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·*Original Article*·

Localization of AKAP4 and tubulin proteins in sperm with reduced motility

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Abstract

Aim: To perform screening, related to A-kinase anchoring proteins 4 (AKAP4) and tubulin proteins, in spermatozoa with absent or severely reduced motility in order to detect the status of the fibrous sheath and the axonemal structure. **Methods:** An immunocytochemical study of tubulin, used as a positive control, and AKAP4 was carried out to detect the presence and the distribution of these proteins in different sperm samples. The morphological characteristics of sperm were studied by transmission electron microscope (TEM) and the results were elaborated using a formula reported in previous studies. PCR was carried out on DNA extracted from peripheral blood lymphocytes to analyse partial sequences of the Akap4 and Akap3 genes. **Results:** Immunolabelling of tubulin and AKAP4 showed different patterns, which led us to divide the patients into groups. In group I, the absence of AKAP4 and tubulin was revealed, although these patients did not show alterations in the Akap4/Akap3 binding site. TEM evaluation highlighted that a high presence of necrosis was associated with total sperm immotility. In group II, a regular AKAP4 and tubulin signal was present, although motility was reduced and TEM analysis revealed the presence of immaturity. In group III, in which a weak AKAP4 label associated with normal tubulin staining and reduced motility was observed, a severe disorganization of the fibrous sheath was highlighted by TEM. **Conclusion:** While the role of AKAP4 in sperm motility is unclear, absent or weak AKAP4-labelling seems to be associated with absent or weak sperm motility. *(Asian J Androl 2007 Sep; 9: 641–649)*

Keywords: AKAP4; immunocytochemistry; motility, sperm; transmission electron microscope

1 Introduction

Male infertility is a significant problem in humans and may be caused by different pathologies, such as anatomical problems, infections, hormonal imbalances, chromosomal alterations and gene anomalies. However, 30% of infertile men are affected by idiopathic oligoasthenoteratozoospermia and the cause of infertility is still unknown as reported by Cavallini [1]. The analysis of sperm motility plays a central role in the evaluation of male fertility because it is known that a high percentage of poorly motile or immotile sperm will not be able to fertilize. The clinical relevance of sperm motility is evident, but the

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molecular mechanisms involved in this process have not been fully understood yet.

Flagellar sperm structure has been extensively described in the published literature, and correct organization of the axonemal pattern and of periaxonemal structures is pivotal for ensuring normal motility. During the past few years, attention has been paid on the fibrous sheath, a cytoskeletal structure surrounding the axoneme and outer dense fibers that defines the extent of the region of the principal piece of sperm flagellum. It consists of two longitudinal columns connected by closely arrayed semicircular ribs that assemble from the distal to the proximal part of the tail throughout the spermiogenetic process.

It is generally accepted that the fibrous sheath plays a role as a mechanical support of sperm flagellum. Fibrous sheath is able to modulate flagellar bending and to define the shape of flagellar beats as described by Fawcett [2].

Recently, extensive studies have been carried out to identify proteins that constitute the fibrous sheath and that are in some way actively involved in sperm motility.

Eddy *et al*. [3] found that in the human fibrous sheath, A-kinase anchoring proteins 3 and 4 (AKAP3, AKAP4) are the most abundant structural proteins, anchoring cyclic-AMP (cAMP)-dependent protein kinase A to the fibrous sheath through the regulatory subunit of kinase. cAMP-dependent phosphorylation of flagellar proteins is involved in the beginning and maintenance of sperm motility. The second messenger cAMP mediates its intracellular effects in spermatozoa through cAMP-dependent kinase (PKA). The intracellular organization of PKA is controlled by its association with AKAPs.

