Human Reproduction vol.13 no.9 pp.2521–2526, 1998

Ultrastructural pathology of the sperm flagellum: association between flagellar pathology and fertility prognosis in severely asthenozoospermic men

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An ultrastructural study of spermatozoa in a series of 247 severely asthenozoospermic patients disclosed two kinds of anomalies. The first was dysplasia of the fibrous sheath, a primary defect of spermatozoa with hypertrophy and hyperplasia of the fibrous sheath, associated axonemal anomalies, familial incidence and chronic respiratory disease. The patients could be divided into two subgroups: the complete form (all spermatozoa affected) and the incomplete form (alterations in 70–80% spermatozoa). There were no spontaneous or in-vitro fertilization (IVF) pregnancies. Intracytoplasmic sperm injection (ICSI) in six patients resulted in successful fertilizations, but only two pregnancies were obtained. These features configure a phenotype that suggests a genetic origin. The second anomaly was non-specific flagellar anomaly (NSFA), random secondary flagellar alterations affecting variable numbers of spermatozoa, without respiratory disease or familial incidence. 54 men with NSFA were followed for 2–6 years. Of these, 18 achieved conception, either spontaneous or by means of assisted fertilization, followed by 14 pregnancies and 12 live births. Their sperm motility significantly increased during the follow-up period. In the remaining 36 men motility did not change during the follow-up period and there were no fertilizations or pregnancies. We conclude that in severe asthenozoospermia, ultrastructural examination of spermatozoa has an effective prognostic value, identifying two syndromes with very different flagellar alterations and fertility potentials.

Key words: asthenozoospermia/fibrous sheath/flagellar pathology/genetic origin/stump tails

Introduction

In the last decade we have extensively reported on the ultrastructure of abnormal spermatozoa in asthenozoospermic or teratozoospermic sterile men (Chemes *et al.*, 1987a, b, 1990; Chemes, 1991, 1993; Brugo Olmedo *et al.*, 1997). In particular, a high incidence of flagellar pathology was found to be the underlying cause of motility disorders in severely asthenozoospermic patients. Ultrastructural studies of spermatozoa in these men disclosed two main classes of alterations: non-specific flagellar anomalies (NSFA), random, secondary alterations that affect variable numbers of spermatozoa in different samples, and dysplasia of the fibrous sheath (DFS), a systematic, primary anomaly affecting most spermatozoa.

In the present paper we present a detailed clinico-pathologic characterization of both syndromes, based on information on a very large series of patients, including fertility follow-up, and results of medical and surgical treatments, classical assisted fertilization methods and microfertilization techniques.

Materials and methods

Patients

The cases included in the present study were men of reproductive age between their third and fifth decades of life. All of them consulted for sterility due to severe asthenozoospermia. The total number of patients studied was 247. Forward progression and rapid linear progression averaged 10 ± 10% (mean ± SD) and 3.2 ± 4.5%, with median values of 5 and 1% respectively. Detailed clinical information is presented in the Results section.

In a subgroup of 88 of these men (54 with NSFA and 34 with DFS), it was possible to obtain clinical and laboratory information for periods ranging from 2–6 years after electron microscopic diagnosis. This information included medical or surgical treatments, the evolution of semen variables (particularly motility), results of in-vitro fertilization (IVF) or microfertilization techniques (intracytoplasmic sperm injection – ICSI), spontaneous or assisted pregnancies, abortions and live births.

Transmission and scanning electron microscopic studies

Semen samples were obtained by masturbation and studied when liquefaction was complete, ~ 30 min after ejaculation, according to methods previously reported (Chemes *et al.*, 1987a). Briefly, the samples were diluted (1:3) with phosphate buffer (0.1 M, pH 7.4) and spermatozoa were separated by centrifugation. The pellets were fixed for 2–4 h with 3% glutaraldehyde in the same buffer, postfixed for 2 h in 1.3% osmium tetroxide and embedded in Epon-Araldite. The blocks were cut in a RMC MT-7000® ultramicrotome (RMC Inc., Tucson, AZ, USA) with glass and diamond knives and the sections were double stained with uranyl acetate and lead citrate and studied on a Zeiss EM 109® electron microscope (Zeiss, Oberkochen, Germany). For studies with the scanning electron microscope, the same fixatives were used. The spermatozoa were fixed in suspension with buffer washes between and after fixation steps. Sperm cells were subsequently sedimented on poly-L-lysine coated glass slide fragments, to assure sperm adherence to the glass, dehydrated in a graded series of ethanols followed by absolute acetone, dried in a Balzers CDP® 030 (Balzers Union Ltd, Balzers, Leichtenstein) critical point drying apparatus using CO_2 as transition fluid, coated with gold-palladium in a Balzers Union SCD® 040, and observed in a

