Acetylated α-tubulin is reduced in individuals with poor sperm motility

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Objective: To determine the status of α-tubulin acetylation and of testis-specific acetylable α-tubulin isoforms in asthenozoospermia.
Design: Research study.
Setting: Research institute and an infertility clinic.
Patient(s): 50 men with normal sperm parameters, and 50 men with asthenozoospermia.
Intervention(s): None.
Main Outcome Measure(s): Western blot analyses of α-tubulin, acetylated α-tubulin, and isoforms TUBA3C, TUBA4A, and TUBA8 in Percoll separated sperm and flow cytometry, real-time reverse-transcription polymerase chain reaction, and immunofluorescent localization.
Result(s): A statistically significant decrease in the expression of acetylated α-tubulin in asthenozoosperm was seen with Western blot analysis, double immunostaining by direct immunofluorescence, and flow cytometric analysis. The transcript and protein of testis-specific acetylable α-tubulin isoform TUBA3C was decreased and TUBA4A was statistically significantly increased in asthenozoosperm as compared with normal spermatozoa. TUBA8 was reduced in asthenozoosperm. Similar observations were noted by indirect immunofluorescent localization. The potential transcription factors involved in the differential expression of TUBA4A and TUBA3C have been identified.
Conclusion(s): Data suggest an association of α-tubulin acetylation with asthenozoospermia. Ours is the first report to demonstrate α-tubulin isoforms in sperm, implicating their role in motility. The differential expression of TUBA3C and TUBA4A suggests that tubulin acetylation may be governed by the isoform of α-tubulin that is expressed or silenced and that this in turn is transcriptionally controlled. (Fertil Steril® 2014;101:95–104. ©2014 by American Society for Reproductive Medicine.)
Key Words: Acetylation, alpha tubulin, asthenozoosperm, isoforms, testis-specific

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Poor sperm motility accounts for almost 50% of cases of male infertility (1). Despite this manifestation being so common, the molecular mechanisms regulating sperm movement remain elusive. Studies of gene knockout mouse models, single-gene defects, and proteomics of the proteins that are differentially regulated in asthenospermia have provided a catalog of proteins that are likely important for sperm motility (2, 3). However, the orchestration of sperm motility is far from clear. We have identified the pathways relevant to sperm motility using a proteomics approach (4). At the cytoskeletal level, sperm flagellar motility is thought to be a result of specific interactions between axonemal microtubular proteins and dynein motors. Flagellar microtubules in association with other proteins make sperm movement a highly stable, organized event (5). Tubulin, the main component of microtubules, is an α, β heterodimer of molecular mass 100 kDa. Both α-tubulins and β-tubulins undergo several posttranslational modifications (PTMs) such as polyglutamylation,

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polyglycylation, tyrosylation/detyrosylation, and acetylation/deacetylation (6). The precise function of acetylated/deacetylated α-tubulin is not known.

Tubulin acetylation, a PTM that occurs on the epsilon amino group of lysine-40 at the N-terminal domain of α-tubulin, is conserved across species (7, 8). Crystal structure analysis of α-tubulin has revealed that this modification is located inside the microtubule lumen (6, 9). Studies in *Drosophila* have illustrated that such PTMs occur after complete assembly of axonemal microtubules (10). Reversible acetylation of α-tubulin has been implicated in regulating microtubule stability and function (11, 12) and is specific to flagellar microtubules (13, 14).

Although the enzymes responsible for tubulin acetylation are not known, HDAC6 and SIRT2 have been identified as tubulin deacetylases (12, 15, 16). Hubbert et al. (12) demonstrated that overexpression of HDAC6 promotes chemotactic cell movement, which supports the idea that HDAC6-mediated deacetylation regulates microtubule-dependent cell motility. Palazzo et al. (17) have shown that the alteration in cell motility observed by Hubbert's group in cells overexpressing HDAC6 is due to alteration in the degree of tubulin acetylation or due to acetylation of some as yet unidentified protein. Our own studies on HDAC6 in the rat (unpublished data) have shown that it is also involved in regulating sperm motility.