In particular, AKAP3 is synthesized in round spermatids, incorporated into the fibrous sheath concurrently with the formation of rib precursors and is reported to be involved in organizing the basic structure of the fibrous sheath [4]. AKAP4 is synthesized and incorporated into a nascent fibrous sheath late in spermatid development and it plays a major role in completing fibrous sheath assembly as reported by Brown *et al.* [4]. A targeted disruption of the Akap4 gene causing defects in mice sperm flagellum and motility has also been demonstrated by Miki *et al.* [5]. Recently, Baccetti *et al.* [6] used transmission electron microscope (TEM) to determine the genetic defect "dysplasia of the fibrous sheath" (DFS) in sperm from a group of infertile men. In these cases, immunolabelling of tubulin confirmed the presence of typical short and thick tails whereas AKAP4 protein staining showed a weak signal, revealing a disorganized and incompletely assembled fibrous sheath. Moreover, polymerase chain reaction (PCR) for detecting the presence of a partial sequence of *Akap4/Akap3* binding regions produced positive results according to Turner *et al.* [7].

Based on our data showing alteration of the fibrous sheath in immotile sperm containing genetic defects, the present study was undertaken to determine if similar alterations were present in sperm with reduced motility but without genetic defects.

As previously described, AKAP4 labeling was performed to detect the typical spatial organization of fibrous sheath components and tubulin staining was carried out in order to check axomene assembly. Morphological characteristics were studied by TEM, a valuable tool for a more detailed evaluation of sperm ultrastructure, and the results were elaborated using the formula by Baccetti *et al.* [8]. TEM analysis was performed to try to explain the structural causes of absent or reduced motility in this group of patients. PCR was carried out on DNA extracted from peripheral blood lymphocytes to analyse partial sequences of the Akap4 and Akap3 genes.

2 Materials and methods

2.1 Patients

Semen samples were obtained from 16 patients (aged 24 to 33 years old) with idiopathic infertility referred to the Regional Referral Center for Male Infertility for semen analysis after 3 years of sexual intercourse without conception. The lymphocyte karyotypes were normal in all cases. Written consent was obtained from all patients donating samples, both infertile men and controls.

2.2 Semen analysis

2.2.1 Light and electron microscopy

Semen samples were collected by masturbation after 3–4 days of sexual abstinence and examined after liquefaction for 30 min at 37ºC. Any delay between ejaculation and sample processing was recorded. Volume, pH, concentration and motility were evaluated according to WHO guidelines [9]. Supravital eosin staining was used for evaluating sperm viability.

For TEM, semen was fixed in cold Karnovsky fixative and maintained at 4ºC for 2 h. Fixed semen was washed in 0.1 mol/L cacodylate buffer (pH 7.2) for 12 h,

postfixed in 1% buffered osmium tetroxide for 1 h at 4ºC and dehydrated and embedded in Epon Araldite. Ultra-thin sections were cut with a Supernova ultramicrotome (Reickert Jung, Vienna, Austria), mounted on copper grids, stained with uranyl acetate and lead citrate and then observed and photographed with a Philips CM10 transmission electron microscope (TEM; Philips Scientifics, Eindhoven, The Netherlands). Three hundred ultra-thin sperm sections (approximately 50% heads and 50% tails) were analyzed for each patient. Major submicroscopic characteristics were recorded, applying the same evaluation criteria, by highly trained examiners who were blind to the experiment. TEM data were elaborated using the mathematical formula by Baccetti *et al*. [8], based on Bayesan's technique. This formula considers 16 selected submicroscopic characteristics of sperm organelles to define sperm function and calculates the number of spermatozoa free of structural defects ("healthy") and the percentages of three main phenotypic sperm pathologies: immaturity, necrosis and apoptosis as demonstrated by Baccetti *et al.* [10]. Moreover Baccetti *et al*. [8] observed that the lowest number of spermatozoa free of defects and assuring normal fertility was slightly over two million.

The controls were five men with normal karyotype who had fathered a child during the previous 1 to 2 years.