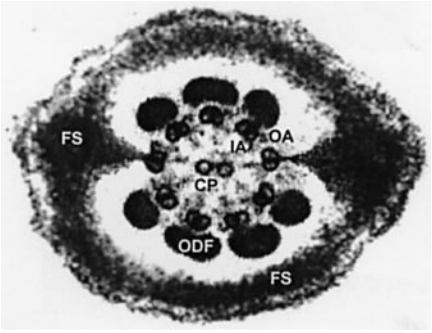

Figure 1. Photocomposition of a cross-sectional view of the sperm flagellum at the principal piece. Semi-schematic rendering of the main axonemal and periaxonemal components. FS: fibrous sheath, ODF: outer dense fibres, CP: central pair of microtubules, IA and OA: inner and outer dynein arms.

Philips 515® (Philips Nederland BV, Eindhoven, The Netherlands) scanning electron microscope. For quantification of axonemal anomalies with the transmission electron microscope, at least 100 flagella were counted. Only transverse sections, where all axonemal structures could be clearly discerned, were considered.

In all cases a small aliquot of fresh semen was studied under phase contrast microscopy, and motility, viability and light microscopic morphology were studied according to standard methods (WHO, 1987).

Statistical analysis

For statistical analysis (Student's *t*-test, one-way analysis of variance, linear regression analysis) the GB-STAT® software program was used (Dynamic Microsystems Inc., MD, USA). Results are expressed as means ± SD.

Results

Ultrastructural studies showed that from the total population of 247 asthenozoospermic men, 205 (83%) had NSFA and 42 (17%) DFS.

Non-specific flagellar anomalies (NSFA)

In this group forward progression averaged 11.6 ± 10.2%, and rapid linear progression 3.6 ± 4.7%. These values were similar to those of the total population.

Light microscopy of sperm smears indicated that flagella were long, slender and undulating, with regular contours, most of them displaying normal morphology or slight alterations. Flagellar diameter and the configuration of the middle piece were within normal limits. Ultrastructural alterations consisted of modifications in the number, topography and organization of axonemal microtubules which determined distortions of the normal 9 + 2 architecture. Typically, microtubules could be missing, partially or completely duplicated, translocated to abnormal sites within the flagellum, or completely disorganized (Figures 1 and 2). These alterations were randomly distributed among different spermatozoa of the same individual, with no predominance of a particular kind. Dynein arms, the fibrous sheath and the middle piece did not show abnormalities. The flagellar diameter was not modified, which explains why the tails appeared normal under light microscopic examination.

