the meiotic divisions, the individual cells –still connected by cytoplasmic bridges – start to develop from round spermatids into testicular spermatozoa. We know the structural changes included in this process (condensation of the nucleus, formation of tail with mitochondria in a mid piece, acrosome covering the apical part of the nucleus, reduction of the cytoplasmic volume etc), but all mechanisms are not known in detail.

The nuclear changes are governed by the successive replacement of the somatic histones by other proteins. Thus there are some testis specific histones described, and also other transitional proteins initially replacing the histones and then finally replaced by protamines. Protamines are basic proteins rich in arginine and cysteine residues which gives a basis for intermolecular linking by salt bridges (involving Zn2+) or disulfide bridges (S-S). It is possible that the protamines have evolved from one sub type of histones, H1. In bull, rat, ram, boar, guinea pig) only one type of Protamine (P1) has been found, while in man and mouse also a second type of protamine (P2) has been found. This P2 contains less cysteine residues than the P1, and the relatively lower degree of stability found in

human sperm has been attributed to the relative abundance of P2 in the chromatin. Protamines form a complex with DNA, linking to the major grove of the DNA double helix and forming a compact "doughnut-like" structure. Between the "doughnuts" there are DNA loops that can be experimentally detached. The availability of DNA to interact with experimental conditions is the basis for the available tests of sperm DNA integrity that will be discussed later. The amount of zinc in the human sperm chromatin has been calculated to correspond to a 1:1 ratio to protamines, indicating a possible role for zinc to link protamine molecules by salt bridges. The alkaline nature of protamines neutralizes the repellent forces of the DNA molecule when binding, thereby allowing the much denser structure of the sperm nucleus. A completely condensed mammalian sperm chromatin has been described as crystalline-like due to the very low content of water. Lack of water in the chromatin would be an important factor for the protection of the DNA during transfer to the oocytes. Furthermore, in contrast to DNA-histone interaction, where DNA methylation and acetylation/methylation status (epigenetic control) regulates DNA transcription and replication, the compacted state of the DNA-protamine complex is believed not to inactivate the DNA. The inactive DNA of the sperm does not seem to have active systems for repair of DNA damages, leaving to the systems available in the oocytes to repair any damage arisen during the final stages of spermatogenesis and transfer. Thus, the higher the frequency of DNA breaks, the more likely that errors will occur when DNA repair takes place in the oocytes. Another event taking place in the oocyte after fertilization – when the sperm nucleus is rapidly decondensed – is the replacement of protamines for somatic cell histones, allowing the DNA to become active again.

Another part of the necessary changes taking place in the testis is the reprogramming of the epigenetic control, whether by DNA methylation or by histone-DNA interaction. In human sperm chromatin the P1 and P2 protamines dominate, but still some 15% of the protein content seems to consist of histones. It has been argued whether presence of histones in the sperm chromatin is pathological, a sign of a generally inefficient testicular function in the human species, or maybe essential for the transfer of epigenetic control to the fertilized oocytes. The presence of histones in human sperm chromatin makes it at least a theoretical possibility that some epigenetic control could be transferred by histone-DNA interaction. Nevertheless, when sperm are formed the somatic pattern must be replaced by a gamete pattern. The gamete pattern should include sperm specific – paternal – imprinting, and possibly control of genes needed early in the embryonic development. For example, genes controlling the development of the placenta are normally only active in the paternal genome. Tesarik recently showed, in a study using donated oocytes, that in a group of men with failure after intracytoplasmic sperm injection (ICSI) paternally derived development problems can occur late in embryogenesis, pointing to post-fertilization processes depending on sperm factors.

