Human Reproduction Update, Vol.9, No.5 pp. 405–428, 2003 DOI: 10.1093/humupd/dmg034

Sperm pathology: a step beyond descriptive morphology. Origin, characterization and fertility potential of abnormal sperm phenotypes in infertile men

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Sperm pathology is presented as the discipline of characterizing structural and functional deficiencies in abnormal spermatozoa. This concept complements that of sperm morphology mainly concerned with the appearance of spermatozoa. These two notions collaborate in providing correlations of prognostic value with sperm fertilizing capacity, explaining the mechanisms of sperm inefficiency, suggesting strategies to improve fertilization and opening a door to molecular genetic studies. Phenotypes of genetic origin involving sperm heads, flagella and the neck region are presented describing their clinical manifestations, sperm structure, cytochemistry and genetic background. When available, animal models are used to highlight possible genetic mechanisms. Sperm pathologies secondary to andrological conditions or environmental factors are described, stressing the non-specific nature of the sperm response to noxious agents. The available literature on the prognostic value of sperm pathologies in ICSI is also reviewed. Flagellar anomalies bear a good prognosis, but those affecting the acrosome, sperm chromatin and the neck region entail an increasing chance of failure, which highlights the differential roles played by specific sperm components in fertilization, implantation and early embryonic development. A final discussion is devoted to genetic counselling and the risks involved in using immotile or abnormal spermatozoa in assisted reproduction.

Key words: fertility prognosis/genetic infertility/ICSI/sperm morphology/sperm pathology

Introduction

Knowledge on the structure of spermatozoa can be traced back to the seventeenth century when Anton van Leeuwenhoek communicated for the first time the existence of numerous *animacula* in the seminal fluid of animals and men. He reported his findings in a letter submitted to the Royal Society of London in November 1677 (Figure 1). In his morphological rendering of spermatozoa he reproduced with precision the main sperm components and documented a striking heterogeneity, which, beyond the accuracy of his observations, is the first account of teratozoospermia. Intensive research during the eighteenth and nineteenth centuries established the testicular origin and fundamental role of spermatozoa in fertilization. The introduction of modern morphological, biochemical and molecular techniques together with advancements in reproductive medicine during the twentieth century resulted in the characterization of various distinct sperm abnormalities of infertile males. It was soon realized that there was a limited amount of abnormal, immotile and dead spermatozoa in the ejaculates of fertile individuals and that these percentages were pathologically increased in numerous cases of male infertility. From these observations evolved the concepts of teratozoospermia, asthenozoospermia and necrozoospermia, all conditions negatively influencing fertility prognosis in spontaneous conditions or with the use of various assisted reproductive techniques including IVF. In all these circumstances, the quality of the single fertilizing spermatozoon could not be established with certainty. The introduction of ICSI allowed the examination of motility and morphology of the very spermatozoon to be microinjected. It then became clear that abnormal and immotile spermatozoa could successfully fertilize oocytes, and the question was raised about the convenience of using them in assisted reproduction technology procedures. Some andrologists stressed the importance of different tools to characterize sperm pathologies and establish a diagnosis, still others were more inclined to use them for assisted reproduction without much attention paid to diagnosis. Recent evidence has indicated that in many of these patients a genetic component is present and that depending on the nature of sperm pathologies, the outcome of IVF–ICSI changes considerably.

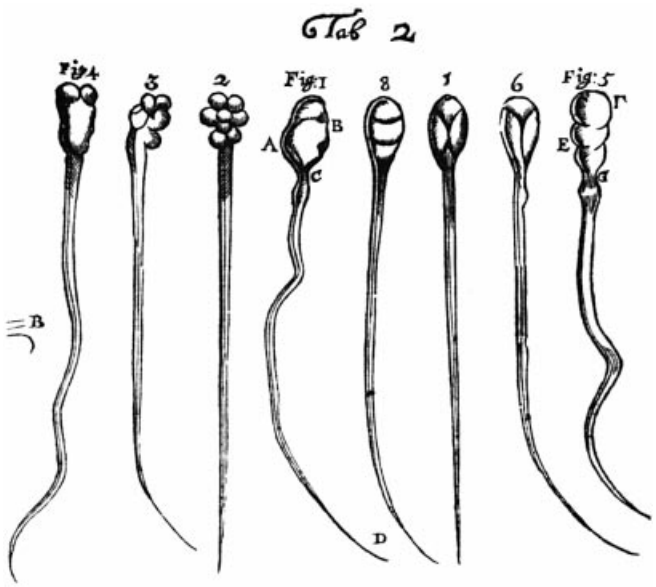


Figure 1. First drawing of spermatozoa made by Anton van Leeuwenhoek after his observations with a primitive light microscope. Note the remarkable heterogeneity of head shapes.

In the this article we will develop the concept of sperm pathology and its association with sperm morphology, review the various genetic and acquired sperm phenotypes, explore their meaning in the study of infertile men and examine available literature on their prognostic value in assisted reproduction.

Sperm pathology: a step beyond descriptive morphology

Sperm morphology is currently examined in semen smears with the main criteria for normalcy relying on morphometric parameters of the sperm head, mid-piece and flagellum. The main alterations, subjectively assessed, have previously been summarized (MacLeod, 1970; World Health Organization, 1992). More recently, manual and computer-assisted objective methods have been proposed that allow a reproducible evaluation of sperm parameters (Calamera *et al*., 1994; Kruger *et al*., 1995; Hofman *et al*., 1996). Correlations of sperm morphology with various biological tests or results of IVF have precisely identified the characteristics of normal spermatozoa (Kruger *et al*., 1986, 1988; Mortimer *et al*., 1986; Jouannet *et al*., 1988; Liu and Baker 1992; Grow *et al*., 1994; Toner *et al*., 1995; Garret *et al*., 1997).

The introduction of strict morphological criteria (Kruger *et al*., 1986; 1988) has proven particularly useful in predicting the fertilizing competence of spermatozoa in assisted reproduction. Abnormal forms are solely defined on the basis of atypical sperm shapes, which, with the exception of acrosome anomalies, do not identify the cellular basis of their functional incompetence because of technical limitations of light microscopy. Ultrastructural evaluation of teratozoospermia coupled with immunocytochemistry and molecular techniques allow a precise characterization of sperm abnormalities including their structural, molecular and functional aspects. This approach goes beyond descriptive morphology of the appearance of spermatozoa. Sperm pathology is therefore a special example of the general concept of cell pathology coined by German pathologist Rudolph Virchow who introduced the idea that the basis of all disease originated with injury to the cell and in particular to the structure and function of cell organelles (Virchow, 1860). It may seem outdated to claim the application of a nineteenth century concept to current reproductive pathology, but the fact is that normal spermatozoa have been characterized recently, and their pathological alterations can only now be understood in their physiopathological complexity.

It is clear that strict morphology correlates with sperm fertilizing capacity and has prognostic value in assisted reproduction. But, what is wrong with wrong sperm shape? What hides behind a head-shape change in amorphous or tapering spermatozoa? In other words, what is it that impairs sperm function in morphologically abnormal sperm? Is it just abnormal shape or is there something wrong with specific sperm components? Sperm pathology is the discipline of characterizing structural and functional deficiencies in abnormal spermatozoa. This is significant because it helps to explain the mechanisms of sperm inefficiency, identifies genetic phenotypes, suggests strategies to improve fertilization and opens a door to molecular genetic studies that will probably lead to the design of the therapeutic tools of the future.

Following the concept of sperm pathology, two main forms of abnormal spermatozoa can be distinguished. In the first and more frequent variety, a heterogeneous combination of different alterations is found randomly distributed in each individual and among different patients. These alterations can be referred to as non-specific or non-systematic sperm defects. The second variety presents with a characteristic anomaly that involves the vast majority of spermatozoa in a semen sample. These alterations may be called systematic in the sense that there is a common sperm phenotype that predominates in a given patient and resembles similar defects in other individuals suffering from the same condition. The first variety is usually secondary to various pathologies that affect the normal function of the testis or the seminal pathway. Systematic alterations tend to show family clustering and have proven or suspected genetic origin.

Pathological sperm phenotypes of genetic origin

Flagellar abnormalities in motility disorders

With the possible exception of the early works by Williams (1950) and Kagan (1963) who reported on specific defects in human spermatozoa, systematic investigations in this area started in the 1970s (Pedersen *et al*., 1971; Ross *et al*., 1971, 1973; Holstein *et al*., 1973; Pedersen and Rebbe, 1974, 1975; Afzelius *et al*., 1975; Bisson *et al*., 1975; Kullander and Rousing, 1975; Afzelius, 1976; Anton Lamprecht *et al*., 1976; Nistal *et al*., 1978; Holstein and Schirren, 1979; LeLannou, 1979). In particular, the classical studies of the Scandinavian school demonstrated that male infertility associated with chronic respiratory disease was caused by genetic-related dynein deficiency in the axonemes of immotile spermatozoa and respiratory cilia (Afzelius *et al*., 1975; Pedersen and Rebbe, 1975; Afzelius, 1976). These patients are infertile due to sperm immotility, suffer frequent episodes of sinusitis and respiratory infections because of impaired mucociliary clearance, eventually leading to bronchiectasia, and have alterations

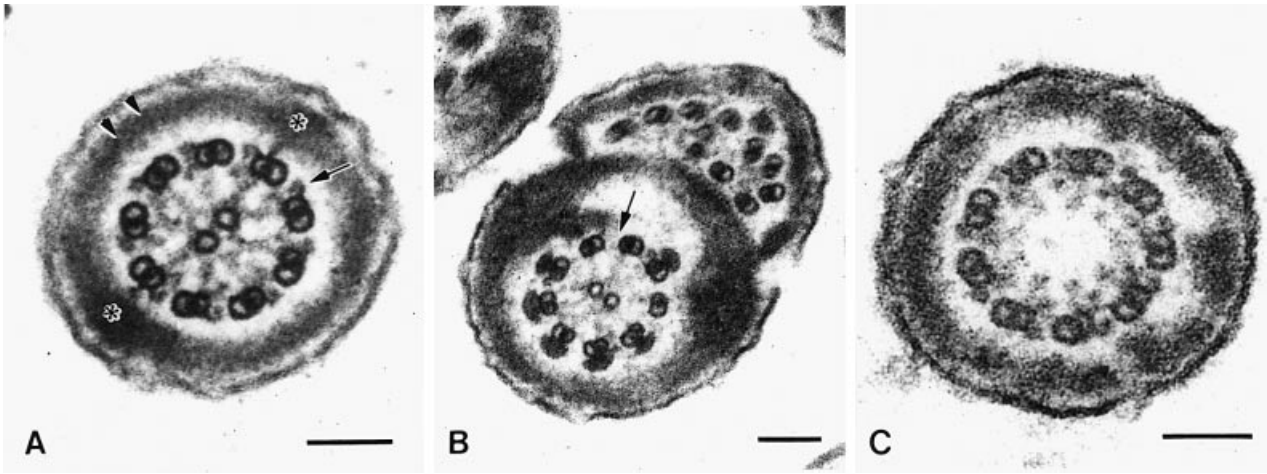


Figure 2. (**A**) Cross-section of a human sperm flagellum at the principal piece. The nine peripheral doublets of the axoneme, central pair, dynein arms (arrow) and radial spokes are clearly seen. The fibrous sheath is composed of two lateral columns inserted in doublets 3 and 8 (asterisks) and semi-circumferential ribs (arrowheads). (**B** and **C**) Spermatozoa from two patients with primary ciliary dyskinesia. There is lack of dynein arms (arrow, **B**) or absence of the central pair (**C**). Bars = 0.1 μm.

in the visceral rotation (situs inversus) with dextrocardia in 50% of the subjects, the so-called Kartagener syndrome (Siewert, 1904; Kartagener, 1935). Alterations in the visceral position are probably caused by immotile cilia in the embryo that would impair normal organ rotation, with chance alone determining whether they will take up the normal or the reversed position (Afzelius, 1976). Men suffering from this association were originally referred to as immotile cilia syndrome (ICS), and more recently renamed as primary ciliary dyskinesia (PCD, Rossman *et al*., 1981) since partial or residual motility are occasionally present in some of these patients (Afzelius and Eliasson, 1979; Camner *et al*., 1979; Jouannet *et al*., 1983; Moryan *et al*., 1986).