The mean value of non viable spermatozoa in these patients

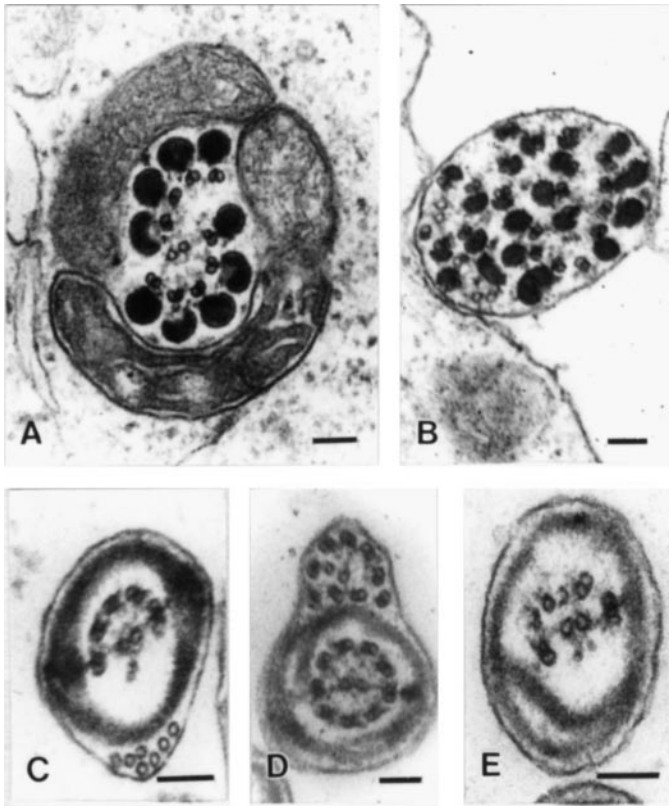

Figure 2. Non-specific flagellar anomalies. There is a heterogeneous variety of microtubular anomalies of the axoneme. **A**: 'fractured' axoneme. **B**: Complete disorganization of microtubular doublets and their associated dense fibres. **C**: Microtubular lack and translocation. **D**: Partial microtubular duplication. **E**: Microtubular disintegration. Flagellar diameter (**C**–**E**) is within the normal range (0.4 to 0.5 μm). Bars represent 0.1 μm.

was 29 ± 17%. The percentages of axonemal abnormalities did not correlate with those of dead spermatozoa (correlation coefficient 0.13). This was confirmed by the similarity between the percentage of abnormal axonemes in patients with values of dead spermatozoa above or below the 40% limit (74 ± 22% and 68 ± 19% respectively). Furthermore, the percentage of axonemal anomalies did not correlate either with sperm concentration (correlation coefficient –0.22), or with total motility, forward progression, rapid linear progression, slow or non linear motility, and non progressive motility (correlation coefficients: 0.28, 0.25, 0.19, 0.22, and 0.21 respectively).

Dysplasia of the fibrous sheath (DFS)

There were 42 patients within this group. Forward progressive motility averaged 1.1 ± 3.1% and rapid linear progression 0.16 ± 0.9%. These values were significantly lower than those corresponding to patients with NSFA ($P < 0.001$). All 42 patients with DFS were adult men with normal sexual development who consulted for primary sterility. Most patients did not suffer from any other andrological conditions. Occasional individual findings included mild hypospadias or varicocele. Approximately 24% of them (10/42) presented respiratory symptoms like rhino-sinusitis, bronchitis and/or bronchiectasis from early childhood. No translocations of thoracic or abdom-