Post-ejaculatory changes in sperm chromatin stability

A common misinterpretation of early studies of human sperm chromatin stability is that the chromatin of the ejaculated human sperm is stabilized by covalent, disulphide crosslinks (S-S). On the contrary, if examined within a few minutes after ejaculation, most sperm heads from healthy men can decondense, provided Zn2+ is blocked. In the absence of zinc, another type of stability, that needs disulphide cleaving agents for disruption, rapidly develops. In the natural course of events, most sperm are ejaculated together with the zinc-rich prostatic secretion (~1000 x blood concentration) onto the cervical mucus and successful spermatozoa are most likely never in contact with the seminal vesicular secretion that dominates the last 2/3 of the ejaculate. Sperm ejaculated together with seminal vesicular secretion have lower nuclear content of zinc. Furthermore, among spermatozoa collected in a laboratory pot, where all sperm are trapped in the coagulate and then released when liquefaction takes place, the nuclear zinc content seems to be more dependent on the abundance of seminal vesicular fluid than the zinc rich prostatic secretion. This could be due to the

changes in bio-availability of zinc that takes place in the laboratory setting. At ejaculation sperm are expelled together with the more acidic, zinc-rich prostatic secretion, where zinc is associated with citrate. Upon admixture of the more alkaline seminal vesicular fluid, rich in proteins of different molecular weight, increase in pH increases the capacity of citrate to bind zinc simultaneously with the appearance of proteins with high affinity for zinc. Thus, from the zinc-providing prostatic secretion, the admixture of seminal vesicular fluid turns the environment for the ejaculated sperm into a zinc-binding environment that can deprive sperm nuclei of zinc.

In vitro experiments have shown that directly when zinc is withdrawn from the nucleus, the chromatin is destabilized – causing easier access to the chromatin for water and water soluble compounds. If the chromatin is left in a zinc deprived state, a disulphide-based stability can develop, that may interfere with normal chromatin decondensation after fertilization. The possible effects of post-ejaculatory causes for abnormal exposure of sperm chromatin to different damaging substances have not been studied directly, but there is accumulating evidence pointing to negative relations between especially seminal vesicular fluid and vulnerable sperm DNA.

Tests for DNA integrity and "DNA damage"

In recent years, some different techniques have been developed to evaluate the status of the sperm chromatin. In some cases the basic principles were investigated many decades ago, but not until recently there has been a clinical interest to assess qualitative aspects of the sperm chromatin. In all techniques, there is a matter of getting access to the sperm DNA and with different staining techniques visualize "abnormal" patterns due to breakes in the DNA molecule. It could, however, be argued that the DNA damages may not be present in the sperm before it is subjected to the test conditions. With that perspective, it would be fairer to name the tests "DNA vulnerability tests". Also, the test do not show any direct link between test results and specific mechanism – studies have discovered relations between the proportion of sperm with vulnerable DNA and many other parameters, including measures of ART outcome. This presentation is limited to the outlines of the most commonly used techniques.

Single cell gel electrophoresis (Comet)

The Comet assay – or single cell gel electrophoresis – consists of two main techniques: the neutral and the alkaline Comet assay, respectively. In both techniques sperm are embedded in an agarose gel after removal of cell membranes and exposure to disulphide breaking agents. To enable release of DNA from the embedded cells extended treatment with enzymes is included; for the alkaline technique the high pH can replace some enzymatic reactions. After applying an electric field to the agarose gel (electrophoresis) forcing charged particles to migrate from the sperm nuclei, DNA compounds are identified by adding a fluorescent dye (ethidium bromide). The main difference between the two techniques is that the neutral detects mainly double-stranded DNA breaks, while the alkaline supposedly detects (but does not distinguish between) double- and single-stranded DNA breaks due to the denaturing effect of the alkaline procedure.

Originally the Comet assay was developed for the assessments of DNA in somatic cells. Considering the considerable difference in chromatin structure between somatic cells and spermatozoa, it could mean the assay should be further adapted to the sperm chromatin to provide more valid results. Still, there are a number of publications pointing to a relation between proportion of sperm with high degree of DNA fragmentation and clinical situations like cryo-preservation and sperm preparation.

Acridine Orange based tests

Almost 40 years ago Gledhill and Rigler published studies DNA stainability with the fluorescent DNA stain Acridine Orange (AO) and related changes to infertility in the bull. A decade later Evenson published a AO based technique including the use of flow cytometry and described differences between fertile and infertile men and bull. The principle for the AO methods is that when the compound binds to double stranded DNA green fluorescence is elicited, while binding to single stranded DNA elicits red fluorescence.