Before reviewing the different pathological phenotypes that have been described in PCD/ICS, the normal features of the tail will be summarized. The human sperm flagellum is a long structure, ~50 μm in length and 0.4–0.5 μm in diameter. It is composed of a central element, the axoneme, which is a cylinder composed by a circumferential array of nine peripheral microtubular doublets surrounding a central pair of microtubules, the so-called 9 + 2 configuration (Figure 2). Each peripheral doublet is composed of two apposed subunits, microtubules A and B, which share part of their wall and are composed by protofilaments of tubulin heterodimers. Extending from subunit A, two arms project toward the B subunit of the next doublet. These arms are composed of dynein, a structural protein with ATPase activity that utilizes ATP as an energy source to generate axonemal movement (Gibbons, 1965, 1977; Baccetti *et al*., 1981). Each peripheral pair is connected to the next one by nexin links and to the central pair by nine radial spokes. Tetkins, a group of proteins related to intermediate filaments, are associated with the tubulin protofilaments in the axoneme (Norrander *et al*., 1996). The axoneme is surrounded by the outer dense fibres (ODF) and the fibrous sheath (FS). The ODF are nine slender cylindrical structures of different lengths associated with the corresponding peripheral doublet. All of them are present at the mid-piece, but fibres 3 and 8 end at the beginning of the main piece where they are continued by the lateral columns of the fibrous sheath (see below). The FS is a sort of flagellar exoskeleton present only at the main piece and organized into two longitudinal columns that run along the length of the principal piece and insert into microtubular pairs 3 and 8. These columns are regularly joined by transverse semicircular ribs.

Immotile spermatozoa in PCD/ICS have morphologically normal but stiff flagella on light microscopy. The discrepancy between normal tail morphology and sperm immotility prompted the interest in finding what was wrong with the tails of these immotile spermatozoa despite their apparently 'normal' morphology. Ultrastructural investigations solved the riddle by disclosing that most anomalies responsible for PCD/ICS were beyond the resolving power of light microscopes. A wide spectrum of axonemal defects has been reported. In the original descriptions, lack of both dynein arms was noted in peripheral doublets (Afzelius *et al*., 1975; Afzelius, 1976; Pedersen and Rebbe, 1975). Numerous other defects were reported thereafter, such as missing outer or inner dynein arms, absence of one or two central microtubules or radial spokes, transposed microtubules, lack of the axoneme, and association of dynein deficiency in cilia with sperm fibrous sheath aberrations (Figure 2) (Afzelius *et al*., 1976; Eliasson *et al*., 1977; Afzelius and Eliasson, 1979; Baccetti *et al*., 1979, 1980; Nistal *et al*., 1979; Sturges *et al*., 1979, 1980; Schneeberger *et al*., 1980; Walt *et al*., 1983; Escalier and David, 1984, Chemes *et al*., 1990; Neugebauer *et al*., 1990). Wilton *et al*. (1985) quantified different axonemal components in cilia and flagella of 10 non-smoker fertile individuals and found that the observed number of dynein arms was lower than the theoretical number of nine. These findings challenge the concept of

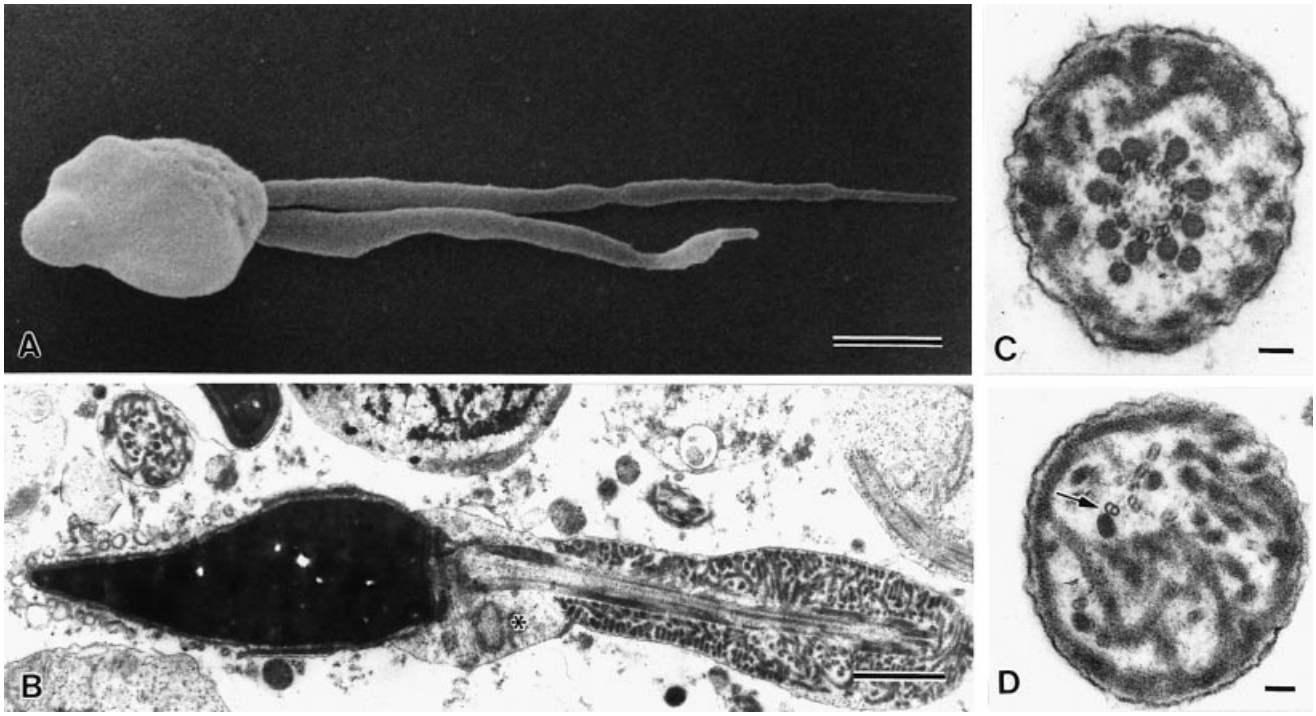


Figure 3. Dysplasia of the fibrous sheath. (**A** and **B**) Short, thick and irregular tails in longitudinal views. In **A** the tail is duplicated. In **B**, note the absence of a mitochondrial sheath (asterisk) and redundant elements of the fibrous sheath. (**C** and **D**) Two cross-sections of pathological flagella with disorganized and hyperplastic fibrous sheaths. In **C** the axoneme is partially preserved but lacks a central pair of microtubules and has abnormal extension and duplication of the outer dense fibres. In **D** the axoneme is almost completely obliterated with few remaining microtubular doublets with missing dynein arms (arrow). Bars = 1 μm (**A**, **B**), 0.1 μm (**C**, **D**). Panels **A**–**D** were originally published in Chemes *et al*. (1998), © European Society of Human Reproduction and Embryology. Reproduced by permission of Oxford University Press/Human Reproduction.

'partial dynein deficiency' and indicate that the diagnosis of PCD/ICS should always be based on actual quantifications and comparisons with the published normal values.

Familial incidence of PCD/ICS, most perhaps due to an autosomal recessive mutation(s), and a high incidence among Maoris and Samoan islanders of New Zealand have been noted (Holmes *et al*., 1968; Guggenheim, 1971; Waite *et al*., 1978, 1981; Wakefield and Waite, 1980). It is now accepted that there is extensive locus heterogeneity, with a number of (related) gene mutations possibly involved in different patients (Schneeberger *et al*., 1980; Afzelius, 1981a; Chao *et al*., 1982; Pennarun *et al*., 1999; Blouin *et al*., 2000; Bartoloni *et al*., 2002). It was suggested that specific genic anomalies may cause lack of synthesis of dynein(s) or of a protein that binds dynein to the microtubules (Afzelius and Eliasson, 1979). More recently, as many as 12 different chromosome loci have been singled out as the genetic basis for PCD (Blouin *et al*., 2000). Spontaneous mutations in genes encoding for heavy dynein chain types 5, 11 (DNAH5 and DNAH11) and intermediate type 1 (DNAI1) have been found in human families with the PCD phenotype (Pennarun *et al*., 1999; Guichard *et al*., 2001; Bartoloni *et al*., 2002; Noone *et al*., 2002; Olbrich *et al*., 2002). Mice models lacking the isoforms for two heavy chain dyneins (MDHC7 and MDNAH5) express respiratory alterations and ultrastructural abnormalities almost identical to the human disease (Neesen *et al*., 2001; Ibañez Tallon *et al*., 2002). Pf20 and Spag6 are two protein components of the axonemal central apparatus that co-localize in polymerized microtubules. Mice lacking Spag6 are infertile because of low sperm motility due to axonemal alterations including lack of the central pair (Sapiro *et al*., 2000, 2002; Zhang *et al*., 2002). This phenotype closely resembles findings in humans with PCD/ICS lacking the central microtubular pair.

Severe asthenozoospermia or total immotility have also been reported in men with dysplasia of the fibrous sheath (DFS; Chemes *et al*., 1987a, 1998; Chemes, 2000; Rawe *et al*., 2001, 2002a). Patients suffering from DFS are young males with serious motility disorders and primary sterility. Spermatozoa display characteristic short, thick and irregular flagella. This particular appearance originated the denomination of 'stump tails' or 'short tails' to refer to this pathology. These terms are misnomers that fail to provide an insight into the underlying nature of these abnormalities and encompass a heterogeneous array of defects having a short and thick tail as the common feature. DFS sperm should not be confused with other alterations secondary to necrozoospermia or sperm aging in men with partial obstruction of the seminal pathway that lead to flagellar disintegration and thickening. The denomination 'dysplasia of the fibrous sheath', introduced by Chemes *et al*. (1987a, 1998), identifies the main alterations in the fibrous sheath and points to a dysplastic development of the tail during spermiogenesis. Individual examples of this pathology, or

morphological descriptions without clinical data, had been reported by Ross *et al*. (1973), Holstein and Schirren (1979), McClure *et al*. (1983) and Williamson *et al*. (1984). Bisson and David (1975) and Escalier and David (1984) have published extensive series with familial incidence and were the first to indicate that the cytoskeleton of the tail is the main component involved. Familial incidence is present in ⩾20% of DFS patients, and geographical clustering has been reported in Northern Africa and South America (Bisson and David, 1975; Bisson *et al*., 1979; Escalier and David, 1984; Chemes *et al*., 1987a, 1998). In this respect, a striking contrast between the high incidence of DFS and low incidence of PCD/ICS has been noted in a population of multi-ethnic origin (Chemes, 2000), which may indicate the interaction between genetic and environmental influences in the generation of this phenotype.

Testicular origin of DFS sperm is ascertained by the presence of similar alterations in immature spermatids found in semen and by the various biopsy studies reported in DFS patients (Ross *et al*., 1973; Barthelemy *et al*., 1990; Rawe *et al*., 2001). The key component of the DFS phenotype is a redundant and haphazardly arranged fibrous sheath that forms thick rings or broad meshes without the orderly disposition in longitudinal columns and transversal ribs. The axoneme, embedded in these hyperplastic fibres shows variable distortion ranging from well-formed axonemes to almost complete obliteration (Figure 3). Microtubular doublets may display partial or total lack of inner/outer dynein arms, and the central pair is absent in about half of the cases. Outer dense fibres 3 and 8, normally restricted to the mid-piece, may extend to the principal piece. The annulus fails to migrate caudally remaining just beneath the connecting piece and mitochondria do not assemble in a normal mid-piece. Rawe *et al*. (2001) have characterized in detail the incidence of different distortions in the fibrous sheath, microtubular doublets and mitochondrial sheath in DFS spermatozoa that also show increased mitochondrial and surface ubiquitination (Figure 4) (Sutovsky *et al*., 2001; Rawe *et al*., 2002a). The ubiquitin tag may indicate the existence of a quality control mechanism for the elimination of defective spermatozoa.

Sperm alterations remain stable during clinical evolution and are not modified by any therapeutic measures. This, together with the familial incidence and association with dynein deficiency strongly suggests a genetic component in the DFS phenotype (Baccetti *et al*., 1975, 1993, 2001; Alexandre *et al*., 1978; Bisson *et al*., 1979; Chemes *et al*., 1998). Analysis of the family trees seems to indicate autosomic recessive inheritance.