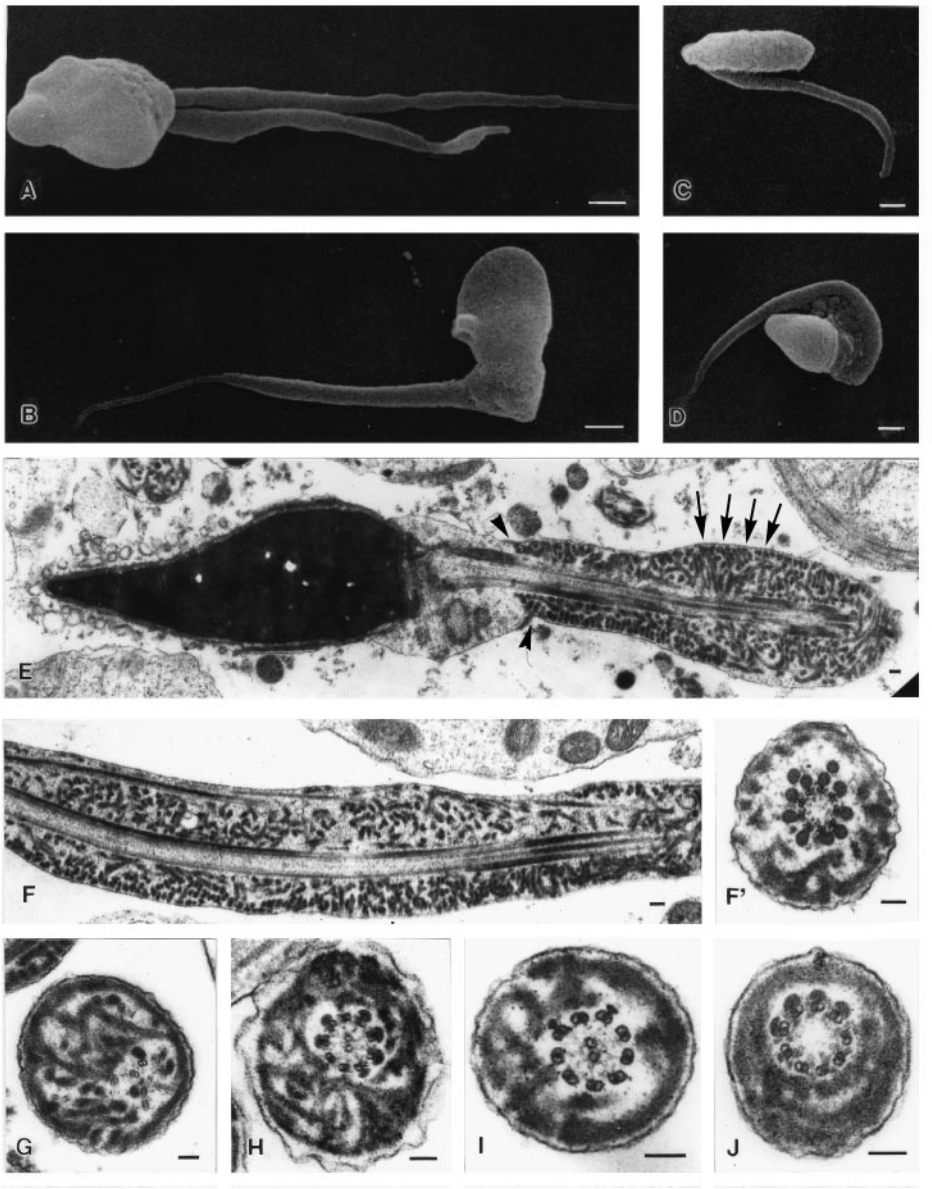

Figure 3. Dysplasia of the fibrous sheath (FS). **A–D:** Scanning electron micrographs depicting short (9–12 μm long; normal is ≅ 60 μm), thick, and irregular flagella. **E**: Lack of mitochondrial sheath with the annulus in a cranial position (opposite arrowheads). Redundancy and disorganization of the fibrous sheath (arrows). **F**: Typical FS changes and lack of central pair. **F′**: FS hyperplasia, lack of central pair, preserved dynein arms and abnormal extension of outer dense fibres 3 and 8 to the principal piece. **G**: Complete axonemal distortion by hyperplastic FS. **H** and **I**: The axoneme is preserved in spite of FS changes. Dynein arms are present in **H** and absent in **I**. **J**: FS changes, lack of central pair and normal dynein arms. Flagellar diameter ranges from 0.5 to 1.3 μm (normal 0.4–0.5). Bars: 1 μm (**A**–**D**) and 0.1 μm (**E**–**J**).

inal organs was reported in any of the patients. There were three pairs of brothers (not twins), and two other patients who had one brother each with sterility (these relatives could not be studied).

Light microscopy of semen smears showed large numbers of spermatozoa with short, rigid, thick and irregular tails, and middle pieces that were frequently missing. These alterations were particularly noticeable under scanning electron microscopy, where the profiles of the tails could be discerned with more resolution (Figure 3A–D). Transmission electron microscopy showed severe axonemal and periaxonemal alterations. In normal spermatozoa the fibrous sheath is a cytoskeletal structure formed by two longitudinal columns that run along the principal piece and insert in microtubular doublets 3 and 8. These columns are regularly joined by transversal semicircular ribs (Figure 1). In patients with DFS the common characteristic was a serious distortion of the fibrous sheath (Figure 3E–J). This consisted of a marked hypertrophy and hyperplasia of random fibrous sheath constituents that formed thick rings or broad meshes without the orderly disposition in longitudinal columns and transversal ribs. Missing axonemal central pairs and/or dynein arms in the microtubular doublets were also apparent in some, but not all, cases. Outer dense fibres 3 and 8, which are normally present only at the middle piece level, extended to the principal piece. The annulus failed to migrate caudally and remained in a cranial position, just

Table I. Clinical forms of dysplasia of the fibrous sheath

	n	FP[a]	RLP[b]	Prevalence	Viability	Respiratory disease
Complete	28	0.2 ± 0.6	0	100%	39 ± 24	9
Incomplete	14	3.0 ± 4.8	0.6 ± 1.4	70–80%	33 ± 18	1

[a]FP = forward progression (%).
[b]RLP = rapid linear progression (%).
Values are means ± SD.

