Use of negative staining technique and electron microscopy for the study of structural anomalies of outer dense fibres of human flagellum

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Motility disorders due to tail defects are often seen in clinical andrology. Sperm motility should be assessed with regard to the morphology of the flagellum. Since suitable longitudinal sections are rarely obtained by routine transmission electron microscopy (TEM) and in view of the importance of dense fibres in modulating sperm motility and providing tensile strength, a detailed, study of human sperm flagellum by negative staining and TEM was attempted. The study was undertaken in two groups of men (I) fertile and (II) asthenozoospermic. The study revealed that outer dense fibres extend to 50–60% of the principal piece. Normal dense fibres were seen in 83% sperms and 23% sperms in groups I and II respectively. The characteristics seen were variation in diameter, breakage or degradation with lacking or extended endpiece. The negative staining method provides an easy and useful analytical tool for identifying the defects of dense fibres and quantifying them.

1. Introduction

Ultrastructural studies have been used extensively to study mammalian spermatozoa. Abnormalities have been described in detail by Zamboni (1987). Human semen is heterogenous in nature with reference to sperm count and morphology even when motility is normal. This, coupled with limitations in measurement of structural alterations, has made the assessment of cause and possible therapy of asthenozoospermia difficult. Different kinds of abnormalities have been reported in patients with asthenozoospermia and/or teratozoospermia (Wiliamsons *et al* 1984). Among them the "Kartegener's" or "Immotile cilia syndrome" has been well documented. This is a congential defect with either lack of dynein arms (Pedersen and Rebbe 1975) and/or lack of radial spokes and central microtubules or transposition of ciliary microtubules (Bacetti *et al* 1979). Ultrastructural studies have reported that defects of fibrous sheath, i.e., hyperplasia, and disorganization and fibrous sheath dysplasia could be responsible for asthenozoospermia (Chemes *et al* 1987). There are a number of case reports on ultrastructural alteration of flagellar structures (Holstein and Schirren 1979; Ross *et al* 1973; Wilton *et al* 1992) but there is no systematic evaluation of particular flagellar defects. Analysis of sperm motility, needs assessment of flagellar substructures in addition to quantitation of motile spermatozoa. Since suitable longitudinal sections are only rarely obtained by routine transmission electron microscopy (TEM) and in view of the importance of dense fibres in modulating sperm motility and providing tensile strength a detailed study of human spermatozoa using negative staining and TEM was attempted.

2. Materials and methods

Semen samples were obtained by masturbation from men enrolling at the male infertility clinic of our Institute (with 3–5 days of sexual abstinence). Routine analysis was carried out for different seminal parameters according

