Expression of beta-tubulin epitope in human sperm with pathological spermiogram

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Objective: To determine the location of the corresponding epitope on the tubulin molecule and to find any differences in its exposition in human sperm with normal and pathological spermiograms. The mature spermatozoon exhibits extraordinary structural compartmentalization that is related to the presence of cytoskeletal proteins and has a functional role in connection with fertilization and motility. Previously, we have shown that anti-β-tubulin antibody TU-12 provided an unexpectedly strong reaction in human and boar sperm head.
Design: A retrospective study.
Setting: Academic research laboratories and private IVF center.
Patient(s): One hundred thirteen men participating in the IVF program.
Intervention(s): Sperm were divided into five categories: normozoospermia, oligozoospermia, asthenozoospermia, teratozoospermia, and asthenoteratozoospermia. Well-characterized monoclonal antibodies were applied for monitoring tubulin epitope distributions in pathological spermatozoa.
Main Outcome Measure(s): Qualitative and quantitative detection of tubulin.
Result(s): TU-12 epitope was located in the β-tubulin region β426–435. Immunoblotting revealed differences in the amount of tubulin among men with normozoospermia and pathological spermiogram. Striking differences were observed in the exposition of TU-12 epitope in heads of normal and pathological spermatozoa.
Conclusion(s): The results suggest that tubulin epitopes could be useful biomarkers of the pathological sperm state. (Fertil Steril® 2007;88(Suppl 2):1120–8. ©2007 by American Society for Reproductive Medicine.)

Key Words: Beta-tubulin, sperm head, pathological spermiogram

Microtubular structures, which are involved in a wide range of functions, display a huge diversity that results from the variety of tubulin isoforms and from associated proteins (1). Microtubules are formed by self-assembly of the tubulin heterodimer and are composed of highly phylogenetically conserved α- and β-subunits, each with a molecular mass of approximately 50 kDa. Tubulin is a highly heterogeneous protein, and more than 20 charge variants can be distinguished by isoelectric focusing. Some of the isoforms result from the expression of multiple tubulin genes since multigene families encode both subunits (2). Tubulin heterogeneity is further increased by different post-translational modifications, mainly in the C-terminal structural domains of the tubulin subunits (3, 4). In contrast, the N-terminal structural domains are less heterogeneous and contain GTP binding sites essential for assembly into microtubules (5). Post-translational modifications play a role in the stability of microtubules and in regulating microtubule interactions with microtubule-associated proteins (6) and motility (7).

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The spermatozoon is the end product of the process of male gametogenesis, and it represents a highly specialized mammalian cell with specific morphology and motility. The mature spermatozoon exhibits extraordinary structural compartmentalization that is related to the presence of cytoskeletal proteins (8–11). This compartmentalization also has a functional role in connection with fertilization and motility (12). The major compartments of the mature spermatozoon are the head and the flagellum/tail. The sperm head consists of the nucleus and the acrosome surrounded by the cytoplasm. The acrosome contains enzymes, which enable sperm to penetrate through the investments of the eggs to achieve fertilization (13). Cytoskeletal proteins were detected in the subacrosomal, para-acrosomal, acrosomal, and postacrosomal regions of the head (14). The flagellum is composed of the connecting piece/neck, midpiece, principal piece, and end piece. The axoneme is composed of a typical 9+2 arrangement of microtubules: nine peripheral doublets and two central microtubules (15). Although the organization of microtubules in the tail is known in great detail (for a review, see 14), data on the tubulin association with the head are more limited (8, 10, 11).

Little is known about the relation of the pathological state of spermatozoa and tubulin changes. The evaluation of tubulin (16) and the state of the acrosome (17, 18) in human pathological spermatozoa were reported as was the influence of the morphological state of the sperm tail on human sperm pathology (19). Previously, we have shown in both human (9)

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and boar (20) spermatozoa that anti-tubulin antibody TU-12 against epitope in the C-terminal structural domain of β-tubulin (9) provided an unexpectedly strong reaction in the sperm head. We therefore tried to find out the localization of the β-tubulin corresponding epitope and whether there were any differences in its distribution between normal human sperm and those with pathological spermiogram. Here we demonstrate that the TU-12 epitope is located in the β-tubulin β426–435 region and that its distribution in the sperm head is strongly dependent on the pathological state of sperm. Our results indicate that tubulins, which are involved in the functional organization of the sperm, could be useful markers of the pathological sperm state.