Besides the patented and license controlled flow cytometry technique called Sperm Chromatin Structure Assay (SCSA), other variants exist. Slide based techniques exist, although unspecific binding of both sorts of AO to glass can make the proper evaluation of the sperm difficult. Fluorescence can be found in three main categories: red fluorescence indicate abundant presence of single stranded DNA and is considered pathological. A normal sperm should have a limited green fluorescence although most men have a varying proportion of "high green fluorescing" sperm – indicating increased access to double stranded DNA for the dye or increased unspecific binding of the dye to sperm structures. From the assessment of red and green fluorescence a DNA fragmentation index (relation between red and green fluorescence) can be calculated.

Studies have showed a relation between SCSA parameters and in vivo fertility. Not surprising, the correlation with traditional semen parameters appears to be weaker – indicating that sperm chromatin accessibility is not directly related to e.g. sperm concentration, motility and morphology. However, a number of studies have pointed to a negative influence of seminal vesicular fluid on sperm DNA accessibility.

Terminal TdT-mediated dUTP-nick-end labelling assay (Tunel)

This assay, named by the acronym Tunel, is based on the principle that free DNA molecule ends can be labelled with modified nucleotides. The labelled ends can then be visualized by fluorescence or in-situ hybridization. In DNA with increased frequency of DNA breaks, there will be more free ends. The sperm assay is based on commercially available tests developed for the detection of apoptotic somatic cells – increased DNA breaks as in indicator for initiation of apoptosis. An interesting, but yet unproven, hypothesis is that increased presence of sperm with DNA breaks is indicative of inefficient mechanisms to dispose of apoptotic sperm during sperm development and maturation within the male reproductive tract. The test has shown a relation between poor semen analysis results and failure of intracytoplasmic sperm injection (ICSI), but not with the formation of a male pronucleus. A general problem, for clinical use, is the lack of useful clinical thresholds.

Conclusions

Not many years ago the mission of the sperm was in general believed to end at the entrance of the sperm in the oocyte. There is now increasing amounts of evidence pointing to the importance of an intact and undamaged sperm delivered to the oocyte. The present techniques mainly demonstrate the accessibility of sperm DNA for compounds that in the assays present as DNA breaks, and results do not give a direct mechanism of action rather a general relation between high degree of accessibility and decreased probability for success of assisted reproduction techniques or fertility in vivo, as well as decreased semen analysis results and negative influence of especially seminal vesicular fluid. Thus, DNA vulnerability appears to be increased in sperm detected by the different assays. An increased vulnerability of the sperm DNA may be due to defects during formation and maturation, but could also be induced by the in-vitro exposure of sperm to seminal vesicular fluid.

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Surgical sperm recovery and sperm DNA damage

L. Ramos
Department of Obstetrics and Gynaecology
Division Reproductive Medicine, (intern post 415)
Radboud University Medical Center
PO Box 9101
6500 HB Nijmegen
The Netherlands
E-mail: l.ramos@obgyn.umcn.nl

Learning objects:

- Diagnosis of obstructive azoospermia
- Importance of an accurate diagnosis
- Epididymal sperm: characteristics
- Chromatin structure and DNA damage of non-ejaculated sperm
- Sperm to be used with ICSI
- Risks of the use of non-ejaculated sperm

1. Diagnosis of azoospermia

The correct classification of azoospermia is not only indicative for a successful sperm retrieval and ICSI outcome, but also for the possible risk of genetic anomalies in children born after ICSI. The classification of azoospermia into obstructive (OA) or non-obstructive (NOA) is rather simplistic and excludes combined defects. This classification is mainly based on elongated spermatid counts in the testicular tubules. When a testicular biopsy is not available for diagnosis, azoospermia is classified using medical history, physical examination and biochemical markers in serum (such as FSH, inhibin B) and semen (such as fructose, a-glucosidase, etc). These parameters have a weak predictive value, being insufficient for an accurate diagnosis. For example: how to classify a post-vasectomy man with a FSH of 21 IU/l? The diagnosis is further complicated by the variability within known OA ethiologies (for example: a congenital bilateral absence of vas deferens (CBAVD) man with a Johnsen score of 7.5) and expected NOA's, for instance, an azoospermic man presenting sperm at a PESA procedure after chemotherapy treatment, thus classifying as an OA.