beneath the connecting piece. As a consequence of this a middle piece was not formed and mitochondria were poorly assembled or absent. In some patients, the 9 + 2 axonemal structure was completely distorted while in some others it was preserved in the centre of dense rings of hyperplastic fibrous sheaths. The fibrous sheath alterations were present in all affected spermatozoa, but the frequency of the other alterations was variable. Dynein arms were present in some cases (Figure 3F, H and J) and absent in all patients with respiratory symptoms (Figure 3I). Central pairs were missing in about half of the cases (Figures 3F and J). Lack of or poorly organized middle pieces and abnormal extension of the outer dense fibres 3 and 8 to the principal piece were present in many (but not all) patients (Figure 3E and F). It was difficult to determine the incidence of these alterations since the distortions imposed by the anomalous fibrous sheaths frequently prevented a clear visualization of other subcellular components.

According to the prevalence of tail abnormalities two groups of patients could be discerned. In some of them all spermatozoa were affected, while in some others the number of abnormal tails was about 70–80% with 20–30% in the normal configuration. These two groups have distinct characteristics and correspond to the complete and incomplete forms of the present syndrome (Table I). The complete form was more frequent than the incomplete form (66 and 33% respectively). Most patients with respiratory symptoms belonged to the complete form (9/10).

Fertility studies

Table II shows fertility outcome during a follow-up period of 2–6 years, and the extent of flagellar pathology and sperm motility at the time of diagnosis and at the end of the follow-up period. Among the 54 men with NSFA there was a similar number of patients with unexplained infertility (26) or suffering from different kinds of andrological conditions (28). These last patients underwent surgery (21 cases for varicocele), hormonal treatments to stimulate spermatogenesis (gonadotrophins, clomiphene or tamoxifen in four cases of testicular pathology, including cryptorchidism and orchitis), antibiotics according to bacterial susceptibility (one patient with infection of the seminal pathway) or corticosteroid therapy (two patients with immune factor). In cases of unexplained infertility empirical treatments included hormonotherapy (mostly gonadotrophins) or stimulants of sperm motility (pentoxifylline). At the end of the follow-up period it was found that 18/54 achieved conceptions either spontaneously or as a result of IVF, followed by 14 pregnancies, 12 live births and two abortions. In this group motility significantly increased during follow-up ($P < 0.01$) and these are referred to as responders to treatment (RT, 33%). In the remaining 36 patients there were no significant changes in motility during the study. There were no fertilizations or pregnancies within this group of non-responders to treatment (NRT, 66%). Furthermore, motility values at the time of fertilization/pregnancy or when last recorded, were higher in responders (15.1%) than in non responders (7.4%) to treatment ($P < 0.01$). Within each of these two subgroups of NSFA there was an equal number of patients with unexplained infertility or infertility secondary to different andrological conditions. The percentage of abnormal flagella, ranging around 70%, was no different in RT and NRT.

Table II. Fertility outcome, flagellar pathology and sperm motility

	NSFA-RT	NSFA-NRT	DFS
Number of patients	18	36	34
Fertilizations[a]	18	0	0
Pregnancies	14	0	0
Abortions	2	0	0
Live births	12	0	0
RLP-I (%)	5.2 ± 7.4[b]	2.3 ± 2.9	0.2 ± 0.9
RLP-II (%)	15.1 ± 8.8[b,c]	7.4 ± 7.0[c]	0.2 ± 0.7
Flagellar pathology (%)	71.7 ± 15.1	69.6 ± 19.4	90.0 ± 14.5

NSFA-RT = patients with non-specific flagellar anomaly (NSFA) responders to treatment.
NSFA-NRT = patients with NSFA non-responders to treatment.
DFS = dysplasia of fibrous sheath.
RLP = rapid linear progression at the time of ultrastructural examination (I) and at fertilization-pregnancy, or otherwise last recorded motility (II).
[a]Spontaneous or after in-vitro fertilization.
[b,c]Values with the same superscript were significantly different ($P < 0.01$).
Values are means ± SD.