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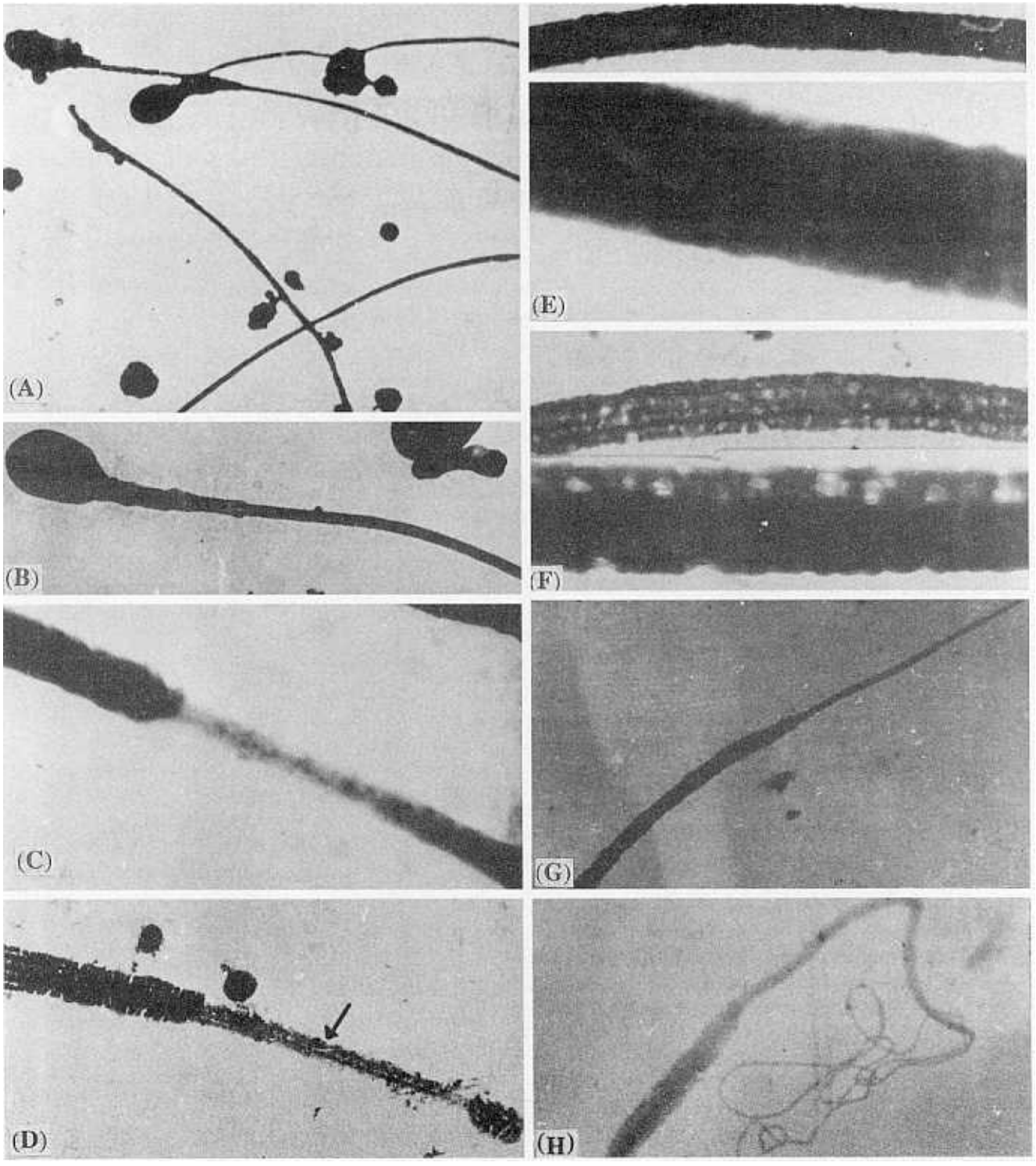

Figure 1. (**A**) Low power view of negative staining of human spermatozoa (×670). (**B**) The normal spermatozoa as seen by negative staining with normal oval head, mid piece and principal piece (×1100). (**C,D**) Structural shaft defects with disruption of outer dense fibres and fibrous sheath, the axoneme remaining intact (**C** ×10,000; **D** ×6700). (**E**) Low and high magnification electromicrograph of normal principal piece (proximal) of human sperm tail (×6700; ×14,000). (**F**) Low and high magnification electronmicrograph of abnormal principal piece of human sperm in which outer dense fibres are missing, the axoneme is clearly visible (×6700; ×14,000). (**G**) Normal end piece of human sperm tail as seen by negative staining (×2400). (**H**) Abnormal end piece of human sperm tail showing membranous extensions of the tail (×2400).