From these examples, a couple of questions arises: i) Does sperm present similar phenotypes and genotypes in all cases of obstructive azoospermia? ii) Can an obstruction impair spermatogenesis or can an azoospermia with mild impaired spermatogenesis be classified as obstructive?

Classifying the type of azoospermia is not an easy task and must be carefully carried out. Not only in cases of NOA but particularly for OA males, as differences in the clinical presentation were observed per aetiology of obstruction (CBAVD, post-vasectomy/failed vaso-vasostomy, infection, unknown, etc) (Ramos et al., 2004c). For men with the clinical symptoms of obstruction, the presence of epididymal sperm (completed spermatogenesis) does not exclude a diminished sperm production (with lower sperm counts per testicular tubule including empty tubules). Patients with a clinical diagnosis of OA, such as post-vasectomy or CBAVD, may present some degree of diminished spermatogenesis, with sufficient motile epididymal sperm to use for ICSI.

Notwithstanding the biological importance of the source of sperm (from testis or epididymis) in case of obstructive azoospermia, there is no consensus on the preferable source of sperm to be used in ICSI. Some pro- and contras for the use of the one or the other will be addressed and discussed in the next session, especially in the context of OA.

2. Obstructive azoospermia: testicular vs. epididymal sperm

Results from the use of testicular and epididymal sperm in cases of OA did not lead to a general consensus on a preference for the one or the other. No differences have been found in the implantation, pregnancy and miscarriage rates between epididymal and testicular sperm in cases of OA (Hovatta et al., 1995; Silber et al., 1995; Rosenlund et al., 1997; Pryor et al., 1997; Palermo et al., 1999; Wennerholm et al., 2000; Tarlatzis et al., 2000; Friedler et al., 2002; Vernaeve et al., 2003; Schwarzer et al., 2003b; Nicopoullos et al., 2004a).

Testicular sperm has been widely used in all cases of azoospermia, as (aged) epididymal sperm may contain higher rates of DNA-breaks (fragmentation) in both nuclear and mitochondrial DNA compared with testicular samples (Steele et al., 1999; O'Connell et al., 2002b). The evaluation of the total sperm population in epididymal samples may lead to misinterpretation of the quality of those single sperm with good morphology and motility that could be used for ICSI, as these are expected to be generally "younger and healthier". In the era of ICSI, sperm quality studies should be restricted to the sperm fraction that satisfies the selection criteria for injection (Ramos et al., 2001; Ramos et al., 2002). Incomplete sperm chromatin condensation (as demonstrated by incomplete protamine thiol crosslinking), as supposed to be found in epididymal (caput) and testicular sperm, does not seem to influence the fertilization and pregnancy rates obtained after ICSI using fresh samples (Silber et al., 1995; Friedler et al., 2002; Nicopoullos et al., 2004b). Likewise, cryopreserved sperm from epididymal samples (but not from testicular samples) achieves pregnancy rates comparable to fresh samples, avoiding unnecessary surgical interventions in OA men (Friedler et al., 1998; Bachtell et al., 1999; Wood et al., 2003; Schwarzer et al., 2003a; Nicopoullos et al., 2004b).

Even if the source of sperm does not affect the ICSI outcome, increased aneuploidy rates in epididymal and testicular sperm have been found in OA (Sukcharoen et al., 2003; Rodrigo et al., 2004). These findings suggest a potentially increased genetic risk in the offspring of OA men.

3. Sperm quality in obstructive azoospermia

a. DNA damage

Differentiated spermatozoa are incapable of repairing their own DNA breaks. The frequencies of DNA-damaged spermatozoa (assessed with the TdT-UTP nick-end labelling, TUNEL) and "abnormal chromatin condensed" (positive for CMA3) in epididymal OA sperm samples do not always show a positive correlation as has been described for ejaculated sperm (Ramos et al., 2002). High and variable percentages of sperm with DNA damage have been found in epididymal sperm (mean 43% and 26%, assessed with the TUNEL assay (Ramos et al., 2004a) or Comet assays (O'Connell et al., 2002b)).