In patients with DFS, the values of rapid linear progression were very low and the percentage of abnormal flagella was very high (90 ± 14.5%, average of all patients including complete and incomplete forms). Various patients were followed with frequent semen analyses for long periods and consistently showed extremely low motility values, always around 0% in the complete form. In one of the patients treatment with testosterone was undertaken to achieve azoospermia, hoping that the 'rebound' effect could reverse at least in part the flagellar pathology due to the possible development of normal clones of germinal cells. However, there were no changes in the characteristics of spermatozoa during testosterone suppression and recovery. We have not observed modifications of the seminal parameters (morphology, motility) during

the follow-up of any of the 42 patients with DFS studied to date. There were no spontaneous pregnancies or successful fertilizations by IVF. In one of the patients with DFS a successful ICSI was followed by a normal pregnancy and a live birth (Brugo Olmedo *et al.*, 1997), but in the same couple, a second fertilization by ICSI did not result in pregnancy. In five other patients successful fertilizations by ICSI were obtained. However, only one singleton clinical ongoing pregnancy was achieved.

Discussion

Asthenozoospermia is a very frequent cause of male infertility. It is also poorly understood, and frequently the reasons for motility disorders remain obscure to the andrologist. Many andrological conditions like varicocele, various testicular pathologies, infections of the male genital tract, antisperm antibodies etc. can lead to diminished sperm motility. However, the mechanism by which these pathologies interfere with sperm movement has not been clearly identified. Even though electron microscopy of spermatozoa in patients with sperm immotility led to the understanding of the immotile cilia syndrome (Azfelius *et al.*, 1975), ultrastructural examination of spermatozoa is not frequently used in the diagnosis of asthenozoospermia. In the present report we show that an accurate ultrastructural diagnosis can identify various sperm abnormalities that can cause asthenozoospermia and lead to a deeper understanding of various conditions of clinical relevance that differ in their response to treatments and fertility potential.

NSFA are the more frequent cause of severe asthenozoospermia. We have previously reported that in 70% of these patients there is a marked increase in the percentage of NSFA (Chemes, 1991). The present findings on the lack of correlation between NSFA and dead spermatozoa indicate that these anomalies are not secondary to necrozoospermia, as is also ascertained by the absence of ultrastructural signs of cell degeneration that may indicate compromised sperm viability. The absence of negative correlation between NSFA and sperm motility indicates that these anomalies do not necessarily lead to immotility, and are compatible with abnormal forms of sperm movement.

NSFA probably arise during spermiogenesis as a consequence of an abnormal testicular function caused by varicocele or other pathologies, or during sperm transit through the male genital tract in cases of infections or presence of antisperm antibodies. In all these conditions they probably represent secondary effects on the structure and function of spermatozoa. These conclusions are supported by the decrease in the percentages of NSFA that we have observed after varicocelectomies or antibiotic treatment of infections (data not shown).

The two subgroups of NSFA, RT and NRT, reflect differences in their response to treatments, as shown by the percentage of rapid linear progressive spermatozoa reached at the end of the follow-up period, that was significantly higher in responder patients, who also achieved fertilizations and pregnancies. This interpretation is supported by the fact that while both responders and non responders had similar percentages of flagellar anomalies and motility values at the time of ultrastructural diagnosis, the percentage of rapid linear progressive spermatozoa reached significantly higher values in the patients of the NSFA-RT group at the end of the follow-up period. This improvement in motility was possibly related to the non-systematic or secondary nature of the flagellar pathology and indicates that NSFA represent a potentially reversible form of flagellar pathology. In accordance with this, Baccetti *et al.* (1997) have recently reported that various alterations in different axonemal components significantly improve after follicle-stimulating hormone (FSH) therapy.

DFS is an altogether different condition. The distortions of the fibrous sheath are present in all or most spermatozoa, the ultrastructural phenotype is typical and consistent, and its prevalence is not modified during the clinical course of the disease or in response to treatments. Very severe asthenozoospermia or total sperm immotility is the rule. This condition has been known in the literature for a number of years as the 'stump tail syndrome' in infertile men and various mammals (reviewed by Barth and Oko, 1989). However, this term, which refers to the peculiar configuration of the tail, is a misnomer that either fails to provide an insight into the underlying nature of these tail abnormalities or encompasses a heterogeneous array of sperm defects having a short tail as the common feature. We therefore propose to use the more comprehensive term, dysplasia of the fibrous sheath, which identifies the main defect involved and refers to it as a developmental anomaly.