MATERAL AND METHODS

Materials

Analytical-grade chemicals were used. Bicinchonic acid (BCA), bovine serum albumin (BSA), and molecular weight standards were obtained from Sigma-Aldrich (Prague). Vecta-Shield mounting medium was obtained from Scandic (Prague).

Antibodies

The following antitubulin monoclonal antibodies were used: TU-01 (IgG1) (21), which recognizes epitope located in the α65–79 region (22) in the N-terminal structural domain of α-tubulin (23); TU-12 (IgM), which is directed against the C-terminal structural domain of β-tubulin (9); TU-06 (IgM), which recognizes epitope located in the β81–95 region (24) in the N-terminal domain of β-tubulin (23); TUB2.1 (IgG1) (Sigma-Aldrich), which recognizes epitope located in the β282–445 region in the C-terminal structural domain of β-tubulin (25); and 18D6 (IgG1), which was raised against the N-terminal peptide of β-tubulin (β1–12) (26). Antibodies TU-06, TUB2.1, and 18D6 served as markers of β-tubulin; antibody TU-01 served as a marker of α-tubulin.

The state of the sperm acrosome was evaluated by monoclonal antibody Hs-14 (IgM) against human intra-acrosomal sperm proteins (17). Anti-mouse Ig conjugated with horseradish peroxidase was purchased from Promega Biotech (Madison, WI). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin IgG (γ-specific) and IgM (μ-specific) were purchased from Sigma-Aldrich. Indocarbonate (Cy3)-conjugated anti-mouse IgM antibody from Jackson Immunoresearch Laboratories (West Grove, PA) was used in colocalization tests. Monoclonal antibody Prog.13 (IgG) against progesterone (27) was used as a negative control.

Cells

Human ejaculated spermatozoa were obtained with the patients' permission from Iscare IVF, Pronatal, and the Institute of Sexology, Prague. The evaluation of semen density, motility, and morphology was carried out in compliance with World Health Organization standards (28) and divided into five categories: normozoospermia (normo); oligozoospermia (with $<20 \times 10^6$/mL) (oligo); asthenozoospermia (with subnormal motility, lower than 50%) (astheno); teratozoospermia (more than 70% of pathological spermatozoa) (terato), and astheno-teratozoospermia (motility lower than 50%, more than 70% of pathological spermatozoa) (A-T). Characteristics of these sperm categories and the number of evaluated samples are summarized in Table 1.

Protein Preparation and Epitope Mapping

Microtubule protein from porcine brain was prepared by three temperature-dependent cycles of assembly and disassembly according to Shelanski et al. (29). Tubulin depleted of microtubule-associated proteins was obtained by phosphocellulose chromatography (30) and stored in small aliquots in liquid nitrogen. To separate α- and β-tubulin subunits, tubulin was carboxyamidomethylated and the subunits were effectively separated by 8% SDS-PAGE according to Laemmli using modifications in separation gel and electrode buffer composition (31). Subunits were isolated from the gel by electroelution using Model 422 Electro-Eluter (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's directions. Electroelution was performed in 50 mM ammonium bicarbonate containing 0.1% SDS. Eluted proteins were concentrated in Speed Vac (SAVANT Instruments, Farmingdale, NY).

Chemical proteolysis of β-tubulin was performed by dissolving the isolated protein in 75% formic acid and incubating the solution in the dark at 37°C for 24 hours (32). After dialysis against water using a dialysis membrane with molecular weight cut off (MWCO) 3,500 Daltons, samples were mixed with five-time concentrated SDS-sample buffer and analyzed by immunoblotting.

For epitope mapping, the synthetic overlapping peptides, a total of 53 linear 15-meric peptides with five amino acid overlaps, were prepared by synthetic peptide array on membrane support (SPOT) synthesis (Jerini Peptide Technologies, Berlin, Germany). Each spot carried an approximately 5-nmol peptide covalently bound to the cellulose-β-alanine membrane. Peptide scans covered the sequence β171–445 in the N-terminal structural domain of porcine brain tubulin (accession no. P02554 in the Swiss-Prot Sequence Database). Epitope mapping was performed according to the manufacturer's directions with chemiluminescent detection of bound antibodies (see below in the section entitled Gel Electrophoresis and Immunoblotting).