The origin of these DNA breaks are probably several and combined factors may also be encountered: a) Incomplete chromatin condensation (incomplete nuclear thiol crosslinking) in the testis or at the level of caput epididymis, rendering an open DNA structure more susceptible to external damage. For example: the high percentage of DNA breaks in OA samples (Ramos et al., 2002), and the

effect of long incubations in vitro of testicular samples (Dalzell et al., 2004);

b) DNA damage in differentiated spermatozoa can be induced by an overload of reactive oxygen species (ROS) produced by: (i) external sources such as cell breakdown and aged spermatozoa in the obstructed epididymis alone or in combination with leucospermia; (ii) internal ROS production generated by metabolic processes in the cell. The most likely damage mechanisms in these cases are peroxidation of membrane lipids and mitochondria dysfunction before any nuclear damage can be measured;

c) The effect of the obstruction on the testis may result in an irreversible damage to spermatogenesis, affecting sperm production (causing hypospermatogenesis) as has been described in rats (Inaba et al., 1998). Another indication for the negative effect of the obstruction on spermatozoa is the large-scale mitochondrial deletions found in testicular sperm in OA (O'Connell et al., 2002a).

Although fertilization (PN formation) is not impaired by using DNA damaged sperm, embryo development is negatively affected (Twigg et al., 1998; Henkel et al., 2003).

b. Chromatin condensation

Sperm chromatin is highly condensed and presents a unique structure and protein content. Protamines (in human P1 and P2) and a variable amount of histones are present in the sperm nucleus. Protamination has been indirectly assessed by the accessibility of various chromophores (like CMA3 and aniline blue) to the condensed nucleus. In a study carried out on epididymal sperm from OA men, we found that the percentage of CMA3 positive sperm showed a large variation between the samples (mean and SD, 53±21%, range 7-97%). Lower chromatin condensation (highest percentage of CMA3 positivity) was especially found in primary obstructions such as in CBAVD and idiopathic samples (60±17% and 65±18% respectively) compared with the post-vasectomy samples (37±17%) or ejaculated sperm from normospermic males (29±12%). Despite the generally normal testicular histology in OA men, chromatin condensation differed between OA patients. The physiological heterogeneity found in chromatin condensation between patient subgroups is probably influenced by the aetiology of the obstruction.

Because only a small fraction from the sperm sample is selected for ICSI, the question to be asked by reproductive biologists is whether the selection procedure applied at the time of injection is adequate for identifying the most “healthy” or “DNA-intact” sperm. Using the two most common selection criteria (motility and morphology at 400x magnification), we found that normally shaped and motile (epididymal) sperm contain a lower level of DNA-fragmentation (TUNEL) compared with the total sample (Ramos et al., 2002).

The different approaches for the study of sperm integrity (defined by the absence of DNA-breaks) show the high heterogeneity and complexity of non-ejaculated spermatozoa. Both from the literature and from our own observations, sperm from the obstructed caput epididymis show a strongly increased heterogeneity. Even in the motile fraction with “normal” morphology, there will be variance in the level of maturation. With our present insight of zygote biology, it cannot be said if these differences in nuclear maturation could affect the processing of the male gamete by the zygote cytoplasm (formation of PN), or the genetic integrity of the resulting embryo.