This condition affects various cytoskeletal constituents of the sperm tail which appears short, thick and irregular. We consider that fibrous sheath modifications are the key component of this pathology since they are present in all affected spermatozoa. Other abnormalities, such as lack of the central pair of microtubules, missing dynein arms, abnormal extension of the outer dense fibres 3 and 8 and unassembled middle pieces, appear with different frequencies. Other authors have considered that the fibrous sheath alterations are secondary to the failure of proper axonemal assembly (Escalier and David, 1984; Phillips *et al.*, 1993). However, this does not seem to be the case in dysplasia of the fibrous sheath since the fibrous sheath anomalies may coexist with well formed axonemes as demonstrated by the material described here. As a consequence of the lack of caudal migration of the annulus, the principal piece extends to the connecting piece and mitochondria do not assemble around the axoneme.

Studies on testicular biopsies have demonstrated that these defects arise during late spermiogenesis as a failure of the fibrous sheath to organize properly (Ross *et al.*, 1973; Barthelemy *et al.*, 1990). We also have presented evidence that the fibrous sheath defect is of testicular origin since it is present in immature spermatids found in semen. Holstein and Roosen Runge (1981) have shown that in immature spermatids the ribs of the fibrous sheath are normally arranged in two layers, and that the characteristic pattern in a single row only appears in mature spermatids. The multilayered disposition observed in patients with DFS may be seen as a sort of immaturity of fibrous sheath development.

The spectrum of ultrastructural abnormalities described here for dysplasia of the fibrous sheath has been also observed in the two previous large series published by Bisson *et al.* (1979, 40 cases) and Escalier and David (1984, 27 cases), where

familial and ethnic incidence, and associated respiratory symptoms have been recorded. Baccetti *et al.* (1993) have reported a series of eight patients and proposed the existence of two forms: 'short biflagellated tails' and 'stump uniflagellated tails'. However, their published data do not seem to support these conclusions. We have not found such a distinction in our present series of 42 patients, where biflagellated forms sometimes existed as a minor percentage, never as the predominant anomaly.

We have previously reported lack of dynein arms in bronchial cilia from two patients with DFS and chronic respiratory disease, and considered that they represent a mosaic variant of the immotile cilia syndrome in which the fibrous sheath alterations are associated with dynein deficiency in respiratory cilia and sperm flagella (Chemes *et al.*, 1990). The 10 patients with this combination described here and those previously reported (Escalier and David, 1984) indicate that DFS and the immotile cilia syndrome are related entities that can either exist in isolated form or association with each other. The classic form of the immotile cilia syndrome with isolated dynein deficiency and normal fibrous sheaths is very rare in our population of infertile men. We have detected only four cases in more than 600 patients studied, while in the same population, DFS without respiratory disease amounted to 32 patients. The fact that these two entities are sometimes associated suggest that the gene(s) responsible for their appearance are linked.

All the patients with DFS presented a homogeneous evolution in relation to fertility. Regardless of various treatments, seminal and clinical characteristics remained stable for extended periods of time. Medical treatments or classical IVF methods consistently failed to achieve fertilizations or pregnancies. These features and the familial incidence reported by us and other authors (Bisson *et al.*, 1979; Chemes *et al.*, 1987b; Baccetti *et al.*, 1993) configure a phenotype that suggests a genetic origin of the syndrome. There have been recent reports on the localization of the AKAP82 gene that encodes for a major fibrous sheath protein to the X chromosome of men (Moss *et al.*, 1997). Whether DFS is a consequence of the mutation/deletion of gene(s) coding for structural proteins of the flagellum, or depends on the failure of a regulatory system controlling proper flagellar assembly, will surely be a matter of investigation in the near future. To date there are no reports on gene anomalies in patients with DFS.

The present results indicate that in a large population of severely asthenozoospermic patients, the ultrastructural study of spermatozoa has a prognostic value identifying two different kinds of flagellar alterations with different fertility potential. In fact, while 33% of the patients with NSFA were able to achieve fertilizations/pregnancies after medical treatments or classical techniques of assisted fertilization, there are no such reports in any of the 42 patients with the DFS. These last patients are good candidates for microfertilization techniques as has been demonstrated by recent reports of live births (Stalf *et al.*, 1995; Brugo Olmedo *et al.*, 1997), and by the observations reported here that in five other patients there were successful microfertilizations, followed in one of the couples by an ongoing clinical pregnancy.

Acknowledgements

The technical help of Yolanda Castaño and Oscar Rodriguez is acknowledged. This work has been supported by grants from the National Research Council of Argentina.

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Received on January 26, 1998; accepted on June 25, 1998