Immunofluorescence

For indirect immunofluorescence, sperm cells were washed in phosphate-buffered saline (PBS; 0.15M NaCl, 0.02M NaH_2PO_4, pH 7.4) and resuspended in PBS to a cell density of 2×10^7 sperm cells/mL. Ten-microliter sample drops were loaded on glass slides and air dried. Slides were fixed 12 minutes with methanol, precooled to −20°C, and then incubated for 6 minutes in acetone at −20°C (9). To detect intra-acrosomal protein Hs-14, the air-dried cells were fixed for 10

TABLE 1

Characterization of spermatozoa groups used in the assays.

Sperm characterization	No. of men	Group	Characterization of the sperm pathology[a]		
			Concentration	Motility	Morphology
Normozoospermia	66	1 (Normo)	$>20 \times 10^6$/mL	>50%	>30% without defect
Oligozoospermia	6	2 (Oligo)	$<20 \times 10^6$/mL	>50%	>30% without defect
Asthenozoospermia	19	3 (Astheno)	$>20 \times 10^6$/mL	<50%	>30% without defect
Teratozoospermia	15	4 (Terato)	$>20 \times 10^6$/mL	>50%	>70% pathol. sperm
Ashenoteratozoospermia	7	5 (A-T)	$>20 \times 10^6$/mL	<50%	>70% pathol. sperm

Note: Spermatozoa used for detection of α- and β-tubulins in ejaculated sperm samples with each group.
[a] World Health Organization 1993.

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minutes with acetone at room temperature (19). The samples were incubated with monoclonal antibodies, in the form of ascitic fluids, diluted in PBS. The antibody TU-01 was diluted 1:80, the antibody TU-12 was diluted 1:20, and Hs-14 was diluted 1:10. Ninety minutes of incubation with primary antibodies took place at 37°C. Washing (3 × 10 minutes) in PBS was followed by 60 minutes of incubation with a secondary FITC-conjugated anti-mouse antibody diluted 1:128 in PBS at 37°C. Afterward, slides were washed again in PBS (3 × 10 minutes) and placed in mounting medium. For appropriate controls, smears were incubated with nonspecific monoclonal antibody, with the supernatant of myeloma cells, and with the FITC-conjugate only.

Acetone-methanol fixation was used for double staining of the acrosomal Hs-14 protein and β-tubulin. Sperm smears were incubated (overnight at 4°C) with Hs-14 antibody. Washed samples were incubated with FITC-conjugated anti-mouse IgM antibody, diluted 1:128, for 1 hour at 37°C. The remaining binding sites on Cy-3-conjugated antibody were blocked by incubation with normal mouse serum (diluted 1:20) for 3 hours at 37°C before incubation with the anti-β-tubulin antibody TU-12. After incubation (overnight at 4°C), secondary Cy-3-conjugated IgG immunoglobulin (1:500) was applied (1 hour at 37°C).

Samples were examined with a Nikon Labophot-2 fluorescent microscope equipped with 40× Nikon Plan 40/0.65 lenses and photographed with a COHU4 CCD camera (COHU Inc., San Diego, CA) with the aid of LUCIA imaging software (Laboratory Imaging, Prague) or with a confocal microscope Leica TCS-SP (Leica, Micro, Prague) with Ar (488, 458 nM) and HeNe (543, 633 nM) lasers equipped with a 60× objective. Negative control antibodies and the conjugate alone gave no detectable staining.

In some immunofluorescence experiments, antibody TU-12 was preabsorbed with porcine brain tubulin or BSA (negative control) in 1:10 and 1:50 ratios. Mixtures of purified antibody and absorbing protein were incubated for 30 minutes at room temperature.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed according to the method of Laemmli. Washed samples (5×10^6 sperm/mL) were pelleted and resuspended in 100 μL of twice concentrated SDS sample buffer. After heating in a boiling water bath (3 minutes), cooling and centrifugation (15,000 *g*, 3 minutes, 4°C), the samples were divided into aliquots and stored at −70°C. Samples were run on 7.5% polyacrylamide gels, and separated proteins were then electrophoretically transferred onto nitrocellulose. Details of the immunostaining procedure are described in the literature (33). The antibodies TU-01, TU-06, and TU-12, in the form of ascitic fluids, were diluted 1:1,000. The antibodies TUB2.1 and 18D6 were diluted 1:2,000 and 1:10,000. Blots were incubated with secondary antibody conjugated with horseradish peroxidase, diluted 1:5,000, and washed with enhanced chemiluminescence reagents according to the manufacturer's directions and detected by autoradiography films X-Omat AR. Exposed films were quantified by densitometry using the AIDA program (Raytest, Straubenhardt, Germany).