c. Morphometry

Next to the evaluation of sperm with CMA3, a DNA-specific stain (Feulgen) in combination with computerized karyometric image analysis (CKIA) system was used to evaluate sperm normality (Ramos et al., 2004b). This technique offers an integral evaluation of morphological and chromatin texture parameters for each spermatozoon. Ejaculated and epididymal sperm show a large variation in almost all parameters measured, which was more accentuated in non-ejaculated sperm. After sperm selection (for motility and morphology at 400x magnification), morphometric parameters showed that the microscopic selection resulted into sperm comparable to normal. However, for those parameters related to the DNA-stainability and chromatin texture (nuclear condensation), significant differences between ejaculated and epididymal sperm were found. This result suggests that the size and form of the sperm do not necessarily predict the internal chromatin structure. Thus, the frequency of "normal shaped" sperm significantly increased after ICSI-selection, but the improvement did not extend to a higher level of chromatin normality. The heterogeneity in the stainability (chromatin condensation) of epididymal samples from OA patients indicates that some of the selected spermatozoa have hypo- or hyper-condensed chromatin (high or low integral optical density respectively), indicating that the present routine ICSI-selection criteria are not sufficient for selecting normally condensed nuclei.

Final remarks

The diagnosis of OA is not only indicative for a higher take-home-baby rate with ICSI compared to NOA (Friedler et al., 2002; Vernaeve et al., 2003; Schwarzer et al., 2003b), but also for counselling about potential genetic risks. The high rates of DNA damage present in epididymal spermatozoa in OA may be a risk factor for the induction of chromosome anomalies (Sun et al., 1997; Lopes et al., 1998; Morris et al., 2002; Ramos et al., 2004a).

Awareness of the use of non-ejaculated sperm, even from men presenting a normal testis histology, has to be considered. Understanding the mechanisms of maturation, damage and repair in the gametes and zygotes will help to predict genetic risks in the ICSI offspring.

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CF and CBAVD

W. Lissens
Center for Medical Genetics
University Hospital of the Free University Brussels
Vrije Universiteit Brussel
Laarbeeklaan 101
1090 Brussels
Belgium
E-mail: willy.lissens@az.vub.ac.be

Cystic fibrosis (CF)

Cystic fibrosis is one of the most common autosomal recessive diseases among Caucasians. It affects about one in 2500 children and about one in 25 individuals is a healthy carrier. CF is mainly characterized by progressive lung disease, exocrine pancreatic insufficiency in about 85% of patients and elevated electrolyte levels in sweat, which serve as a diagnostic parameter for the disease. CF remains a life-threatening disease although symptomatic therapy has considerably increased the survival rate: 50% survival to around one year in the 1950's to around 30 years now.

CF is caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene, which codes for a 1480 amino acid transmembrane protein that functions as a chloride channel in the plasma membrane of epithelial cells. One mutation, a deletion of 3 bp from exon 10 of the gene that results in the absence of the amino acid phenylalanine at position 508 of the CFTR protein (F508del), is common in CF patients from European descent. Nevertheless, more than 1300 other mutations have been identified. These mutations can be classified into several classes according to different molecular mechanisms that lead to defects in the CFTR protein. In general, mutations that result in the complete functional loss of the CFTR protein are termed 'severe' mutations while 'mild' mutations retain some residual CFTR function. The majority of CF patients have 2 severe mutations (around 90%), while the remaining have one severe and one mild mutation. The latter are mostly patients with a milder phenotype (patients with pancreatic sufficiency)

Cystic fibrosis and congenital bilateral absence of the vas deferens

The majority (97-99%) of CF males are also infertile because of congenital bilateral absence of the vas deferens (CBAVD). Obstructive azoospermia due to CBAVD in otherwise healthy males accounts for up to 2% of male infertility and for up to 10% of obstructive azoospermias. Because of the similarity of the fertility problem between these healthy males and males with CF, it was already suggested more than 30 years ago that isolated CBAVD might be a mild form of CF. In that case, mutations in the same gene should be at the basis of both conditions. However, this hypothesis could not be tested until the isolation of the CFTR gene.

Congenital bilateral absence of the vas deferens

Mutation analysis of the CFTR gene in Caucasian males with isolated CBAVD indeed indicated increased numbers of mutations. However, there was a clear difference in the type of mutations found in CBAVD patients and in CF patients. In contrast to patients with CF, none of the CBAVD

patients carried two severe mutations. Rather, they had either a severe and a mild mutation (almost 90% of patients) or two mild mutations. In addition, most of the mild mutations were not found in CF patients but are typical for isolated CBAVD. In particular, a variant called 5T (5T allele) was extremely frequent in these males: the combination of a 5T allele in combination with a CF mutation in the other copy was the most common cause of isolated CBAVD. Even in Asian and Oriental populations in which CF is presumed to be rare, the frequency of the 5T allele was extremely high in CBAVD patients, with a considerable number of patients being homozygous for this allele. This indicates that the 5T allele is a common cause of CBAVD, even in populations where CF is rare. Because of the presence of CF mutations in these patients just like in CF patients, this form of CBAVD is considered as a genital form of CF.