2.2.2 Immunofluorescence

Semen samples were washed twice in phosphate buffered saline (PBS), smeared on glass slides, air dried, rinsed in PBS and fixed for 15 min in methanol at –20ºC. Slides were then treated with blocking solution (PBS, 1% bovine serum albumin, 5% normal goat serum) for 20 min at room temperature (RT) and incubated overnight at 4ºC with mouse monoclonal anti-tubulin (Sigma Chemical, St. Louis, MO, USA) and mouse monoclonal anti-AKAP82 (BD Biosciences, Erembodegem, Belgium) specific for human AKAP4 protein, diluted at 1:100 and 1:50 respectively in PBS, 0.1% BSA, 1% NGS. After three washes in PBS, the samples were treated with goat anti-mouse IgG-Texas Red conjugated antibody (Southern Biotechnology, Birmingham, AL, USA). Finally, the samples were washed three times in PBS and mounted with Vectashield (Vector Labs, Burlingame, CA, USA). Incubation in primary antibodies was omitted in control samples. Observations and photographs were made with a Leitz Aristoplan light microscope (Leica, Wetzlar, Germany) equipped with a fluorescence apparatus. A total of 200 spermatozoa from each sample were counted and scored as either labelled or not labelled with the respective antibody. For the AKAP4 experiment, only those spermatozoa stained throughout the length of the principal piece were counted. The same five samples from healthy men of proven fertility were examined and used as controls.

2.3 PCR analysis

DNA was extracted from peripheral blood lymphocytes using the QIAamp DNA Blood Kit (QIAGEN, Valencia, CA, USA).

PCR products corresponding to a region of hAkap4 involved in binding to *Akap3* (site 1) and to a region of *Akap3* involved in binding to *hAkap4* (site 2) were amplified according to Turner *et al.* [7]. Oligonucleotide primers flanking the respective binding sites were used:

site 1: sense primer 5'-TCAGTGCCCTTATAGGTGAG-3', antisense primer 5'-GCAGAGCTTCATCACAGATTC-3';

site 2: sense primer 5'-TTGAGGAATCTCCACAGCG-3'; antisense primer 5'-CCAACGGTCTTTCACACAACTTC-3').

Control DNA was extracted from the blood of five fertile men.

3 Results

Sixteen semen samples from men with idiopathic infertility were examined by light and electron microscopy to determine sperm concentration, motility and morphology. In all samples, immunocytochemistry for the localization of AKAP4 and tubulin was performed.

Among the group of infertile patients, sperm concentration was normal in eight out of 16 patients according to WHO guidelines [9]. Rapid (a) and slow (b) progressive motility was absent or severely reduced in all analysed samples. These parameters are reported in Tables 1, 2 and 3.

Immunolabeling of AKAP4 protein and tubulin, allowed us to separate the patients into three groups (Tables 1–3):

Group I: patients 1–5 in whom the label was negative for both antibodies and motility was totally absent (Table 1);

Group II: patients 6–10 in whom the AKAP4 signal (Figure 1A, 1B) and the tubulin label (Figure 1C, 1D) were 100%, except in patient 9 in whom AKAP4 label-

Table 1. Sperm parameters, immunocytochemical screening for A-kinase anchoring proteins 4 (AKAP4) and tubulin proteins and transmission electron microscope (TEM) analysis of spermatozoa from five infertile patients. These patients showed absence of motility and negative immunolabelling for both proteins. *Rapid (a) and slow (b) progressive motility.

Cases	Number of sperm/mL ($\times 10^6$)	Motility (%) (a + b)*	AKAP4 labelling (%)	Tubulin labelling (%)	Number of healthy sperm	Apoptosis (%)	Necrosis (%)	Immaturity (%)
1	8.0	0	0	0	0	15.560	99.790	56.780
2	57.8	0	0	0	57	11.880	91.970	58.440
3	15.0	0	0	0	3 221	16.382	72.049	58.359
4	69.0	0	0	0	0	15.779	96.186	57.297
5	30.8	0	0	0	0	0.692	85.228	25.575
Mean	36.1	0	0	0	655.6	12.059	89.045	51.290
SD	26.5	0	0	0	1434.3	6.596	10.936	14.392

Table 2. Sperm parameters, immunocytochemical screening for A-kinase anchoring proteins 4 (AKAP4) and tubulin proteins and transmission electron microscope (TEM) analysis of spermatozoa from five infertile patients. These patients showed reduced motility and positive immunolabelling for both proteins. *Rapid (a) and slow (b) progressive motility.