Quantification of Proteins

The concentrations of proteins in whole sperm lysates were evaluated with modification of Smith's method (34). SDS sperm extracts were diluted 1:10 in distilled water. Three hundred microliters of BCA were added to 15 μL of solution. The samples were incubated 30 minutes at 37°C. After incubation, the absorbance was measured with an ELISA Reader Tecan Sunrise at 570 nm. For calibration, the solution of BSA (0, 5, 10, 20, 30 μg) was used. The concentrations of proteins were subtracted from the calibration curve.

Statistical Analysis

BMDP Statistical Software (Los Angeles, CA) was used to analyze the statistical differences among the compared groups. For statistical purposes, immunofluorescence of 200 cells in each sample was evaluated. $P<.01$ and $<.05$ were considered statistically significant.

RESULTS

Epitope Mapping

Tubulin is a highly conserved protein and brain tubulin, which can be prepared in very high purity; it is often used for the location of epitopes recognized by specific antitubulin monoclonal antibodies. Previous experiments have shown that the TU-12 epitope is located in the C-terminal structural domain of porcine brain β-tubulin (β282–445) (9). It is known that specific chemical proteolysis of aspartic-proline bonds (consisting of 75% formic acid), generates a small number of proteolytic fragments in tubulin dimers (32). We therefore performed immunoblotting analysis of porcine brain β-tubulin fragments after formic acid cleavage, using anti-β-tubulin antibodies with known epitope location as a marker. β-tubulin has two aspartic-proline bonds at positions β31–32 and β304–305 (P02554 in the Swiss-Prot Sequence Database) (35). Formic acid cleavage generates five fragments denoted β1, β2, β3, β1+β2, and β1+β3 as outlined in Figure 1A. Subunits were effectively separated by electrophoresis (Fig. 1B, lane 1), and the β-tubulin subunit was isolated from gel by electroelution (Fig 1B, lane 2). When purified β-tubulin was used for formic acid proteolysis, fragments β1+β3, β1, and β2 were discernible after the staining of blotted proteins by SYPRO Ruby Protein Blot Stain (Fig. 1C, lane 1). Antibody 18D6, recognizing an epitope in the region β1–12, served as a marker of the β1+β3 fragment (Fig. 1C, lane 2); antibody TU-06, which recognizes an epitope in the region β81–95, served as a marker of the β1 fragment (Fig 1C, lane 3), and antibody TUB2.1, which recognizes an epitope in the region β282–445, served as a marker of the β2 fragment (Fig. 1C, lane 4). Immunoblotting with TU-12 antibody revealed reactivity with both β2 and β1+β2 fragments (Fig. 1C, lane 5). This indicates that the corresponding epitope is located in the region β305–445. For a more accurate epitope location we used a peptide scan of immobilized overlapping peptides, 53 linear 15-meric peptides with five amino acid overlaps, covering the region β171–445. The results of immunostaining with the TU-06 antibody are shown in Figure 1D. Using this approach, the epitope was located into the β-tubulin region β426–435.

Tubulin Quantification

In the first set of experiments, the comparison of α- and β-tubulin amounts in sperm with normal and pathological spermiograms was performed by immunoblotting using antibodies TU-01 against α-tubulin and TU-12 against β-tubulin. In whole-cell lysates, the antibodies stained only proteins with their relative electrophoretic mobility corresponding to the position of the α-tubulin and β-tubulin subunits. Quantitative immunoblotting revealed that the amount of α-tubulin detected in normozoospermia and tested pathological samples was different. A typical example of immunoblotting analysis is shown in Figure 2. Taking the intensity of staining in the sperm with normozoospermia as 100% (Fig. 2A, lane 1), the other groups have the following staining intensities: astheno (Fig. 2A, lane 2), 85.4%; terato (Fig. 2A,