CFTR mutations are not found in all patients with CBAVD. Two different groups of CBAVD patients without CFTR mutations can be recognized. In a first group representing 10 to 20% of patients, CBAVD is associated with urinary tract malformations and renal anomalies such as unilateral aplasia, ectopy or horseshoe kidney. In general, no mutations are found in these patients. However, a few patients have been found to be carriers of a CF mutation but this is probably purely coincidental because of the high frequency of CF mutations in the populations studied. This form of CBAVD is not considered to be a genital form of CF. The etiology of these two forms of CBAVD is probably different. Because both the seminal and renal parts of the mesonephric ducts are affected in the group of patients with urinary tract malformations and renal anomalies, damage must occur before the separation of the 2 mesonephric duct derivatives at seven weeks of gestation. In contrast, the genetic defect in patients with CBAVD associated with CFTR mutations appears to affect the fetus after separation of the mesonephric ducts at seven weeks because only the seminal tract is involved. From studies in male CF patients it seems that CFTR mutations probably do not affect the embryological development of the Wolffian duct from which the ureter and ducts of the epididymis and vas deferens are derived. In childhood, atresia can then occur secondary to luminal obstruction from viscous secretions.

The second group of CBAVD patients without CFTR mutations, and without renal anomalies, also accounts for 10 to 20% of patients. It is not known yet whether or not these represent another sub-population of CBAVD patients with a different etiology. It cannot be excluded at the moment that at least part of these patients belongs to the other categories of patients because of unidentified and more complex CFTR mutations or more subtle renal anomalies.

CBAVD and the CFTR 5T variant

Soon after the CFTR gene was identified, studies at the mRNA level showed that in epithelial cells of normal individuals variable amounts of CFTR transcripts were present without exon 9 of the gene. The molecular basis of this exon 9 skipping was inversely correlated with the number of T bases present in exon 8 just in front of exon 9: 7Ts (25%) or 9Ts (10%) gave only a small proportion of exon 9 skipping, while 5Ts produced up to 90% of abnormal mRNA. The resulting CFTR protein lacks 60 amino acids and is no longer able to function as a chloride channel. In addition, there is variability in the exon 9 skipping between different individuals but also between different tissues of a same individual. Especially, genital tissues produce less functional CFTR mRNA than epithelial cells. This observation could explain, at least in part, why the genital tract is affected in isolated CBAVD, but not the respiratory tract. The 5T variant is a mild mutation since it leaves some normal CFTR mRNA. The presence of this variant together with a severe CF mutation on the other allele will not always result in CBAVD. This is called ‘incomplete penetrance’, and the degree of penetrance was calculated to be between 0.56 and 0.60 by comparing the frequency of the 5T variant in combination with a CF mutation in fathers and in mothers of CF children. In these

parents, the genotype will have no effect in females (mothers of CF children), but in males only these ones without CBAVD will have been able to father children.

The 'incomplete penetrance' of the 5T variant probably points to other factors that are involved in the determination of the phenotype. Indeed it was recently shown that the number of TG bases in front of the 5T variant also influences the proportion of full-length CFTR mRNA: the more TGs, the lower the proportion of full-length mRNA. In addition, other common polymorphisms might have functional consequences on CFTR mRNA and protein, for instance by lowering the amount of functional CFTR, as is the case for a valine at amino acid position 470 as opposed to a methionine at this polymorphic position. These observations make it difficult to estimate the genotypic effect of these variants on the phenotype.