About 20% of DFS patients have recurrent sino-bronchial infections, eventually leading to bronchiectasia. This association is clinically identical to that seen in PCD/ICS, the distinguishing features being the presence of sperm fibrous sheath distortions in addition to lack of dynein in sperm and ciliary axonemes. This combination represents a different variant of the classical forms of PCD/ICS (Chemes *et al*., 1987a, 1990). Previously published cases by Camner *et al*. (1979), Williamson *et al*. (1984) and Escalier and David (1984) probably belong to this category. Absence of the central pair of axonemal microtubules has been reported as an isolated cause of PCD/ICS. However, critical reading of the literature shows that in most cases the 9 + 0 configuration is associated with DFS-like anomalies (Eliasson *et al*., 1977; Afzelius and Eliasson 1979; Baccetti *et al*., 1979; Nistal *et al*., 1979; Escalier and David, 1984; Neugebauer *et al*., 1990; Zamboni, 1992; Chemes *et al*., 1998).

In recent years, extensive work has been carried out on the protein composition of the fibrous sheath. A number of proteins have been isolated and characterized that predict a role for this structure beyond that of a mechanical framework of the flagellum, as had been originally hypothesized (reviewed by Eddy *et al*., 2003). Among these proteins, three members of the AKAP family (A-kinase anchor proteins) have been characterized in spermatozoa: AKAP4, AKAP3 and TAKAP-80 (Carrera *et al*., 1994; Fulcher *et al*., 1995; Turner *et al*., 1998; Mandal *et al*., 1999; Vijayaraghavan *et al*., 1999). AKAP3 and -4 are the most abundant structural proteins of the FS and bind to one another. They function to anchor cAMP-dependent protein kinase A (PKA) to this structure via the regulatory subunit of the kinase. The genes that code for both AKAP have been sequenced and the regions of the respective binding sites between both AKAP as well as that for PKA have been identified (Turner *et al*., 1998; Mandal *et al*., 1999). Immunohistochemical localization of AKAP3 and -4 at the light and ultrastructural levels in various DFS patients indicates their abundance in sperm tails where they localize to the amorphous fibrous sheaths. One and two-dimensional gel electrophoresis, immunoblotting and binding of the regulatory subunit of PKA do not show differences between normal controls and DFS patients. Sequence analysis of the AKAP3 and AKAP4 binding sites did not reveal mutations (Turner *et al*., 2001), but targeted disruption of the AKAP4 gene in mice results in sperm immotility and abnormally short flagella (Miki *et al*., 2002), with localized aggregations of FS material somewhat reminiscent of the DFS phenotype (E.M.Eddy, personal communication). Sperm-specific thioredoxins concerned with disulphide bond reduction are present in the lateral columns of the fibrous sheath and in pathological flagella of DFS patients (Miranda-Vizuete *et al*., 2001; Yu *et al*., 2002; H.E.Chemes, personal unpublished observations). Phenotypes similar to DFS have been described in mice with defects in hybrid sterility loci 6 and 7 (Pilder *et al*., 1993, 1997). It is possible that DFS is a multigenic disease caused by alterations in several different gene products.

There are other forms of axonemal pathologies of genetic origin. Increased abnormalities in respiratory cilia and sperm flagella have been found in patients with genetically determined retinitis pigmentosa (Hunter *et al*., 1988; Ohga *et al*., 1991; van Dorp *et al*., 1992; Bonneau *et al*., 1993). We have recently found dynein-deficient sperm axonemes in an asthenozoospermic patient with albinism (H.E.Chemes, personal unpublished observation).

Male infertility has been reported in a form of flagellar dyskinesia characterized by abnormal extension of outer dense

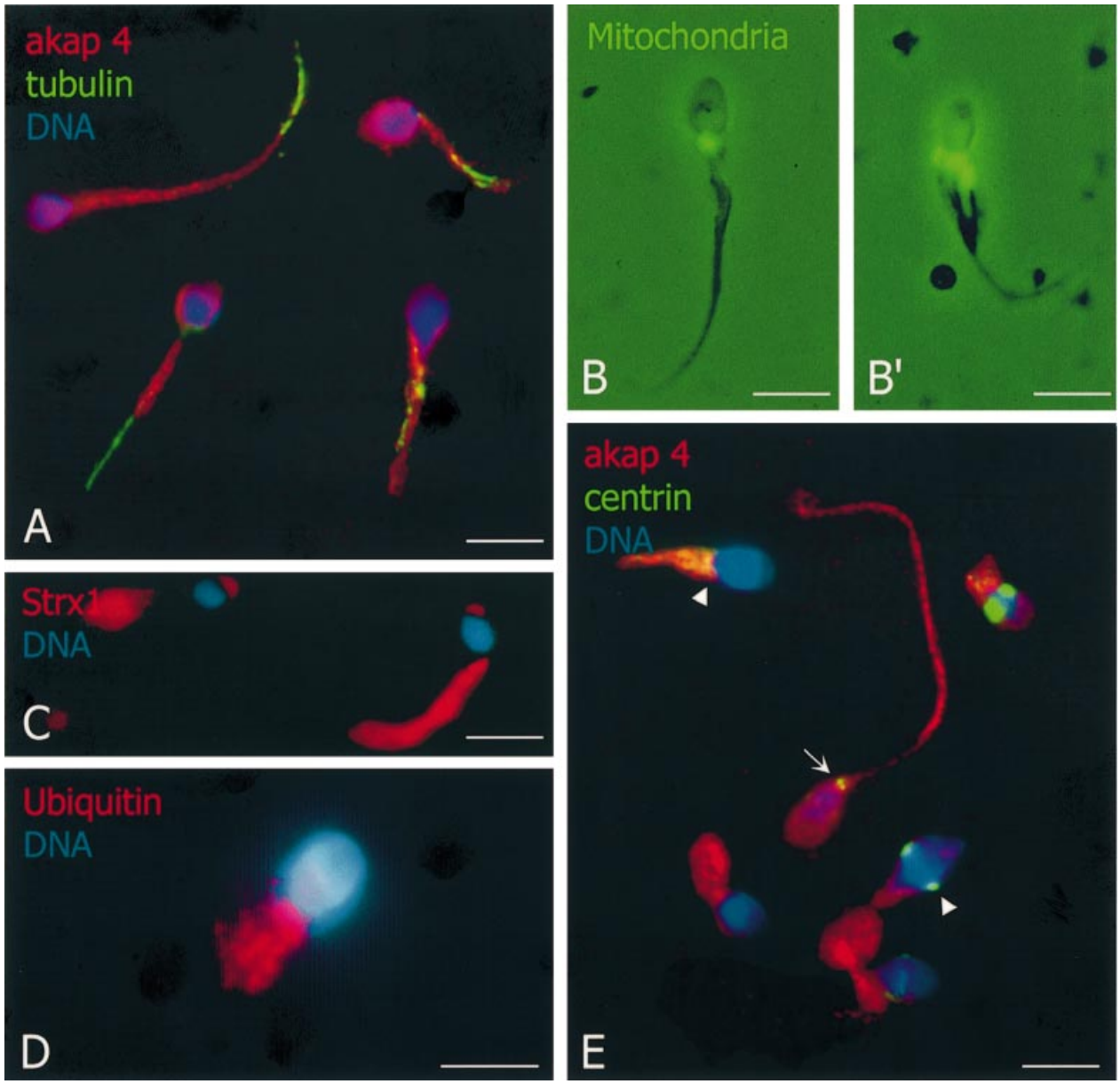


Figure 4. Different cellular markers in spermatozoa with dysplasia of the fibrous sheath (DFS). (**A**) Various DFS spermatozoa with short and irregular tails. Immunolabelling with anti-A-kinase anchor proteins (AKAP)4 (red) and anti-tubulin (green). Most of the dysplastic tails are labelled with AKAP4 antibody that reaches the sperm head, no mid-piece is discernible. There is a relatively weak and discontinuous green fluorescence (tubulin) over the principal and end pieces. (**B**) MitoTracker Green FM™ staining (green) shows a single mitochondrion (**B**) or a 'necklace' (**B'**) formed by few mitochondria surrounding the connecting piece. Phase contrast and fluorescence microscopy. (**C**) Sperm thyoredoxin (Strx) immunolocalization is shown in red in the dysplastic tails and apical region of the sperm head (acrosome). (**D**) Ubiquitin was immunodetected by an anti-ubiquitin monoclonal antibody, coupled with a secondary antibody labelled with a red fluorochrome. Mitochondria at the mid-piece show strong ubiquitination. (**E**) Lack or ectopic localization of centrin (arrow heads) in sperm with a severe DFS. When the FS hyperplasia is reduced, the centrin pattern appears as one or two dots in the pericentriolar area as expected for normal sperm (arrow). Sperm DNA was counterstained using Hoechst 33258 (blue). Bars = 5 μm. Panels B and B' reproduced from Rawe *et al*. (2001), © European Society of Human Reproduction and Embryology. Panel D reproduced from Rawe *et al*. (2002a), © European Society of Human Reproduction and Embryology. Reproduced by permission of Oxford University Press/Human Reproduction.

fibres and lack or abnormal spatial distribution of lateral columns of the fibrous sheath (Feneux *et al*., 1985; Serres *et al*., 1986; David *et al*., 1993). The presence of this defect in brothers has been incidentally mentioned (Escalier, 2003) and we have observed this condition in the brother of a patient with the classical DFS phenotype (H.E. Chemes, personal unpublished observations). Missing or poorly developed outer dense fibres have also been reported as the cause of sperm motility disorders but there are no clear indications to support a genetic versus an acquired origin (Haidl and Becker, 1991; Haidl *et al*., 1991).

The lack of mitochondria in the sperm mid-piece is another rare sperm pathology of possible genetic aetiology that includes two variants (reviewed by Zamboni, 1992). In the