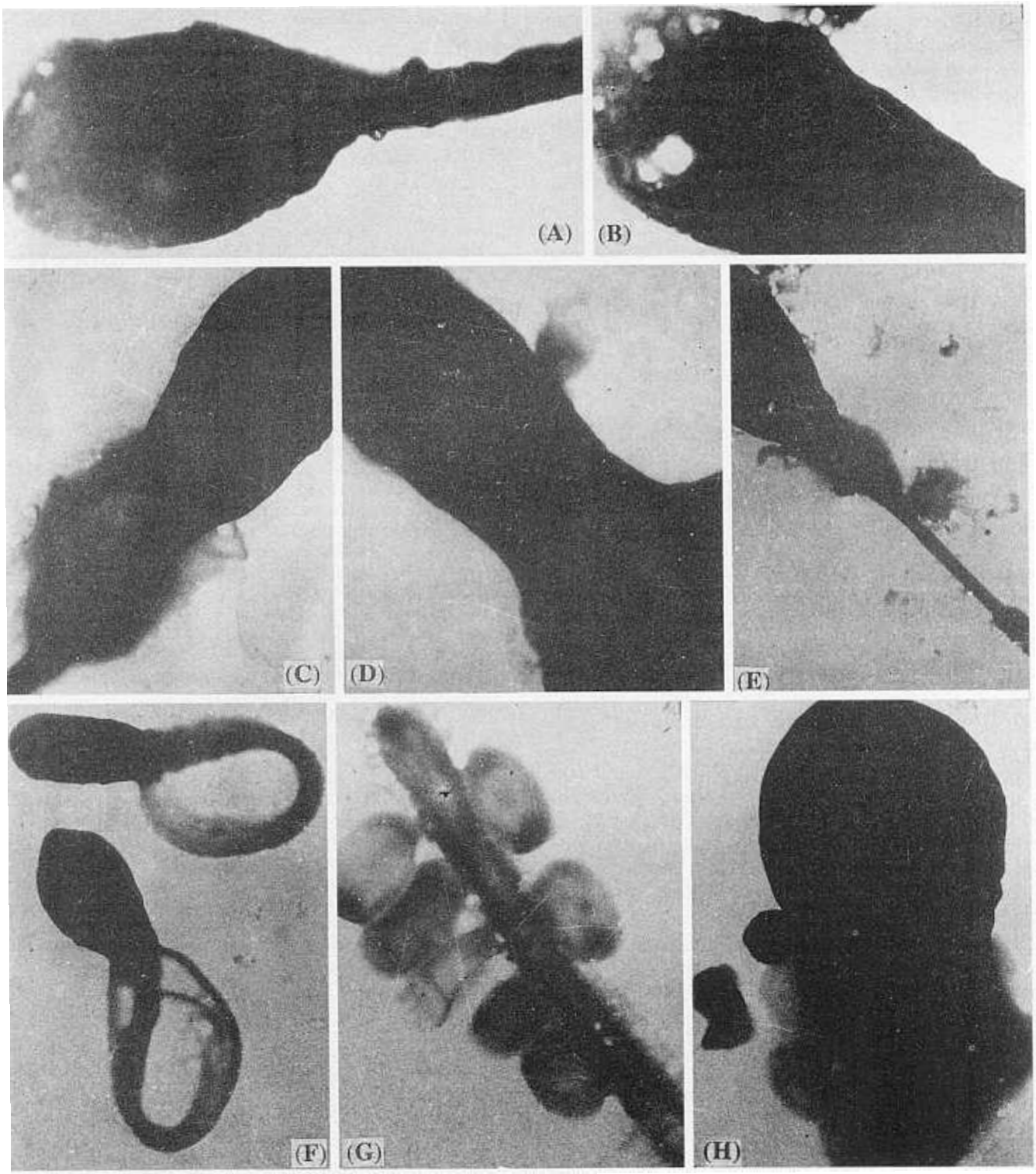

Figure 2. (**A**,**B**) Human sperm head as seen by negative staining showing vacuoles in the acrosomal region (**A** ×6000; **B** ×10,000). (**C**,**D**,**E**) Mid piece abnormalities of human spermatozoa showing cytoplasmic droplet (**C** ×4000; **D** ×10,000) mitochondrial abnormalities (**E** ×2400). (**F**) Tail abnormalities of human spermatozoa showing coiled tail (×1400) as seen by negative staining. (**G**,**H**) Negative staining of human spermatozoa showing bacterial attachment over the principal piece region (**G** ×4000) and at the post-acrosomal region (**H** ×5200).

to the WHO Manual (1992). After initial analysis the samples were fixed in toto in Karnovsky's fluid washed in cacodylate buffer, post fixed in osmium tetroxide and laid on fomvar coated grids. The grids were stained with 1% aqueous phosphotungstic acid for 5 min. The grids were dried and observed under Philips TEM-400T.