Cases	Number of sperm/mL ($\times 10^6$)	Motility (%) (a + b)*	AKAP4 labelling (%)	Tubulin labelling (%)	Number of healthy sperm	Apoptosis (%)	Necrosis (%)	Immaturity (%)
6	12.5	2+15 (17)	100	100	1 498	10.860	25.030	78.410
7	4.5	1+7 (8)	100	100	28	10.163	30.553	81.141
8	123.8	7+9 (16)	100	100	770 793	5.052	26.110	71.130
9	41.0	6+10 (16)	95	100	1 078 709	4.620	31.550	78.170
10	10.0	3+8 (11)	100	100	1 059	9.150	30.090	93.320
Mean	38.4	13.6	99.0	100	370 417.4	7.969	28.667	80.434
SD	49.8	3.91	2.2	0	517 613.1	2.927	2.901	8.098

Table 3. Sperm parameters, immunocytochemical screening for A-kinase anchoring proteins 4 (AKAP4) and tubulin proteins and transmission electron microscope (TEM) analysis of spermatozoa from six infertile patients. These patients showed strongly reduced motility and positive immunolabelling for tubulin, but they were negative for AKAP4. *Rapid (a) and slow (b) progressive motility.

Cases	Number of sperm/mL ($\times 10^6$)	Motility (%) (a + b)*	AKAP4 labelling (%)	Tubulin labelling (%)	Number of healthy sperm	Apoptosis (%)	Necrosis (%)	Immaturity (%)
11	10.3	1 + 2 (3)	10	90	0	6.820	32.074	74.498
12	6.0	1 + 2 (3)	20	80	1	10.862	34.596	71.343
13	4.9	3 + 5 (8)	10	100	193	4.197	25.317	86.584
14	102.5	4 + 6 (10)	20	100	94 938	4.503	24.713	63.722
15	40.5	3 + 5 (8)	20	100	345 987	5.786	28.123	57.988
16	120.0	0 + 4 (4)	20	100	15 069	0.746	25.870	84.210
Mean	47.4	6	16.7	95.8	76031.3	5.486	28.449	73.058
SD	51.5	3	5.2	8.0	137304.6	3.342	4.036	11.194

Table 4. Sperm parameters, immunocytochemical screening for A-kinase anchoring proteins 4 (AKAP4) and tubulin proteins and transmission electron microscope (TEM) analysis of spermatozoa from five men of proven fertility. *Rapid (a) and slow (b) progressive motility.

Cases	Number of sperm/mL ($\times 10^6$)	Motility (%) (a + b)*	AKAP4 labelling (%)	Tubulin labelling (%)	Number of healthy sperm	Apoptosis (%)	Necrosis (%)	Immaturity (%)
A	204.7	46	95	95	70 695 966	0.854	20.134	44.110
B	85.0	49	95	100	3 038 128	4.340	21.580	47.290
C	93.0	50	100	100	53 710 141	4.125	18.827	39.876
D	183.7	70	100	100	6 425 404	2.985	19.765	40.007
E	89.0	76	100	100	45 891 075	0.933	19.975	37.777
Mean	131.1	58.2	98.0	99.0	35 952 143	2.647	20.056	41.812
SD	58.2	13.8	2.7	2.2	29 901 600	0.991	0.991	3.826

ling was 95%. Sperm motility was reduced in all patients (Table 2);

Group III: patients 11–16 in whom the AKAP4 signal (Figure 1E, 1F) was weaker (at least 20%), the tubulin label ranged from 80% to 100% (Figure 1H) and motility was strongly reduced.