CBAVD and spermatogenesis

Histological examination of testis biopsies in men with CBAVD reveals either normal spermatogenesis (70-90%) or hypospermatogenesis (10-30%). Sometimes CBAVD occurs in combination with other defects such as a Robertsonian translocation, a pericentric inversion or a microdeletion of the Y chromosome. In these cases the additional genetic defect might or might not influence spermatogenesis.

CF/CBAVD and fertility treatment

The development of assisted reproductive techniques (ART) has allowed fertility treatment in patients with obstructive azoospermia due to CBAVD. Microsurgical techniques are used to obtain sperm for intracytoplasmic sperm injection (ICSI). These techniques include percutaneous epididymal sperm aspiration (PESA), microsurgical epididymal sperm extraction (MESA), testicular sperm extraction (TESE) and epididymal or testicular fine needle aspiration (FNA).

A recently published meta-analysis comparing the outcome of ICSI as a function of the cause of obstruction (congenital or acquired) showed a significantly increased fertilization rate with acquired versus congenital (CBAVD) causes. However, no difference in live-birth rate was observed, but a significantly higher miscarriage rate in the congenital group existed. These results are not affected by the fact that the sperm is epididymal or testicular of origin and is fresh or frozen.

In couples with the male having CBAVD associated with CFTR mutations the risk of having children with CF or CBAVD (in male offspring) is increased. This risk is of course dependent on whether or not the female partner is a carrier. If she is not a carrier the risk is probably inexistent, while the risk is high when she is a carrier. Since the CF carrier frequency is high in the Caucasian population (around 1/25) it is highly recommended that genetic testing for CFTR mutations be offered to these couples prior to ICSI treatment. While prediction of the risk may sometimes be very obvious, e.g. both partners are carriers of a severe CF mutation that in combination will certainly lead to a CF child (1/4 risk for the couple), other situations might be much more complicated. Partial penetrance associated with some variants in the CFTR gene such as the 5T allele, or the identification of a rare (CBAVD-specific) mutation can make the outcome unpredictable. Patients should also know that technology does not allow the detection of all mutations and that a negative mutation screening will reduce the risk of being a carrier but will not completely exclude it. A genetic counselor should discuss all of these items in advance with the couple. The couple should also be informed about the option of prenatal diagnosis in case of a high-risk situation. However, preimplantation genetic diagnosis (PGD) might probably be more advantageous for these couples than prenatal diagnosis at 11 weeks, or later in pregnancy, because they already have to undergo ICSI treatment: embryos can

be analyzed for CFTR mutations and healthy embryos can be replaced in the uterus.

In couples in which CBAVD is not related to CF, the risk of having children with CF is probably the same as in the general population. However, it is not known whether or not there is a risk for infertility in their (male) offspring. Finally, in the group of patients without CFTR mutations and renal anomalies, the risk for their offspring of having CF or infertility problems is not known. As the other couples, these couples should also be informed about their situation.

Recommendations / conclusions

The condition of isolated CBAVD is most often associated with mutations in the CFTR gene. However, at least one of the mutations involved is specific for CBAVD and is not found in CF. The 5T variant is a typical example of such a CBAVD-specific mutation with an incomplete penetrance.

Since 10 to 20% of patients have a condition of CBAVD that is non-CF related but that is associated with urinary tract malformations and renal anomalies, the diagnosis of CBAVD should not only be based on scrotal palpation. Surgical exploration (when partial vasa are present), transrectal ultrasound (to visualize ejaculatory ducts, seminal vesicles and prostate) and renal ultrasound should also be performed. Other mild symptoms of CF can also be present in the patient, and these should also be looked for. The testicular volume is normal, and semen analysis shows reduced pH, volume and fructose; sperm is absent.

Most of the males with CBAVD will be able to have children using ICSI. However, if treatment by assisted reproductive techniques is considered, the integrity of spermatogenesis should be checked by testicular biopsy. This material can be used for both histology and for extraction and cryopreservation of sperm.

Mutation analysis of the CFTR gene is important for the CF-associated form of CBAVD, especially since these couples are at risk of having CF children or children with atypical forms of CF. All possibilities should be discussed with all couples, including those with non-CF related CBAVD.

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