The study was conducted in two groups, I normal fertile ($n = 25$) and II samples showing asthenozoospermia (motility $< 10\%$) ($n = 30$).

3. Results and discussion

Suitable longitudinal sections were seen in sufficient numbers to perform a quantitative comparison (figure 1A). The course and extension of inner structures could be easily studied. The study reveals that outer dense fibres extend to 50–60% of the principal piece (figure 1B) which is similar to the reports of Haidl (1993) and Serres *et al* (1986). In group I the normal dense fibres were seen in 83% of samples whereas in group II it was seen only in 23%. The disturbances of outer dense fibres could be easily characterized by the abnormal staining pattern. The normal dense fibres of the principal piece could be seen as uniformly darkly stained band (figure 1E) whereas characteristics seen in the abnormal principal piece were breakage or degradation, malformed or missing dense fibres (figure 1C,D,F) along with variation in diameter and surface irregularity. Flagellar changes were isolated or in association with head abnormalities. The extension and course of inner flagellar substructures were clearly seen by negative staining. The study of ultrastructural features and quantification of the results becomes much less time consuming, specially in a large group of samples and with a large number of spermatozoa. Apart from the flagellar defects the negative staining also gives a clear picture of head abnormalities (figure 2A,B) (vacuolation), mid piece abnormalities (cytoplasmic droplet, bent necks and mitochondrial abnormalities) (figure 2C,D,E), tail abnormalities (figure 2F), endpiece details (extensions) (figure 1G,H) and bacterial attachment to spermatozoa (figure 2G,H). The study revealed that poorly developed or missing outer dense fibres seen in group II might also contribute to poor motility in the group apart from mitochondrial and axonemal defects (Gopalkrishnan *et al* 1995). The negative staining method normally used for identification of viruses provides us a useful analytical tool for identifying and quantifying the defects of outer dense fibres.

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References

Bacetti B, Burrini A G, Maller A, Pallini B and Renieri T 1979 "9 + 0" immotile spermatozoa in an infertile man; *Andrologia* **11** 437

Chemes H E, Carrere C, Brugo S, Lavieri J C and Zanchetli F 1987 Dysplasia of fibrous sheath: an ultrastructural defect of human spermatozoa associated with sperm motility and primary sterility; *Fertil. Steril.* **48** 664–669

Gopalkrishnan K, Padwal V, D'Souza S and Shah R 1995 Severe asthenozoospermia: a structural and functional study; *Int. J. Androl. (Suppl. 1)* **18** 67–74

Haidl G 1993 Outer dense fibres: functional or structural elements; *Andrologia* **25** 13–17

Holstein A F and Schirren C 1979 Classification of abnormalities in human spermatids based on recent advances in ultrastructure research on spermatid differentiation; in *The spermatozoon* (eds) D W Fawcelt and M Bedford (Baltimore: Munich Urban and Schwarzenberg) p 341

Pedersen H and Rebbe H 1975 Absence of arms in the axoneme of immotile human spermatozoa; *J. Cell Biol.* **66** 225–232

Ross A, Christie S and Edmond P 1973 Ultrastructural tail defects in the spermatozoa from two men attending a subfertility clinic; *J. Reprod. Fertil.* **32** 243–251

Serres C, Feneux D and Jouannet P 1986 Abnormal distribution of the periaxonemal structures in human flagellar dyskinesia; *Cell Motil Cytosk.* **6** 68–76

Wiliamsons R A, Koethler J K, Smith W D and Stenchever M A 1984 Ultrastructural sperm tail defects associated with sperm immotility; *Fertil. Steril.* **27** 836–847

Wilton L J, Temple-Smith P D and de Krester D M 1992 Quantitative ultrastructural analysis of sperm tails reveals flagellar defects associated with persistent asthenozoospermia; *Human Reprod.* **7** 310–316

World Health Organization 1992 *Laboratory manual for the examination of human semen and semen – Cervical mucus interaction* (Cambridge: Cambridge University Press)

Zamboni 1987 The ultrastructural pathology of the spermatozoa as a cause of infertility: the role of the electron microscope in the evaluation of semen quality; *Fertil. Steril.* **48** 711–734

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