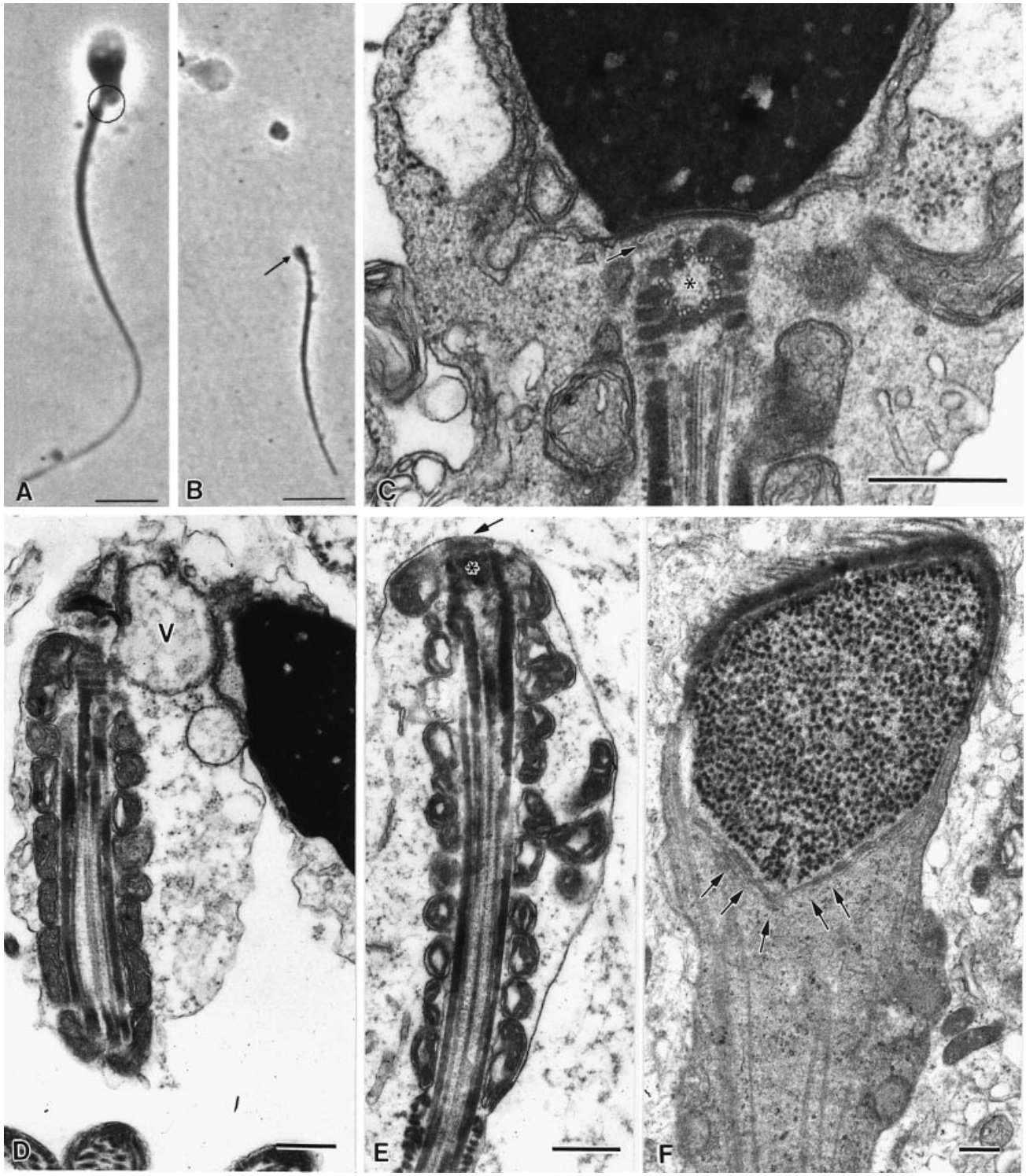


Figure 5. Abnormalities of the connecting piece (head–tail junction). In **A** the head and the tail are not aligned along the same axis (abaxial implantation of the tail). (**B**) Acephalic spermatozoon with minute thickening (arrow). (**C**) Normal configuration of the connecting piece. The tail is lodged in the concave implantation fossa (arrow). Note the triplets of the proximal centriole (asterisk) and the beginning of the axoneme. (**D**) The head and mid-piece are not properly attached and a vesicular structure (V) separates them. (**E**) Acephalic spermatozoon. The plasma membrane (arrow) covers the connecting piece (asterisk). The mid-piece is well formed. (**F**) Elongating spermatid in testicular biopsy. Note lack of attachment of the tail anlagen to the caudal pole of the nucleus (arrows). Bars = 5 µm (**A**, **B**), 0.5 µm (**C**–**F**). Panels **A** and **B** were originally published in Rawe *et al*. (2002) and panels **C**–**F** in Chemes *et al*. (1999), © European Society of Human Reproduction and Embryology. Reproduced by permission of Oxford University Press/Human Reproduction.

first, mitochondria are not present around the axoneme and the mid-piece appears very thin and frequently bent. Severe asthenozoospermia is the rule. The condition is exceedingly infrequent. The second variety of spermatozoa lacking mitochondria is part of the DFS phenotype previously described (see flagellar abnormalities). An abnormal fibrous sheath extends up to the neck region so that mitochondria cannot assemble around the axoneme in a normal mid-piece. Sperm immotility is also the rule because of the combined effect of anomalies in mitochondria and fibrous sheaths. Sperm mitochondrial DNA (mtDNA) encodes for various genes whose products are involved in oxidative phosphorylation and generation of ATP that is used as an energy source for sperm motility. Thangaraj et al. l2003) have communicated a two nucleotide deletion in the sperm mitochondrial COII gene (mitochondrial cytochrome oxidase II) introducing a stop codon and a truncated protein possibly responsible for abnormal motility in their patient. Other single nucleotide polymorphisms and mutations in mitochondrial genes have been found in men with poor semen parameters (Kao *et al*., 1998; Holyoake *et al*., 2001). No structural correlates of these anomalies have been described so far.

Abnormalities of the head–neck attachment and acephalic spermatozoa

The region of head–neck attachment or connecting piece derives from the interaction of the centrioles with the spermatid nucleus. Early in spermiogenesis the sperm flagellum grows from the centriolar complex that approaches the nucleus and attaches to its caudal pole ensuring a linear alignment of the tail with the longitudinal axis of the head.

Abnormalities of the head–neck attachment include varying degrees of alterations in the relationship between these two structures. LeLannou (1979), Perotti *et al*. (1981) and Baccetti *et al*. (1984) reported individual patients with headless flagella in semen and identified them as 'decapitated spermatozoa'. More recently, Baccetti *et al*. (1989a), Holstein *et al*. (1986), Chemes *et al*. (1987b, 1999) and Toyama *et al*. (2000), reported 15 more cases, including familial incidence, and introduced the name of 'acephalic spermatozoa'. The term 'pin heads' (Zaneveld, 1977) has been used in reference to this peculiar appearance, but this denomination adds confusion since there is no nuclear material in these minute globular 'heads'. These spermatozoa are present in very small numbers in seminal samples from fertile individuals and can increase up to 10–20% in subfertile men (Chemes *et al*., 1987b; Panidis *et al*., 2001). In some teratozoospermic patients, 90–100% of the sperm population is constituted by acephalic spermatozoa ending cranially in a normal middle piece, or in globular cytoplasmic droplets (1–5 μm in diameter) that may be confused with the sperm head. However, no traces of chromatin are found in any of these cephalic thickenings as ascertained by a negative Feulgen reaction (Chemes *et al*., 1987b). Sperm motility is variable and loose heads in semen range from abundant (Baccetti *et al*., 1984) to scarce (Perotti *et al*., 1981; Chemes, 1987b, 1999). A somewhat similar condition has also been described in bulls (Bloom and Birch Andersen, 1970).

Ultrastructural studies show a normal configuration of the tail with a well-structured proximal centriole and other elements of the connecting piece, surrounded by a cytoplasmic droplet of variable size. The cephalic end is directly covered by the plasma membrane (Figure 5). Acephalic spermatozoa are of testicular origin and develop from a failure of the centriole–tail anlagen to attach normally to the spermatid nucleus. As a consequence of this, heads and tails develop independently and separate at the moment of spermiation, with the heads being usually phagocytosed by Sertoli cells or along the epididymis (Le Lannou, 1979; Perotti and Gioria, 1981; Baccetti *et al*., 1984; Chemes *et al*., 1987b; Toyama *et al*., 2000). In some patients, acephalic spermatozoa mix with other forms that have heads abnormally implanted in the middle piece (Lüders, 1976; Chemes *et al*., 1999). These two variants express a different degree of abnormality of the head–neck junction with acephalic forms representing the most extreme situation, hence the more inclusive denomination of alterations of the head–neck attachment (Chemes *et al*., 1999; Rawe *et al*., 2002b; Porcu *et al*., 2003). The heads attach either to the tip or to the sides of the mid-piece without a linear alignment with the sperm axis. This misalignment ranges from complete lack of connection to a lateral positioning of the nucleus at a 90–180° angle (Figure 5). These alterations result from a dysfunction of the sperm proximal centriole that is unable to migrate normally to the caudal pole of the spermatid nucleus and fails to nucleate a functional sperm aster in the developing zygote, impairing normal syngamy and cleavage (Chemes *et al*., 1999; Saias Magnan *et al*., 1999; Rawe *et al*., 2002b). These pathological findings reinforce the physiological role of paternal inheritance of the centriole for human fertilization and early embryo development (Schatten, 1994; Hewitson *et al*., 1997; Sutovsky *et al*., 1999).

Holstein *et al*. (1986) and Baccetti *et al*. (1989a) have reported a patient and two brothers in whom the cleavage takes part between the proximal and distal centriole or along the mid-piece, but in most reported cases the separation occurs at the head–neck interface (Perotti *et al*., 1981; Chemes *et al*., 1987b, 1999; Toyama *et al*., 2000). These non-coincident reports indicate that there are various mechanisms responsible for the formation of acephalic spermatozoa. Increased fragility of the head–tail connection has been reported by Chemes *et al*. (1999) and Kamal *et al*. (1999a).

The uniform pathological phenotype, its origin as a consequence of a systematic alteration during spermiogenesis, the fact that seminal characteristics remain constant during clinical evolution even when a pharmacological germ cell depletion–repopulation has been induced, and the familial incidence in men and bulls, indicate that this condition is very likely of genetic origin.

Very little is known about the nature of the centriolar failure in spermatozoa with faulty head–neck attachments. Proximal centrioles are structurally normal (Perotti *et al*., 1981; Chemes *et al*., 1987b, 1999; Baccetti *et al*., 1989a). Proteins such as

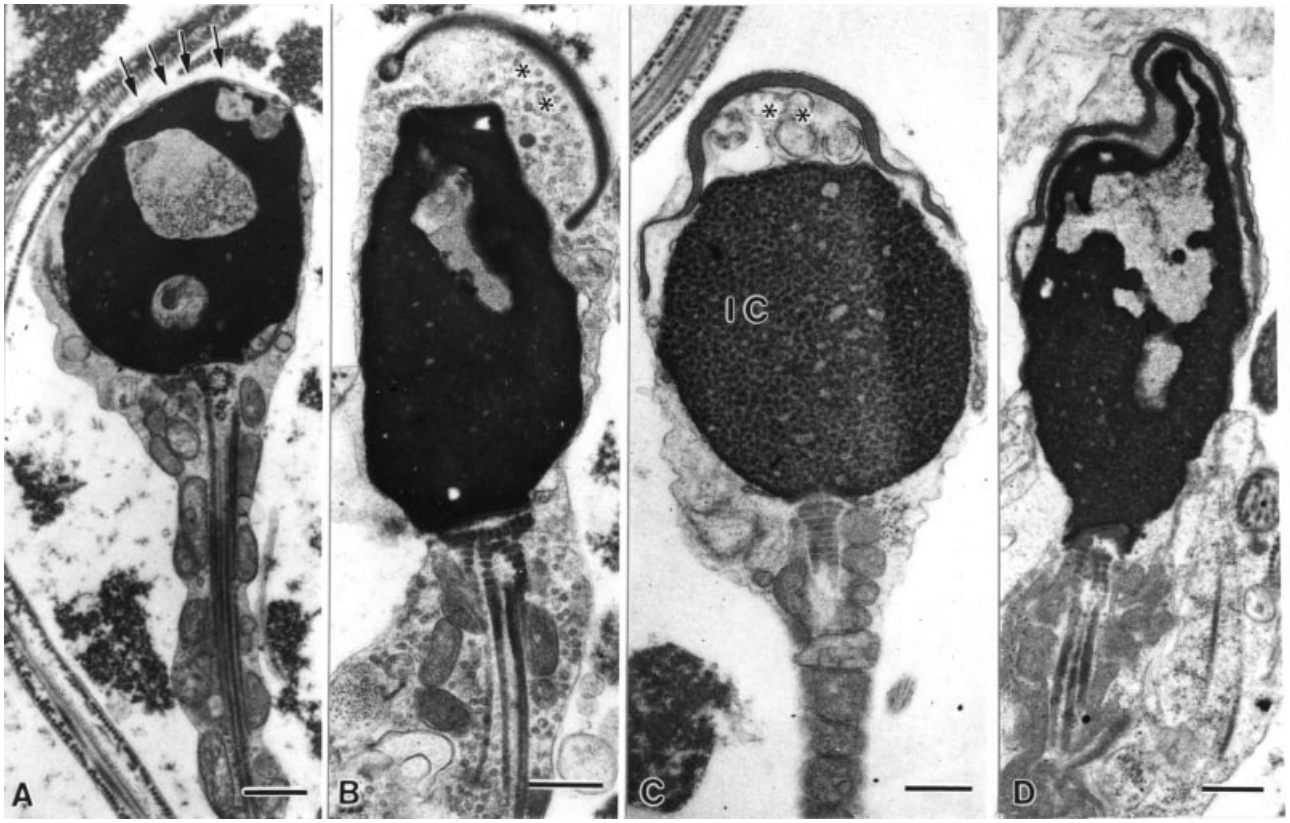


Figure 6. Acrosome and chromatin anomalies. (**A**) Acrosomal agenesis: round-headed spermatozoon lacking the acrosome (arrows). There is also a marked lacunar defect of the chromatin. (**B**) Acrosomal hypoplasia: small and detached acrosome (asterisks). (**C**) Acrosomal hypoplasia (asterisks) in a round-headed spermatozoon with immature, granular chromatin (IC). (**D**) Severe lacunar defect of the chromatin in a grossly distorted amorphous head. Bars = 0.5 μm.

centrin, pericentrin, γ-tubulin, speriolin and that recognized by mitotic protein monoclonal antibody-2 have been localized to the sperm centrosome and connecting piece but no studies are available that show their (possible) significance in the pathogenesis of this syndrome (Manandhar and Schatten, 2000; Goto *et al*., 2003; Porcu *et al*., 2003). The release of the sperm centriole after fertilization probably involves the action of sperm proteasomes recently localized to the neck region of human spermatozoa (Wojcik *et al*., 2000). Azh mice (abnormal spermatozoa head shape) display altered head and tail morphology and decapitated spermatozoa. A mutation in the Hook1 gene has been shown to be responsible for the azh phenotype (Mendoza-Lujambio *et al*., 2002).