The five patients with proven fertility, who were used as controls (Table 4, A–E), showed normal immunofluorescent staining for tubulin (Figure 1H) and AKAP4 (Figure 1G) in 95%–100% of tails (Table 4).

All sperm samples from infertile and fertile men were analysed by TEM and the data obtained were processed using the mathematical formula by Baccetti *et al.* [8].

Mathematically elaborated TEM analysis (Tables 1–4) confirmed that 16 examined patients were infertile (Tables 1–3), showing a number of healthy sperm of < 2 000 000. Necrosis (Figure 2A) was extremely high (Table 1) in group I (89.045± 10.936) versus the other groups (Tables 2 and 3) and controls (Table 4). This sperm pathology is characterised by absent or reacted acrosomes, misshapen nuclei with disrupted chromatin, swollen and disassembled mitochondria and altered axonemal and periaxonemal structures. In these cases, eosin Y staining confirmed the presence of dead sperm at > 80%.

Group I also showed a high percentage of sperm with apoptotic characteristics (12.059 ± 6.596) such as misshapen acrosomes, nuclei with marginated chromatin and swollen mitochondria irregularly organized into large cytoplasmic residues with translucent vacuoles (Figure 2B).

Group II showed a mean of percentage of sperm necrosis (Table 2) very similar to that of controls (Table 4), which was considered normal. However, a very high presence of apoptosis and immaturity (Figure 2C) compared to controls (Table 4) was observed. Immature spermatozoa generally showed altered acrosomes and round or elliptical nuclei with uncondensed chromatin. In particular, a high percentage of spermatozoa with large cytoplasmic residues, often embedding coiled axonemes (Figure 2C, 2D) was highlighted although the axonemal and periaxonemal structures, including the fibrous sheath, were generally normal (Figure 2D), as also revealed by immunocytochemical analyses.

Group III showed a mean percentage of sperm pathologies (Table 3) similar to the previous group (Table 2). Moreover, despite reduced motility, TEM analysis showed "9 + 2" pattern axonemes with structural defects, particularly involving the fibrous sheath, that appeared poor and badly assembled (Figure 2E).

PCR products corresponding to a region of hAkap4 involved in binding to Akap3 (site 1) and to a region of Akap3 involved in binding to hAkap4 (site 2) were present in all examined patients and controls.

4 Discussion

Although sperm motility is one of the most important predictors of fertilizing ability, the mechanisms underlying motility abnormalities are still poorly understood. Recent research performed by Li *et al.* [11] has furnished new clues regarding the key molecular mediators of sperm motility. However, it is well known that sperm motility is regulated by the cAMP-dependent protein ki-