Microtubule-binding assays using ciliary axonemes from wild-type *Tetrahymena* and strains carrying α-tubulin or β-tubulin mutations that eliminate sites of specific PTMs have shown that loss of α-tubulin acetylation at Lys-40 affects the binding and motility of kinesin-1 in vitro (18). This signifies the importance of acetylation in the interaction of other proteins with tubulin. The observation of missing and/or misplaced doublets in 46% of sperm axonemes and hypoacetylation of α-tubulin on ultrastructural and biochemical analysis of sperm flagella from an asthenozoospermic man with retinal degeneration also highlight the role of tubulin acetylation in sperm motility (19).

We investigated the possible role of tubulin acetylation in sperm movement. Our observations show a significant decrease in tubulin acetylation in the spermatozoa of asthenozoospermic individuals. We further show that the transcripts as well as the protein for α-tubulin isoforms TUBA3C and TUBA8 are decreased and TUBA4A is increased in asthenozoospermia. Our observations based on their localization and expression pattern on the sperm flagella suggest that these isoforms perform specialized functions. We have further elucidated the cause of reduced expression of acetyl α-tubulin and the contrasting expressions of the acetylable isoforms TUBA3C and TUBA4A in asthenozoospermic individuals.

MATERIALS AND METHODS

Sample Collection

Human semen ejaculates from 50 normozoospermic and 50 asthenozoospermic men were collected by masturbation after 3 days of abstinence. After liquefaction of the semen, we evaluated the samples according to World Health Organization criteria for semen analysis (20). Patients with a history of long-term medication or of infection, as indicated by a high number of leukocytes (more than 2 to 3 leucocytes per high-power field), were excluded from the study. Samples that were hyperviscous and necrozoospermic were also excluded from the study. The sperm parameters for the recruited samples are described in Supplemental Table 1 (available online).

Prior informed consent was obtained from every participant before semen collection. These individuals were recruited from the Infertility Clinic at the National Institute for Research in Reproductive Health (NIRRH), Mumbai, India, and ethics clearance was obtained from the NIRRH Ethics Committee for Clinical Studies for this purpose.

Sample Preparation

The semen sample on routine analysis was centrifuged at 800 × *g* for 30 minutes and resuspended in 1 mL 0.1 M phosphate-buffered saline (PBS, pH 7.4). This was then overlaid on a discontinuous 45% and 90% (v/v; 1 mL each) Percoll gradient and centrifuged at 800 × *g* for 30 minutes on a swing-out rotor, and the 90% fraction was collected (21). This fraction was then examined under phase-contrast microscope to ensure that it was absolutely pure sperm preparation free from contaminating cell types. It was then washed three times with 0.1M PBS, pH 7.4, and pelleted at 800 × *g* for 30 minutes at 4°C for further use. Depending on the individual total sperm count, the number of sperm available from each sample after Percoll gradient centrifugation, and the number of spermatozoa required for each assay, all the samples were used in more than one analysis/assay.

SDS-PAGE and Western Blot Analysis

For Western blot analysis, sperm pellets from the normal spermatozoa and asthenozoospermic groups were washed twice in 0.1 M PBS, pH 7.4, suspended in two-dimensional lysis buffer containing protease inhibitor cocktail (Roche Diagnostics) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich), incubated overnight at 4°C followed by sonication (10 pulses of 30 seconds each) at 20% amplitude, and centrifuged at 12,000 × *g* for 30 minutes at 4°C. The supernatant was quantified for protein content using the Bradford method (22). The 20 μg of protein lysates thus obtained were electrophoresed on a 12% one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted on a nitrocellulose membrane at 100 V for 1 hour (23). The blots were then processed for Western analysis of α-tubulin, acetylated α-tubulin, and tubulin isoforms as described.

Nonspecific binding was blocked by incubating the blots with 5% (wt/vol) nonfat dry milk in 0.1 M PBS for 1 hour. For investigating the α-tubulin isoforms, the transblotted nitrocellulose were probed with antibodies to TUBA3C (1:2,000; monoclonal), TUBA4A (1:1,000; polyclonal), and TUBA8 (1:2,000, monoclonal; Sigma Aldrich). To study the status of acetylated α-tubulin, we probed the blots by use of monoclonal antibodies to α-tubulin and acetylated α-tubulin (1:20,000; Sigma-Aldrich). The blots were incubated with