Pathology of the sperm head: acrosome and chromatin anomalies

The acrosome of mature spermatozoa derives from transformations of the Golgi apparatus during spermiogenesis. In early spermatids the acrosomal vesicle and granule form inside the Golgi complex that progressively approaches the spermatid nucleus and attaches to it at a site marked by previous changes in the nuclear envelope (Chemes *et al*., 1979; Holstein and Schirren, 1979). This contact defines the anterior or cranial pole of the spermatid nucleus (the future anterior tip of mature spermatozoa). After attachment, the acrosomic vesicle and granule spread as a cap over the nucleus which progressively elongates while the chromatin begins to condense. The acrosome of mature spermatozoa covers the anterior two-thirds of the nuclear surface and is a flattened sac filled with dense contents rich in hydrolytic enzymes. The acrosome is very regular (0.1 μm thick) in most of its extension, but thins in its caudal part known as the equatorial segment. Distal to this segment, the sperm plasma membrane that covers the acrosome attaches directly to the nuclear envelope forming the post-acrosomal dense lamina or post-acrosomal sheath.

Two acrosomal anomalies causing infertility are the lack or insufficient development of the acrosome. The first condition is widely known as globozoospermia or round head acrosomeless spermatozoa due to the peculiar round shape of sperm nuclei. Since not all acrosomeless spermatozoa have round heads (see below), acrosomal aplasia or agenesis, is a more appropriate denomination for this syndrome. Small and detached acrosomes characterize acrosomal hypoplasia.

Spermatozoa lacking acrosomes can be found in small numbers (≅0.5 %) in the semen of fertile individuals, and may increase up to 2–3% in cases of infertility (Kalahanis *et al*., 2002). In acrosomal aplasia, they constitute the predominant anomaly in the vast majority of spermatozoa (up to 100% of ejaculated spermatozoa). The syndrome has been recognized and described in detail during the last 30 years (Schirren *et al*., 1971; Holstein *et al*., 1973; Pedersen and Rebbe, 1974; Bisson

et al., 1975; Kullander and Rousing, 1975; Anton Lamprecht *et al*., 1976; Baccetti *et al*., 1977; Castellani *et al*., 1978; Nistal *et al*., 1978; Holstein and Schirren, 1979; Florke-Gerloff *et al*., 1984, 1985). Sperm heads are characteristically round, the acrosome is either absent or exceedingly small and detached, and there is no post-acrosomal dense lamina (Figure 6). Immunohistochemical studies have demonstrated absence of acrosomal proteins such as acrosine, outer acrosomal membrane antigen and acrosine inhibitor (Florke-Gerloff *et al*., 1985). Most reports of acrosomeless spermatozoa describe insufficiently condensed chromatin due to a failure of the histone–protamine transition and increased rates of DNA fragmentation (Baccetti *et al*., 1977; Vicari *et al*., 2002). Studies on testicular biopsies have clarified the morphogenesis of this anomaly. Very early in spermiogenesis the Golgi complex fails to attach normally to the nucleus in coincidence with an irregular secretory activity and faulty development of the acrosomic granule. The forming acrosome never spreads over the nucleus, stays away from it in a cytoplasmic lobule and is frequently phagocytosed by Sertoli cells (Figure 6). The manchette and post-acrosomal dense lamina do not differentiate (Kullander and Rousing, 1975; Baccetti *et al*., 1977; Castellani *et al*., 1978; Holstein and Schirren, 1979; Florke Gerloff *et al*., 1984, 1985). In some patients the mechanism is not an independent maturation of acrosomes and nuclei, but rather a lack of development that results in a similar phenotype of acrosomeless spermatozoa (Anton Lamprecht *et al*., 1976; Holstein and Schirren, 1979). The lack of acrosome associates with anomalies of the perinuclear theca, a subacrosomal structure of the sperm head that contains various proteins involved in head shape changes, acrosomal–nuclear docking and oocyte activation after fertilization (Longo *et al*., 1987; Sutovsky *et al*., 1997, 2003; Oko *et al*., 2001). Ultrastructural and immunocytochemical studies in acrosomeless spermatozoa have demonstrated absence of the perinuclear theca and calicin (a basic protein of the perinuclear theca; Escalier, 1990). These abnormalities probably explain the defective head modelling during spermiogenesis and the failure of oocyte activation after microinjection of human acrosomeless spermatozoa into oocytes (see below).

Family incidence has been reported in men suffering from acrosomal aplasia, and a mono- or polygenic origin has been suggested but not proven (Kullander and Rousing, 1975; Nistal *et al*., 1978; Florke Gerloff *et al*., 1984; Baccetti *et al*., 2001). Various animal models with similar characteristics have been recently described. Mice carrying the blind sterile mutation and disruptions of the GOPC or the Ck2 genes (Golgi-associated protein and casein kinase II α' catalytic subunit) display abnormal sperm head shapes and failure of acrosome formation (Sotomayor and Handel, 1986; Xu *et al*., 1999; Yao *et al*., 2002). Similar results were obtained by van der Spoel *et al*. (2002) in mice injected with NB-DNJ, an alkylated iminosugar that interferes with the synthesis of sphingolipids. Other experimental examples of acrosomal anomalies include the ebo (ebourifee) and Hrb null mutations or the disruption of the cell-adhesion protein nectin-2 gene in mice(Lalouette *et al*., 1996; Bouchard *et al*., 2000; Kang-Decker *et al*., 2001). Most of these experimental models show an alteration in the mechanisms of Golgi-nuclear recognition and docking.

As seen in the previous sections, acephalic spermatozoa derive from the inability of the spermatid nucleus to adequately define its caudal pole, while acrosomeless spermatozoa result from the lack of proper attachment of the Golgi complex to the anterior pole of the spermatid nucleus. The unusual case described by Aughey and Orr (1978), with acephalic spermatozoa and acrosomeless loose heads in the same patient, indicates that these two abnormal mechanisms have combined, suggesting that this pathology is due to an abnormal differentiation of the bipolar nature of the spermatid nucleus.

Zamboni (1987) has described acrosomal hypoplasia in sperm with small acrosomes over nuclei with a round apex and no post-acrosomal sheath (Figure 6). Their characteristics are very similar to those of acrosomal aplasia, from which hypoplasia may be a variant. The lack of acrosomes frequently associates with round nuclei, and less often with amorphous or oval heads. Moreover, not all round forms are acrosomeless, which implies that the association between absence of acrosome and round nuclei is not an absolute rule. This is illustrated by the 35 patients with acrosomal abnormalities reported by Chemes (2000). From the seven cases with acrosomeless spermatozoa, the classical round heads were observed in four, while the other three had a mixture of round, amorphous and oval heads. The remaining 28 patients had mostly small acrosomes and some acrosomeless forms. Acrosomal hypoplasia should be investigated in cases of severe teratozoospermia and can be readily recognized with the electron microscope (Zamboni, 1992) or after a careful light microscopic examination. In the classification of spermatozoa by strict criteria these abnormalities are included among the severe amorphous varieties that have a poor fertility prognosis (Kruger *et al*., 1988). Acrosomal hypoplasia has been reported in brothers (Baccetti *et al*., 1991, 2001), but may also be an acquired and reversible condition (Camatini *et al*., 1978; Sauer *et al*., 1989).

Another form of acrosome defect has been reported in 10 unrelated men from couples with long-standing infertility. Spermatozoa from these patients bind normally to zonae pellucidae but their ability to undergo an acrosome reaction is reduced to 10% of control values, and they fail to fertilize *in vitro* (Liu and Baker, 1994). Rarer and poorly characterized defects of the acrosome include the 'crater defect' (Baccetti *et al*., 1989b) and acrosomal inclusions (Zamboni, 1992). In both cases, fertility is compromised by the inability of these spermatozoa to normally penetrate oocytes.

The process of differentiation that gives rise to mature spermatozoa involves chemical and macromolecular changes in the chromatin organization of early spermatids. Histones are the characteristic proteins associated with DNA in somatic cells and germ cells up to round spermatids. During nuclear elongation these proteins leave the nucleus and their place is occupied by transition proteins which in turn are interchanged

with protamines that bind to DNA (Courtens and Loir, 1975; Brewer *et al*., 2002; Dadoune, 2003). Histone–DNA complexes form nucleosomes that associate with each other in a super-coiled structure which is the unit of the chromatin fibre. In mature spermatids and spermatozoa, protamines associate side-to-side with the groove of the DNA helix. This macromolecular organization results in a linear, parallel packaging of nucleoprotein fibres which is stabilized by disulphide bonds (Balhorn, 1982; Ward and Coffey, 1991). This is reflected in the compaction of chromatin, visualized through the electron microscope as the appearance and progressive increase of a granular pattern that eventually reaches a dense, compacted state where individual granules cannot be discerned (Holstein and Roosen Runge, 1981). Condensed chromatin in normal spermatozoa display very small (0.1–0.2 μm), hypodense areas throughout the nucleus.