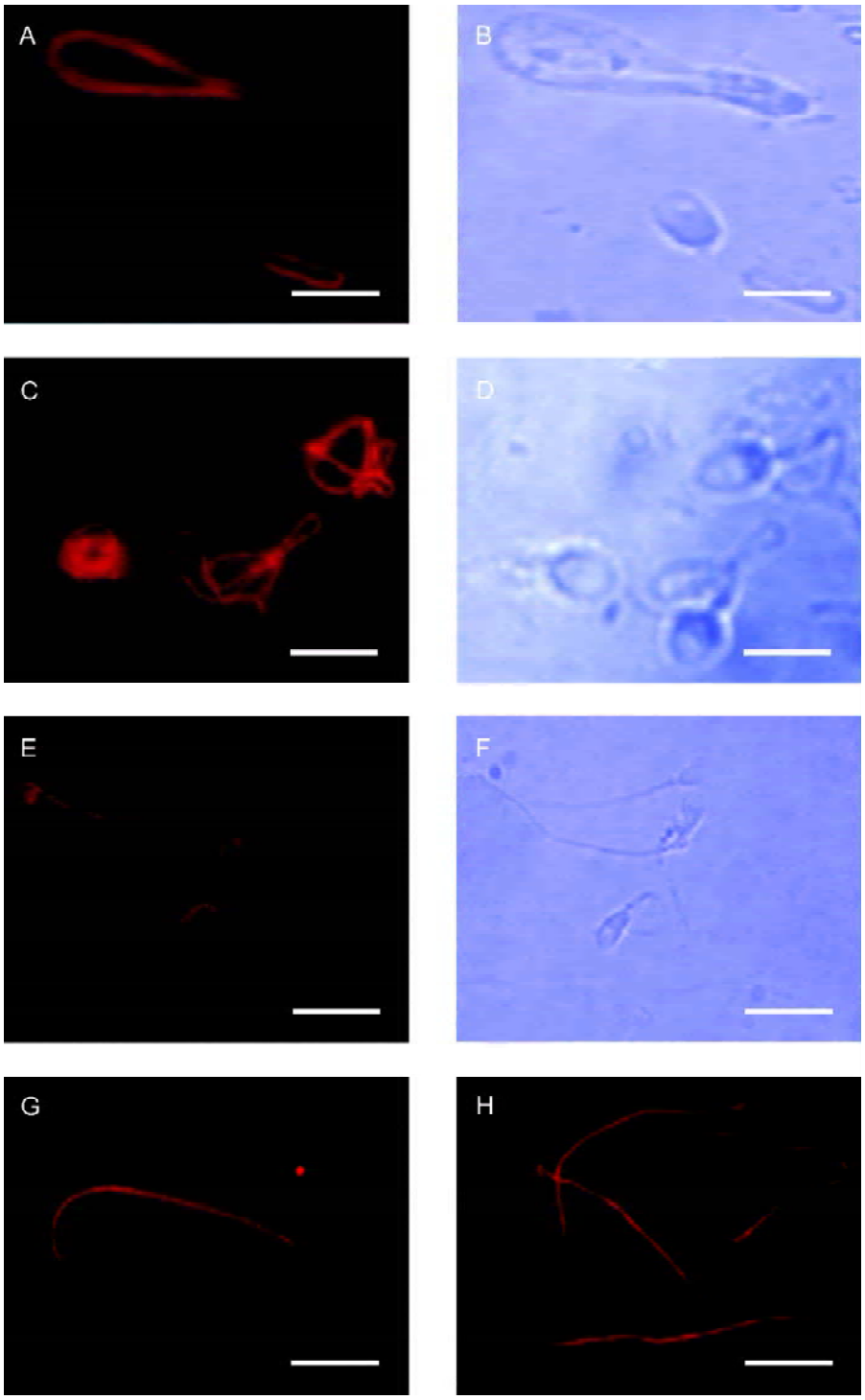

Figure 1. Ultraviolet and light micrographs of ejaculated spermatozoa. (A), (B): Staining with monoclonal anti A-kinase anchoring proteins 4 (AKAP4) antibody highlighted the presence of coiled tails in sperm from patients 6–10, also confirmed by the anti-tubulin label (C, D). (E, F): Immunocytochemical labelling of sperm from patients 11–16 performed with AKAP4 monoclonal antibody highlighted a weak signal. Normal staining of AKAP-4 (G) and tubulin (H) was also shown. (A), (B): scale bar = 22 μm; (C), (D): scale bar = 21 μm; (E), (F): scale bar = 34 μm; (G): scale bar = 30 μm; (H): scale bar = 41 μm.