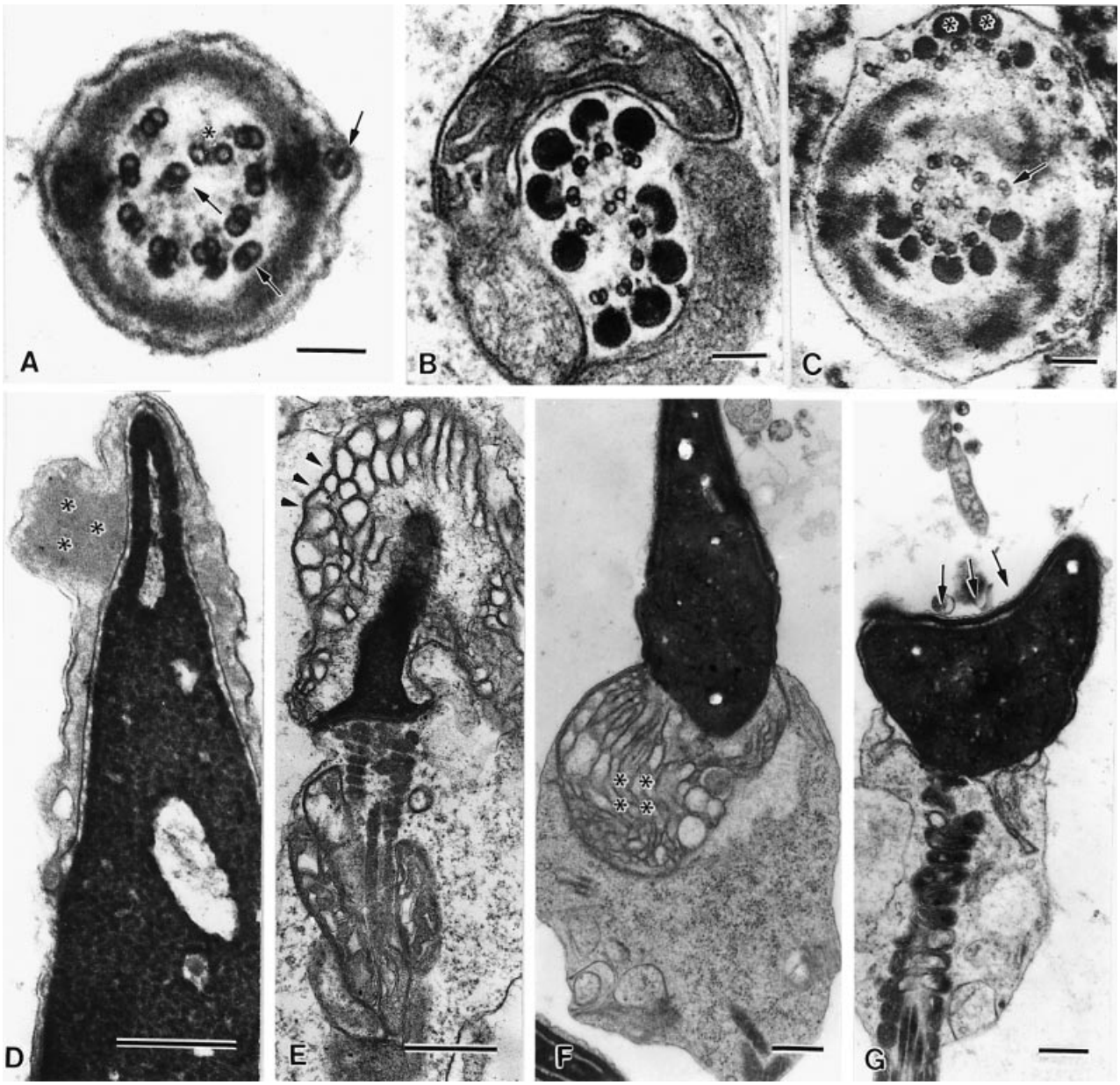
Holstein (1975) and Zamboni (1987) have described deficiencies in the process of chromatin maturation that result in big 'lacunar' defects (2–3 μm in diameter) where the compact arrangement of the chromatin is replaced by granulo-fibrillar or 'empty' areas that occupy as much as 20–50% of the nucleus. These defects frequently coexist with granular immature chromatin and have been referred to as abnormalities in chromatin maturation and compaction (Figure 6). They originate in the testis as a consequence of abnormal spermiogenesis as confirmed by their presence in immature spermatids found in testicular biopsies and semen. Baccetti et al. l1996) have reported similar findings in sterile individuals and suggested that they represent apoptotic changes, but in subsequent studies no association between sperm DNA fragmentation and these 'apoptotic-like' nuclei was found (Muratori *et al*., 2000). Spermatozoa with chromatin abnormalities frequently display abnormal head shapes, have diminished fertility potential or associate with abortions of the first trimester (Chemes, 2000). Various methods have been used to detect these anomalies, such as Aniline Blue staining of histones, flow cytometry after staining with Acridine Orange, TUNEL assays for apoptosis and ultrastructural examination of spermatozoa (Zamboni, 1987, 1992; Baccetti *et al*., 1996; Evenson *et al*., 1999; Chemes 2000; Muratori *et al*., 2000). Single-stranded DNA, DNA breaks, abnormal histone–protamine transition or apoptotic changes have been reported, as well as insufficient chromatin condensation, immaturity and intranuclear lacunae that are their ultrastructural correlates. There is not much information about the genetic constitution of morphologically abnormal spermatozoa. Martin and Rademaker (1988) and Rosenbusch *et al*. (1992) analysed sperm chromosome complements from fertile men after penetration into hamster oocytes and found no significant correlation between abnormal morphology and numerical chromosomal anomalies. High rates of aneuploidy or chromosomal structural aberrations have been found in teratozoospermia, but a clear association with alterations in chromatin maturation and compaction has not been demonstrated (Lee *et al*., 1996; Calogero *et al*., 2001; Kovanci *et al*., 2001). Recent fluorescence in-situ hybridization (FISH) studies of infertile men with poor semen quality have shown increased aneuploidy in spermatozoa despite a normal blood karyotype (Templado *et al*., 2002; Lewis-Jones *et al*., 2003; Vicari *et al*., 2003), which suggests that the same factor(s) causing aneuploidy may also induce teratozoospermia. These findings coincide with reports by Harkonen *et al*. (2001) in 20 teratozoospermic men studied by multicolour FISH. Severe teratozoospermia (<10% normal forms) was associated with higher frequency of disomy 7, 18, YY, XY and diploidy, which led these authors to suggest that severely teratozoospermic men might be at an increased risk of producing aneuploid offspring.

Abnormal patterns of chromatin condensation have been found in mice with targeted disruptions of the Camk4 (a Ca-calmodulin-dependent protein kinase) and transition protein 1 genes (Wu *et al*., 2000; Yu *et al*., 2000). To date, no significant genetic aetiology for chromatin abnormalities has been found in humans. There have been reports of abnormal removal of histones and transition proteins from sperm nuclei, selective absence or incomplete processing of protamine P2, and altered ratios between protamines P1–P3 in spermatozoa from infertile individuals (Balhorn *et al*., 1988; Blanchard *et al*., 1990; Belokopytova *et al*., 1993; de Yebra *et al*., 1993, 1998; Bench *et al*., 1998). However, no mutations in protamine genes have been found in 36 patients with disturbed chromatin condensation, and only one mutation leading to transcription termination was described in a population of 153 males with non-obstructive azoospermia (de Yebra *et al*., 1993, Schlicker *et al*., 1994; Tanaka *et al*., 2003). Nuclear 'vacuoles' have been reported in spermatozoa from individuals with seminal infections, varicocele, fever, testicular tumours and inflammatory bowel disease, where they seem to be due to the disease itself rather than secondary to sulfasalazine therapy as had been previously suggested (Hrudka and Singh, 1984; Baccetti *et al*., 1996; Evenson *et al*., 2000; reviewed by Zamboni, 1992). This indicates that chromatin anomalies may be genetic or secondary to different andrological conditions, but since genetic studies are scarce, no definitive conclusion can be drawn.

Human spermatozoa with large heads and multiple flagella were reported as the predominant anomaly in certain infertile individuals (Nistal *et al*., 1977; Escalier, 1983). High rates of aneuploidy/polyploidy were found in these sperm nuclei and the defect attributed to a failure of nuclear cleavage in meiosis. Familial incidence is documented in a detailed pedigree (Benzacken *et al*., 2001; Devillard *et al*., 2002). This is an infrequent sperm anomaly with few reports in the literature.

Acquired sperm abnormalities secondary to andrological conditions and endogenous or environmental factors

Non-specific or non-systematic sperm defects comprise a heterogeneous array of randomly distributed anomalies. They have no family incidence, are usually secondary to andrological disorders and other endogenous or exogenous factors and are potentially responsive to different treatments (Afzelius, 1981b; Chemes, 2000). The most characteristic finding in non-systematic defects is that multiple head or flagellar anomalies associate simultaneously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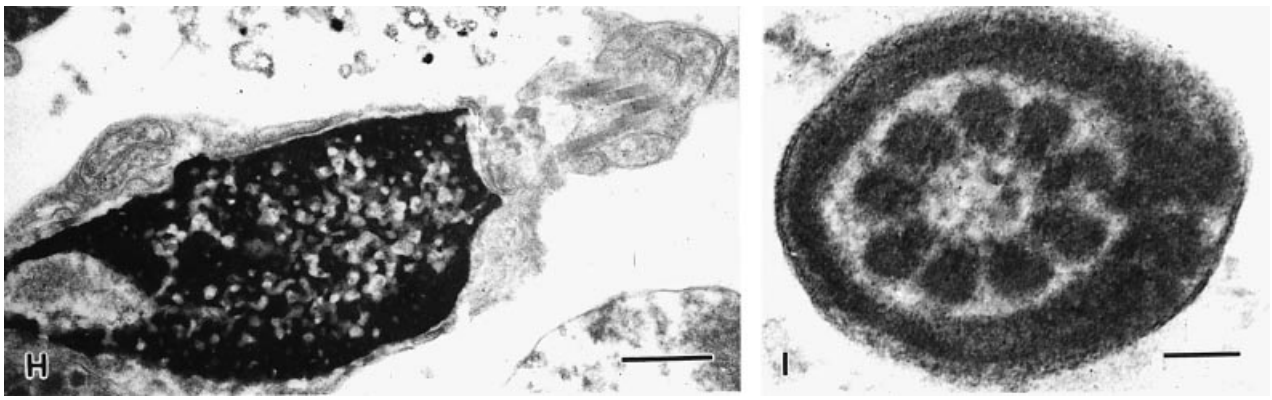
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with no definite pattern and fluctuate in their incidence during clinical evolution and among different patients (Figure 7).

Non-specific flagellar anomalies (NSFA) have been described in control and infertile populations (Wilton *et al*., 1985; Hunter *et al*., 1988; Chemes, 1991). They mainly consist in alterations in the number (lack or duplication), topography (dislocations/transpositions) and general arrangement in the 9 + 2 organization of axonemal microtubules and outer dense fibres. Affected flagella appear normal in light microscopy because their diameter is not modified, and are only identified by ultrastructural examination. Their increment is responsible for deficient motility in 70% of severely asthenozoospermic patients (Williamson *et al*., 1984; Ryder *et al*., 1990; Chemes, 1991; Hancock and de Kretser, 1992; Wilton *et al*., 1992; Chemes *et al*., 1998; Courtade *et al*., 1998). A thorough quantification of their incidence in each patient is essential for diagnosis, since they are also present in lower numbers (up to 40%) in fertile men. These findings demonstrate that severe asthenozoospermia is mainly due to structural abnormalities of the tail, and have challenged the concept that most sperm motility disorders have a 'functional' basis. Longitudinal follow-up revealed that NSFA patients can experience improved sperm motility as a result of various aetiological or empirical treatments (Chemes *et al*., 1998).

Non-specific head anomalies are the most frequent finding in teratozoospermic patients. They are easily detected in smears as variations in head shape and size that are the basis of different classifications of sperm morphology including those based on strict criteria (Kruger, 1986, 1988; World Health Organization, 1992). However, the diagnosis of most of these shape/size aberrations does not identify the underlying pathologies in the two head components most affected in teratozoospermia: the chromatin and acrosome. Alterations in chromatin maturation and compaction and insufficient development or vacuolization of the acrosome are a frequent finding in amorphous sperm heads (Figure 7) (Zamboni, 1987, 1992). They have been described in detail when dealing with pathologies of genetic origin because there are reports of familial incidence of acrosomal hypoplasia and occasional mutations in protamine genes (see previous sections). However, they have also been found associated with inflammatory bowel disease (reviewed by Zamboni, 1992), varicocele (Muratori *et al*., 2000; Reichart *et al*., 2000), administration of alkylated imino sugars or pesticides to mice (Bustos-Obregon and Diaz, 1999; Bustos-Obregon *et al*., 2001; van der Spoel *et al*., 2002), and other acquired conditions (Camatini *et al*., 1978; Sauer *et al*., 1989). Chromatin and acrosomal anomalies are probably heterogeneous disorders including genetic and/or acquired aetiologies.

Andrological conditions and endogenous or environmental factors have been variously mentioned as causative agents of non-specific head and flagellar abnormalities. Some authors have described tapered forms as characteristically found in varicocele patients (MacLeod, 1970; Naftulin *et al*., 1991). However, they have been found associated with other pathologies and are not specific to varicocele. Increased abnormal forms (strict criteria), chromatin immaturity or insufficient compaction and acrosome distortions have been reported in varicocele patients, their incidence diminishing after ligation (Vazquez-Levin *et al*., 1997; Muratori *et al*., 2000; Reichart *et al*., 2000). Among infective agents, *Escherichia coli*, *Pseudomonas aureuginosa* or *Candida albicans* incubated *in vitro* with human spermatozoa are responsible for alterations in sperm heads and tails, plasma membranes and acrosomes, while *Enterococcus* or *Staphylococcus saprofiticus* have no deleterious effects (Teague *et al*., 1971; Huwe *et al*., 1998; Diemer *et al*., 2000). Men with seminal infections by *Ureaplasma urealyticum* and *Chlamydia trachomatis* or antisperm antibodies have astheno- and teratozoospermia and various non-specific sperm tail defects (Williamson *et al*., 1984; Megory *et al*., 1987; Purvis and Christensen, 1993; Menkveld and Kruger, 1998). Increased non specific flagellar anomalies that reverted after antibiotic therapy were observed in patients with leucocytospermia (personal non published observations).

Spermatozoa with double heads and flagella were reported in a patient with hyperprolactinaemia (Baccetti *et al*., 1978). Among hormones with influence on spermatozoa, Ben-Rafael *et al*. (2000) and Bartoov *et al*. (1994) have shown morphological improvements in sperm subcellular components (chromatin, acrosomes, axonemes) after chronic treatment with FSH. Also, administration of vitamins E and C preserves the integrity of sperm DNA by neutralizing oxidative damage by reactive oxygen species (Kodentsova *et al*., 1994).

Toxic and environmental factors cause reversible alterations in sperm structure. ElJack and Hrudka (1979) studied the pattern and dynamics of teratozoospermia in rams treated with ethylene dibromide and found reversible pathological changes in sperm acrosomes, chromatin and mitochondrial sheaths but not in axonemes. Parathion, malathion and chlorinated compounds induce anomalies in sperm heads, mid-pieces and flagella when administered to mice (Krzanowska, 1981; Bustos-Obregon and Diaz, 1999; Contreras and Bustos-Obregon, 1999; Sobarzo and Bustos-Obregon, 2000; Bustos-Obregon *et al*., 2001). Epidemiological studies on the influence of various work environments and contact with different toxic substances have shown important increases in sperm defects in farmers and graziers (exposed to various pesticides) and men working in motor, mechanical and welding trades, chemical and petroleum workers (exposed to fuels, oils, organic solvents, exhaust fumes and hydrocarbons) (Whorton and Meyer, 1981; Harrison *et al*., 1998). Unusually large increases in the mean percentage of abnormal spermatozoa in smokers compared with non-smokers were

Figure 7. Non-specific anomalies. The various flagellar and nuclear defects depicted here are mixed in different proportions in each patient, with no particular predominance of any single sperm defect. (**A**–**C**) Non-specific flagellar anomalies. In **A** the central pair is displaced (asterisk) and there is microtubular translocation (arrows). In **B** the axoneme is 'fractured' and laterally displaced at the mid-piece. (**C**) Supernumerary doublets (arrow) and partial duplication outside of the fibrous sheath (asterisks). (**D**) Acrosome irregularities and diminished density (asterisks). (**E**) The acrosome is replaced by a multilamellar structure (arrowheads) over a very small head. (**F**) A multimembranous structure covers the caudal pole of the nucleus (asterisks). (**G**) A grossly distorted sperm head covered by a small acrosome (arrows). (**H** and **I**) Dead spermatozoa with disintegration of the chromatin, mid-piece mitochondria (**H**) and axonemal microtubules (**I**). Panel **B** was originally published in Chemes *et al.* (1998), © European Society of Human Reproduction and Embryology. Reproduced by permission of Oxford University Press/Human Reproduction. Bars = 0.1 μm (**A**–**C**, **I**), 0.5 μm (**D**–**G**, **H**).

reported by Banerjee *et al*. (1993) and Sofikitis *et al*. (1995), although the significance of these findings has been put in doubt because of small sample sizes and the use of different definitions of abnormal sperm morphology.

Various physical agents have deleterious influences in sperm quality. Ionizing radiation effects on sperm structure have been studied in humans exposed to high radiation doses after nuclear reactor accidents and in mice experimentally subjected to X-rays or radioisotopes. The main observations were nuclear and chromatin structural defects, decreased motility and sterility (Sailer *et al*., 1995; Schevchenko *et al*., 1989; Bartoov *et al*., 1997; Fischbein *et al*., 1997). Cryopreservation of human spermatozoa adversely affects sperm morphology, motility, mitochondrial function and viability (O'Connell *et al*., 2002). Exposure to any factor that compromises the thermoregulatory function of the scrotum will adversely influence semen parameters. Lifestyles including posture and clothing, excessive use of sauna, high ambient temperatures and intensity of activity can induce higher scrotal temperatures and reversible sperm abnormalities (Mieusset, 1998; Saikhun *et al*., 1998; Thonneau *et al*., 1998).

An account of non-specific sperm pathologies would not be complete without mention of necrozoospermia, the increase of non-viable spermatozoa above the higher limits found in fertile individuals (25–50% dead spermatozoa; World Health Organization, 1992, 1999). This is a poorly known seminal condition associated with infections, toxic agents, congenital or acquired obstructions of the genital tract, spinal cord injury, etc. (Singer *et al*., 1987; Wilton *et al*., 1998; Nduwayo *et al*., 1995; Brackett *et al*., 1998; de Kretser *et al*., 1998; Lohiya *et al*., 1998; Vicari *et al*., 1999; Halder *et al*., 2003). Various bacterial agents affecting the prostate, seminal vesicles or the epididymis or some of their chemical constituents have been singled out as causative agents (Singer *et al*., 1987; Vicari *et al*., 1999; Hosseinzadeh *et al*., 2003). The percentage of dead sperm in semen decreases with shorter storage times and increased transport speeds through the epididymis, which may indicate the involvement of unknown epididymal factors ('epididymal necrozoospermia') (Wilton *et al*., 1998; de Kretser *et al*., 1998). In these situations, frequent ejaculations or testicular sperm extraction have been advocated to obtain better quality spermatozoa (Tournaye *et al*., 1996; Rybouchkin *et al*., 1997a). Non-viability can be detected by means of vital dyes such as eosin or with the hypo-osmotic swelling test. Post-necrotic changes include fragmentation leading to short and irregular flagella that may be confused with 'stumpy spermatozoa' in the case of DFS (see discussion of this point in the section dealing with genetic-related pathologies of the sperm tail). Ultrastructural examination reveals disintegration of mid-piece mitochondria or flagellar microtubules, vesiculization of the chromatin (Figure 7), and widespread dissolution of the plasma and acrosomal membranes (false acrosome reactions that can be differentiated from genuine ones in which the equatorial segment is preserved). Irreversible chromosomal damage has been reported in dead spermatozoa, which explains the generalized poor results obtained in assisted reproduction (Tournaye *et al*., 1996; Rybouchkin *et al*., 1997a). In the case of 100% immotile spermatozoa, some workers mistakenly equate complete asthenozoospermia with total necrozoospermia. This creates unnecessary confusion in view of the very different nature and fertility potential of immotile (but live) and dead spermatozoa (see following section).

Table I. Fertility in severe asthenozoospermia: spontaneous, low-complexity assisted reproductive technology and IVF[a]

	NSFA-RT	NSFA-NRT	DFS
No. of patients (n = 88)	18	36	34
Fertilizations/pregnancies	18/14	0/0	0/0
Live births	12	0	0
Flagellar pathology (%)	72 ± 15	70 ± 19	90 ± 14
Motility I (%)	5.2 ± 7.4[b]	2.3 ± 2.9	0.2 ± 0.9
Motility II (%)	15.1 ± 8.8[b,c]	7.4 ± 7.0[c]	0.2 ± 0.7

[a]Data from Chemes *et al*. (1998).
[b,c]Statistically significant differences between same superscripts ($P < 0.01$).
NSFA = non-specific flagellar anomalies; RT = responsive to treatment; NRT = non-responsive to treatment; DFS = dysplasia of the fibrous sheath.

Evidence has been gathered in recent decades on the role of antisperm antibodies in the pathogenesis of infertility. Spermatozoa have numerous surface antigens and antibodies have been found both in men and women that bind to spermatozoa and alter their function. Diminished sperm motility, defective cervical mucus penetration and alterations and sperm–oocyte interaction and fusion have been reported but no specific pathological phenotypes associated with sperm autoimmunity have been described so far (Verpillat *et al*., 1995; Wolf *et al*., 1995; Lombardo *et al*., 2001).

Sperm pathology and fertility prognosis. The significance of sperm pathology in the study of infertile males

Sperm motility and morphology have long been recognized as indicators of the fertility potential of human spermatozoa. The recent introduction of microfertilization techniques provides access to the structural and functional features of spermatozoa that are being used for fertilization. This possibility can be used to evaluate the relationship between sperm quality and fertility outcome so that a more objective picture is emerging of the differential roles played by specific sperm components in fertilization, early embryonal development and implantation.

Asthenozoospermia: flagellar pathologies and fertility prognosis

As described in previous sections, flagellar structural abnormalities are responsible for most cases of severe asthenozoospermia. To examine their value as indicators of fertility potential, two groups of men, 54 with NSFA and 34 with DFS were followed for 2–6 years and information was obtained on medical or surgical treatments, changes in motility and fertility outcome using conventional methods or IVF (Chemes *et al*., 1998). At the end of the follow-up period it was found that 18/54 NSFA patients (33%) improved motility after various empirical or aetiological treatments (Table I, NSFA-RT) and obtained 18 fertilizations, 14 pregnancies and 12 live births. The other 36 men with NSFA neither improved motility nor obtained fertilizations/pregnancies (NSFA-NRT). The 34 patients with DFS had very low motility that did not change

Table II. ICSI outcome with immotile spermatozoa

	No. of patients	Fertilization rate (%)	Pregnancies/ abortions	Outcome
Primary ciliary dyskinesia/immotile cilia syndrome[a]	11	54 ± 12	5/1	7 live births
Dysplasia of the fibrous sheath/stump tails/short tails[b]	12	63 ± 16	10/2	14 live births

[a]Data from Bongso *et al*. (1989), Papadimas *et al*. (1997), von Zumbusch *et al*. (1998), Gallo *et al*. (1999), Kamal *et al*. (1999b), Cayan *et al*. (2001).
[b]Data from Stalf *et al*. (1995), Brugo Olmedo *et al*. (1997, 2000), Chemes *et al*. (1998), Favero *et al*. (1999), Kamal *et al*. (1999b).

during evolution and no fertilizations/pregnancies occurred. These findings indicate that 1/3 of NSFA are reversible and can obtain fair fertility results, while the DFS does not respond to conventional fertility treatments or IVF, as confirmed by the lack of other positive results in the literature. One single publication by Kay and Irvine (2000) has documented a live birth after IVF using sperm with no progressive motility from a patient with primary ciliary dyskinesis, a pathology related to DFS.

The practice of ICSI has shown that fertilization could proceed after injection of abnormal or immotile spermatozoa. Payne *et al*. (1994) treated 18 severe male factor patients with both classical IVF and ICSI and obtained a much higher fertilization rate with ICSI (76%) than with IVF (15%), which indicates that many sperm functional impairments were overcome by direct injection into oocytes. In retrospective studies of 966 ICSI cycles and 76 fertilization failures, Nagy *et al*. (1995) and Liu *et al*. (1995b) reported that ICSI results were not influenced by alterations in any of the three classical sperm parameters (sperm count, motility and morphology) with the exception of acrosomal aplasia or immotile spermatozoa. However, in their 'immotile' population, viability was always <10%, which makes it very likely, as also noted by the authors, that their poor results were due to injection of dead spermatozoa (rather than live immotile). Although dead spermatozoa are obviously immotile, the distinction between necrozoospermia and total asthenozoospermia is an essential prognostic factor in ICSI as shown by numerous reports on successful fertilizations/pregnancies in patients with immotile, but live spermatozoa (Nijs *et al*., 1996; Barros *et al*., 1997; Kahraman *et al*., 1997; von Zumbusch *et al*., 1998). The difficulty in distinguishing between dead and completely immotile but live spermatozoa has been circumvented by various methods including the hypo-osmotic swelling test, stimulation of motility with pentoxifylline, or retrieving testicular spermatozoa (Kahraman *et al*., 1996; Ved *et al*., 1997; Wang *et al*., 1997; Terriou *et al*., 2000). In 23 reported cases of PCD or DFS/stump/short tails, microinjection of immotile or in-situ motile spermatozoa has resulted in fair to good fertilization and pronuclear formation rates, numerous pregnancies and 21 live births (Table II) (Bongso *et al*., 1989; Stalf *et al*., 1995; Papadimas *et al*., 1997; Brugo Olmedo *et al*., 1997, 2000; Chemes *et al*., 1998; von Zumbusch *et al*., 1998; Gallo *et al*., 1999; Favero *et al*., 1999; Kamal *et al*., 1999b; Cayan *et al*., 2001). Therefore, flagellar pathologies causing sperm immotility do not compromise ICSI outcome if sperm viability is not affected.

Since PCD/ICS and the DFS are genetic conditions, the question arises as to their possible transmission to the next generation. Most of the successful pregnancies are very recent and therefore evaluation of fertility in the offspring will not be possible for some years. On the other hand, in the available literature there are no reports of respiratory disease (a common finding in PCD and some DFS) in any of the children born so far. Even though an autosomal recessive mode of inheritance is most likely, a thorough genetic counselling will only be possible when all the genes (and possible mutations/deletions) involved are fully characterized. Until then, it seems reasonable to make patients aware of the potential risks involved in using abnormal spermatozoa to attain fertilizations that would not have taken place if the natural mechanisms of sperm selection had operated as happens in spontaneous conceptions. Most couples would take the chance if infertility for their progency were the only risk involved, with the understanding that treatments are already available and may become more effective in the future.