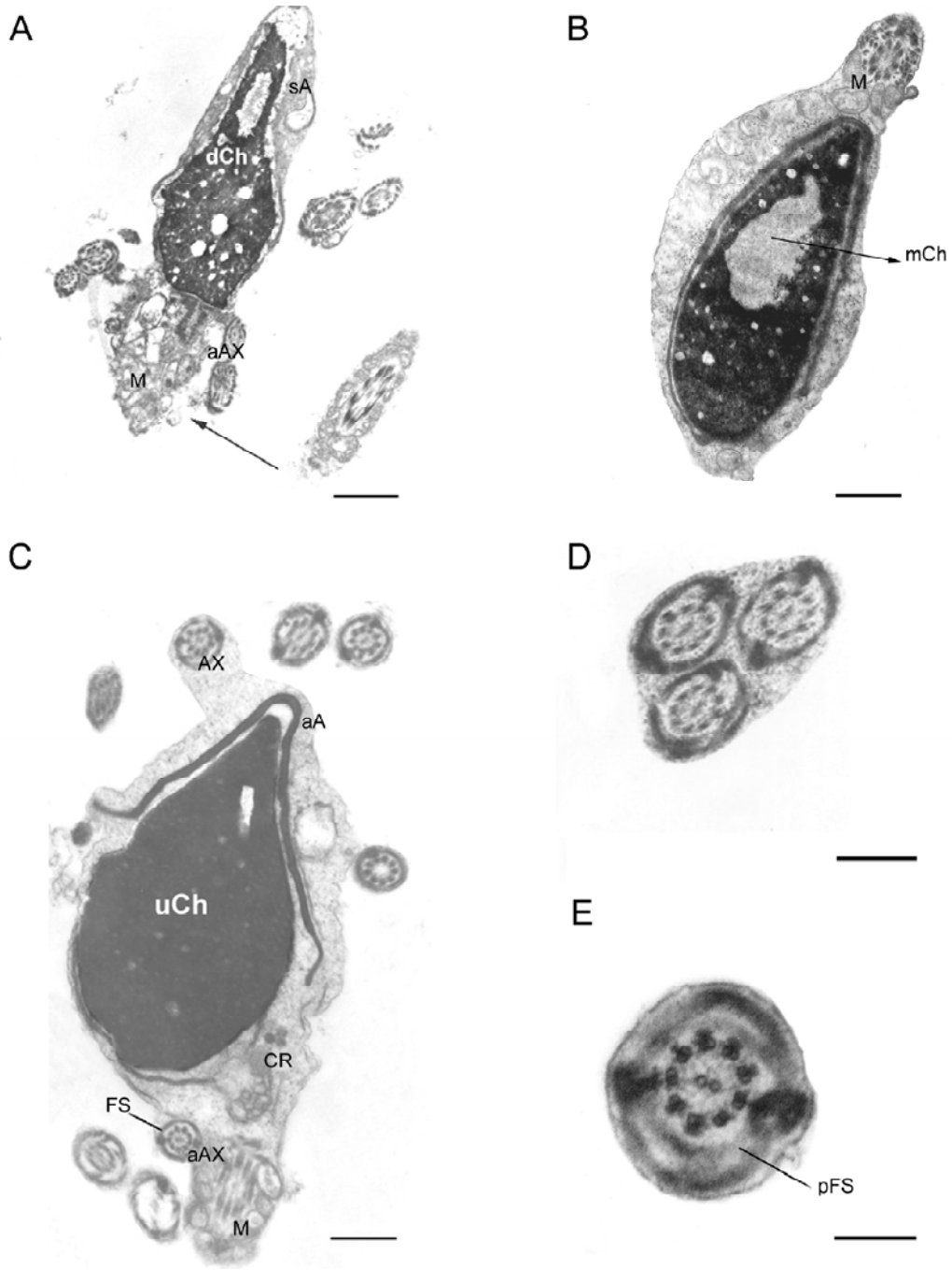

Figure 2. Transmission electron microscope (TEM) micrographs of longitudinal and cross sections of sperm. (A): A necrotic spermatozoon characterized by acrosome with sparse content (sA) and a misshapen nucleus with disrupted chromatin (dCh). The axoneme is rolled up and altered (aAX) and mitochondria (M) are swollen and dispersed. The plasma membrane is broken (arrow). Scale bar = 1 μm. (B): An apoptotic sperm characterized by misshapen nucleus with marginated (mCh) chromatin. A cytoplasmic residue embedding swollen mitochondria (M) and a coiled axoneme is present. Scale bar = 0.74 μm. (C): An immature sperm characterized by an irregular nucleus with uncondensed chromatin (uCh). The altered acrosome (aA) appears far away from the nucleus. Cytoplasmic residue (CR) embeds swollen mitochondria (M) and coiled axoneme (aAX), showing a regular "9 + 2" pattern and an almost assembled fibrous sheath (FS). Scale bar = 0.7 μm. (D): A rolled up axoneme with a normal pattern. Scale bar = 2.5 μm. (E): A cross section of the principal piece with normal pattern and poor fibrous sheath (pFS). Scale bar = 0.17 μm.