Fertility prognosis in teratozoospermia

Teratozoospermia is a very heterogeneous condition comprising alterations in the shape of different sperm components. There is a close relationship between deviations of normal shape and fertilizing potential because structures of mature spermatozoa provide the best organization to serve specific functions. Teratozoospermia should be understood as the combination of morphological abnormalities with the corresponding impairments in sperm function. Consequently, abnormally shaped heads express different alterations in the organization and function of the chromatin, the perinuclear theca, the acrosome or the cytoskeletal influences that model a normal sperm nucleus. High rates of fertility in bulls positively correlate with certain nuclear configurations which, in turn, are highly dependent on chromatin stability (Ostermeier *et al*., 2001). Investigations on IVF results and a meta-analysis of six studies on intrauterine insemination show a significant improvement in the pregnancy rate in coincidence with morphology values above the 4% threshold (Kruger *et al*., 1988; Van Waart *et al*., 2001). Sperm abnormalities adversely influence results of assisted reproduction treatment as shown by their presence in 61.5% of 52 failed IVF cycles (Oehninger *et al*., 1988), and by the low fertilization rates after

Table III. ICSI outcome utilizing spermatozoa with anomalies of the head–neck junction

	No. of patients/ no. of cycles	Fertilization rate (%)	Outcome
Chemes *et al*. (1999); Rawe *et al*. (2002)	2/6	80	Fragmented embryos; good embryos with no pregnancies; 2 chemical pregnancies
Saias-Magnan *et al*. (1999)	1/4	70	Good embryos with no pregnancies
Kamal *et al*. (1999)	16	48	1 live birth, 2 ongoing pregnancies, 13 failures
Porcu *et al*. (2003)	2/5	88	1 chemical pregnancy, 2 pregnancies with 4 live births

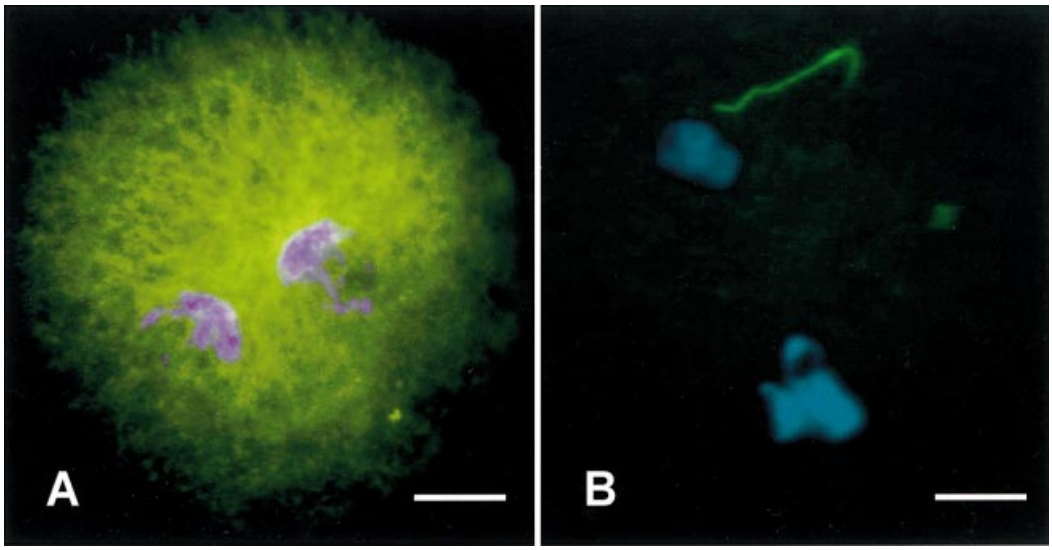

Figure 8. Bovine oocytes injected with human spermatozoa from a normal donor (**A**) or from a patient with centriolar alterations and abnormalities of the connecting piece (head–tail junction, (**B**). In **A** there is a well-developed sperm aster (green fluorescence of beta tubulin). In **B** both pronuclei are formed (blue) but no sperm aster is formed from the centrosome of the sperm pronucleus (green sperm tail). Bars = 25 μm. Panels **A** and **B** were originally published in Rawe *et al*. (2002b), © European Society of Human Reproduction and Embryology. Reproduced by permission of Oxford University Press/Human Reproduction.

ICSI in 17 men with 100% abnormal head morphology (Tasdemir *et al*., 1997). Nagy *et al*. (1995) and Liu *et al*. (1995b) claimed that abnormal morphology does not influence ICSI results, but in 10/15 of their patients with total fertilization failure, strict morphology was ⩽2% and they also reported failed fertilization in six patients with acrosome-less spermatozoa. The correlation of high-resolution light microscopy and electron microscopy with ICSI results stresses the importance of normal acrosome and chromatin structure, head–neck junction and centrosomes for adequate fertilization and pregnancy (Nikolettos *et al*., 1999; Chemes, 2000; Bartoov *et al*., 2002; Rawe *et al*., 2002b).

Anomalies of the neck region with increasing fragility of the head–mid-piece junction have a wide phenotypic manifestation that ranges from different degrees of misalignment between heads and tails to complete separation, acephalic spermatozoa and loose sperm heads in semen. In most reported cases, acephalic forms predominate, which makes impossible any attempt at assisted reproduction (LeLannou, 1979; Perotti *et al*., 1981; Holstein *et al*., 1986; Chemes *et al*., 1987b; Baccetti *et al*., 1989a; Toyama *et al*., 2000). In another variant of the syndrome, acephalic forms are less frequent and spermatozoa with abnormal head–mid-piece alignment predominate (Lüders, 1976; Chemes *et al*., 1999; Saias Magnan *et al*., 1999; Kamal *et al*., 1999a; Rawe *et al*., 2002b; Porcu *et al*., 2003). This prompted various recent attempts to achieve pregnancies with microfertilization techniques (Table III). In the first report (Chemes *et al*., 1999) four metaphase II oocytes were fertilized by ICSI but remained at the pronuclear stage and degenerated after failure to undergo syngamy and cleavage. Saias Magnan *et al*. (1999) reported four ICSI cycles in a similar patient with little embryo fragmentation but no pregnancies. Similar results were communicated by Rawe *et al*. (2002b) in five ICSI cycles with two chemical pregnancies. These findings suggested a malfunction of the sperm centriole, which was confirmed by the inability of spermatozoa with abnormal head–mid-piece junction to assemble an aster when injected into bovine oocytes (Figure 8) (Rawe *et al*., 2002b). Two successful pregnancies were reported by Porcu *et al*. (2003), and Kamal *et al*. (1999a) announced three pregnancies using spermatozoa from 16 men with 'easily decapitated' spermatozoa, a condition that is possibly a variant of the syndrome of abnormal head–neck attachment. Therefore, even though some pregnancies have recently been

obtained using spermatozoa with abnormalities of the head–mid-piece connection, their fertilizing potential is seriously compromised as shown by their inability to induce pregnancies in the other patients reported.

Spermatozoa lacking acrosomes (acrosomal aplasia, globozoospermia) are unable to fertilize oocytes in IVF because they fail to bind to the zona pellucida (Schmiadi *et al*., 1992). Microfertilization techniques bypass sperm–oocyte binding and penetration and seem to be the ideal technique to be applied to this condition. Unsuccessful ICSI attempts in nine cases of acrosomal aplasia were reported by Bourne *et al*. (1995), Liu *et al*. (1995b), Battaglia *et al*. (1997) and Edisiringhe *et al*. (1998). Failures have been attributed to deficient oocyte activation, since acrosomeless spermatozoa have alterations in the perinuclear theca and associated proteins that are probably responsible for oocyte activation after fertilization (Longo *et al*., 1987; Escalier, 1990; Sutovsky *et al*., 1997; Oko *et al*., 2001). When acrosomeless spermatozoa from GOPC knockout mice or humans are microinjected into mouse oocytes, activation is not achieved unless it is induced by electroporation or treatment with 8% ethanol (Rybouchkin *et al*., 1996; Yao *et al*., 2002). Following this experience, Rybouchkin *et al*. (1997b) and Kim *et al*. (2001) obtained successful pregnancies with acrosomeless spermatozoa by means of Ca^{2+} ionophore activation of the oocytes. However, artificially induced oocyte activation is not always followed by pregnancy (Battaglia *et al*., 1997). Besides these failures there are also various reports of ICSI successes after microinjection of acrosomeless spermatozoa, but fertilization rates were low (10–50%, Lundin *et al*., 1994; Liu *et al*. 1995a; Trokoudes *et al*., 1995; Stone *et al*., 2000; Nardo *et al*., 2002; Zeyneloglu *et al*., 2002). These results indicate that even though human acrosomeless spermatozoa are able to fertilize human or hamster oocytes (Lanzendorf *et al*., 1988) and achieve pregnancies in numerous couples, they bear abnormalities responsible for unsuccessful fertilizations, low fertilization rates or the need for artificial activation.

Defects of chromatin maturation and compaction are frequently found in severe teratozoospermia, sometimes associated with acrosomal hypoplasia. Their incidence in spermatozoa fluctuates along clinical evolution. Infertility or abortions during the first trimester have been reported in these patients (Zamboni, 1987, 1992; Francavilla *et al*., 1996; Hamamah *et al*., 1997; Evenson *et al*., 1999; Chemes, 2000). Francavilla *et al*. (2001) have recently reported on 21 ICSI cycles in a series of azoospermic males with late spermatogenic maturation arrest. Increased numbers of spermatids with abnormal chromatin condensation were found in ultrastructural examination of testicular biopsies. Fertilization rates were normal, but the delivery rate/cycle was 44% lower than that of a control population. Kahraman *et al*. (1999) have also reported normal fertilization rates and low pregnancy rates in a study of 17 males with megalohead multi-tailed spermatozoa that have been shown to be polyploid (Nistal *et al*., 1977; Escalier 1983; Devillard *et al*., 2002). Sakkas *et al*. (1996), Evenson *et al*. (1999) and Egozcue *et al*. (2000) found that abnormal chromatin packaging or sperm disomy were responsible for low fertility and increased risk of pregnancy loss.

Concluding remarks

We have developed the concept of sperm pathology as the discipline that characterizes structural and functional deficiencies in spermatozoa. It allows an understanding of abnormal function that goes beyond that provided by classical sperm morphology classifications that are mainly based on descriptions of abnormal sperm shapes. These two notions do not compete with each other. They cooperate in providing a correct diagnosis, a prognostic tool, and a deeper understanding of the mechanisms of abnormal reproduction in the sterile male.

Special effort was made to highlight each pathological phenotype with a denomination that identifies the organelles involved and the pathogenic mechanisms. The problem of nomenclature is not a trivial one: the way we speak and write conditions the way we think. If descriptive terms are used, thoughts will not go beyond appearances. It is essential to distinguish a dead (immotile) from an immotile (live) spermatozoon and to use denominations that give us the basic understanding of each pathology. A ‘stump tail’ can either belong to a DFS spermatozoon or be the result of tail disintegration in ageing spermatozoa; an ‘‘amorphous’’ head can correspond to acrosomal agenesis or to abnormal chromatin maturation and compaction.

A correct identification of sperm pathologies indicates different fertility potentials and outcomes in assisted reproduction technology. It also serves to assess the genetic risk in each case. With the availability of many therapeutic tools, patients are ready to take the chances if infertility is the only risk involved for their offspring. However, from the medical point of view, the possible enrichment in pathological genomes in future generations evokes ethical and evolutionary considerations on the social role of current assisted reproductive technologies and those yet to come. The possibility of inherited sterility is certainly one of the most perplexing paradoxes of our times.

Acknowledgements

We wish to express our acknowledgement to the Centro de Ginecología y Reproducción (CEGYR), where one of us (V.Y.R.) formerly worked, and particularly to S.Brugo Olmedo MD and medical staff for previous collaborative work. The collaboration of Susana Mancini MSc in the literature search, Pablo Winitzky MSc with the organization of the manuscript and Oscar Rodriguez with excellent photographic work are fully acknowledged. Special thanks are due to Jeffrey L.Salisbury PhD, Tumor Biology Program, Mayo Clinic, Rochester, for the 20H5 anti centrin antibody, Mitch Eddy PhD, Gamete Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental, Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina for the AKAP4 antibody, and to Antonio Miranda Vizuete PhD (Center for Biotechnology, Department of Biosciences at Novum, Karolinska Institute, Huddinge, Sweden) for the thyoredoxin antibody. Supported by Grants from the National Research Council (PIP 0900 and 4584) and ANPCyT (PICT 9591). We acknowledge the generous support of the Reproductive Sciences for the Americas Network (RSANET) to Vanesa Rawe.

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