nase (protein kinase A)-mediated phosphorylation of groups of flagellar proteins. In mouse, human and bull spermatozoa, two major fibrous sheath proteins, AKAP4 (also called AKAP82 or fibrous sheath component 1) and its precursor proAKAP4, have been identified as members of the A-kinase anchor protein (AKAP) by Carrera *et al.* [12, 13]. The hypothesis is that, by anchoring the activity of PKA in the fibrous sheath, AKAPs play central roles in the regulation of normal sperm motility. Turner *et al.* [14] did not find evidence of an association between the degree of processing of pro-hAKAP4 and increases or decreases in motility in spermatozoa from normal men.

Recently, Brown *et al.* [4] reported that AKAP4 anchors AKAP3 and two novel spermatogenetic cell specific proteins, Fibrous sheath interacting proteins 1 and 2 (FSIP1; FSIP2).

Miki *et al.* [5] demonstrated that targeted disruption of the Akap4 gene causes the absence of sperm motility together with a total lack of fibrous sheath on the principal piece of mature mice sperm.

Baccetti *et al.* [15] described a rare sperm tail defect characterized by absence of the fibrous sheath in humans. AKAP4 labelling was present at the testicular level in cytoplasmic residues and residual bodies, yet it was totally absent in ejaculate spermatozoa. Moreover, in a case of disorganized and incompletely assembled fibrous sheath, such as fibrous sheath sperm, Baccetti *et al.* [6] found moderate and diffused immunofluorescent staining of AKAP4.

The aim of this study was to assess the status of the fibrous sheath and the axonemal structure by performing screening, related to AKAP4 and tubulin proteins, in spermatozoa with absent or severely reduced motility.

Immunolabelling of tubulin and AKAP4 in sperm flagella showed different patterns, leading us to divide the patients into groups. In group I, in which sperm motility was 0%, no AKAP4 or tubulin labelling was detected. When sperm motility was greater than 0%, a variable pattern of AKAP4 and tubulin staining was observed (groups II and III).

In group I, TEM evaluation highlighted that a high presence of necrosis was associated with cases of total immotility and the absence of AKAP4 and tubulin, indicating a loss of antigenicity caused by post-necrotic protein degradation. In order to exclude a genetic origin of the absence of AKAP4, PCR analysis was performed to detect the presence of a partial sequence of *Akap4/Akap3* binding regions and it produced normal results.

In group II, despite reduced motility, regular AKAP4 and tubulin signals were observed. This apparent inconsistency was justified by TEM analysis that revealed the presence of sperm immaturity. We observed numerous cytoplasmic residues, typical markers of this pathology and responsible for the decrement of motility. The axonemal and periaxonemal structures embedded in these cytoplasmic residues, including the fibrous sheath, were generally normal as also revealed by immunocytochemical analyses.

In group III in which a weak AKAP4 label was observed, associated with good tubulin staining, TEM analysis showed a severe disorganization of the fibrous sheath and a normal "9 + 2" axonemal pattern. This pattern was quite similar to that observed in cases of dysplasia of the fibrous sheath (DFS) as already described by Baccetti *et al.* [6]. However, none of these patients were affected by this genetic sperm defect, characterized by a typical ultrastructural feature as highlighted by Chemes *et al.* [16].

In conclusion, while the role of AKAP4 in sperm motility is unclear, absent or weak AKAP4 labeling seems to be associated with absent or weak sperm motility.

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