FIGURE 1

Reactivity of TU-12 antibody with β-tubulin fragments and synthetic overlapping peptides. Schematic representation of the β-tubulin peptide map after formic acid cleavage. The positions of formic acid cleavage sites (formic) and generated proteolytic fragments (β1, β2, and β3) are shown on the first line. Sizes of the fragments after incomplete cleavage are outlined below this line (**A**). Coomassie blue staining of carboxyamidomethylated tubulin heterodimer (lane 1; 12 μg) and isolated β-tubulin (lane 2; 6 μg). α and β denote positions of tubulin subunits; 7.5% SDS-PAGE (**B**). Immunostaining with antibodies to β-tubulin fragments generated by formic acid. Lane 1: protein staining of blotted fragments; lanes 2–5: immunostaining with antibodies 18D6 (marker of β3), TU-06 (marker of β1), TUB2.1 (marker of β2), and TU-12. Positions of β-tubulin fragments are indicated on the right margin; 12.5% SDS-PAGE (**C**). Molecular mass markers (in kDa) are indicated on the left of **B** and **C**. Immunostaining of immobilized peptides covering porcine brain sequence β171–445. Peptide scan was formed by 53 linear 15-meric peptides with five amino acid overlaps. Numbers at the top and bottom denote peptide spots in the upper and lower row of the scan, respectively (**D**).

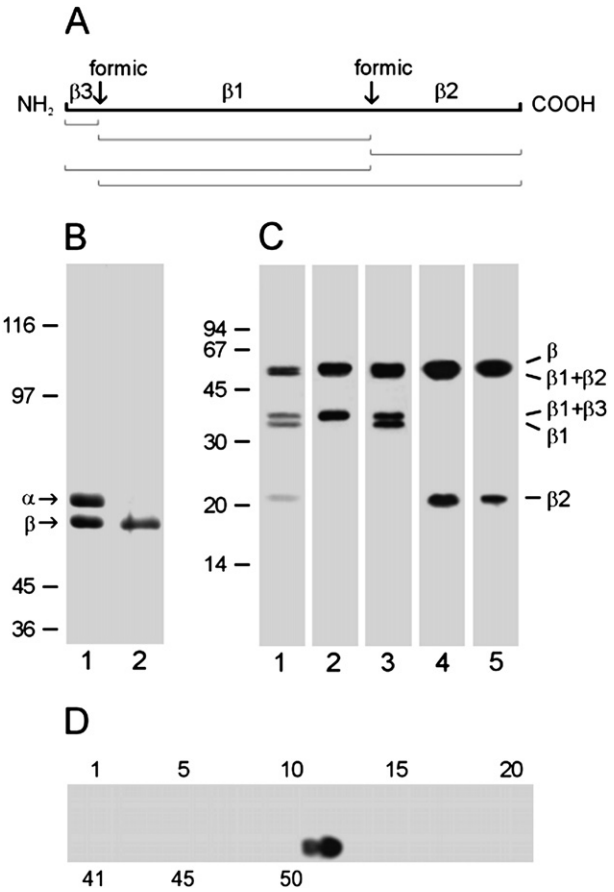

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FIGURE 2

Quantitative immunoblotting of the α-tubulin (**A**) and β-tubulin (**B**) in the sperm of different experimental groups. Normo, Lane 1; astheno, lane 2; terato, lane 3; A-T, lane 4.

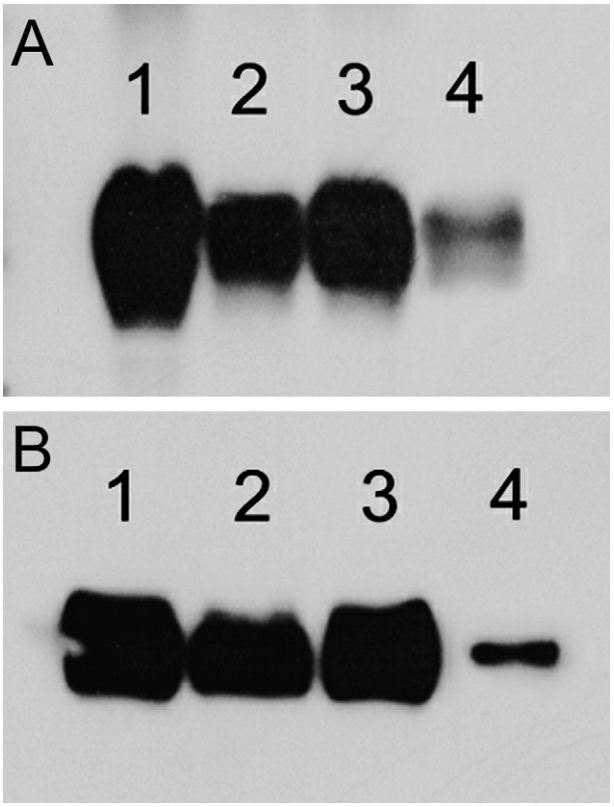

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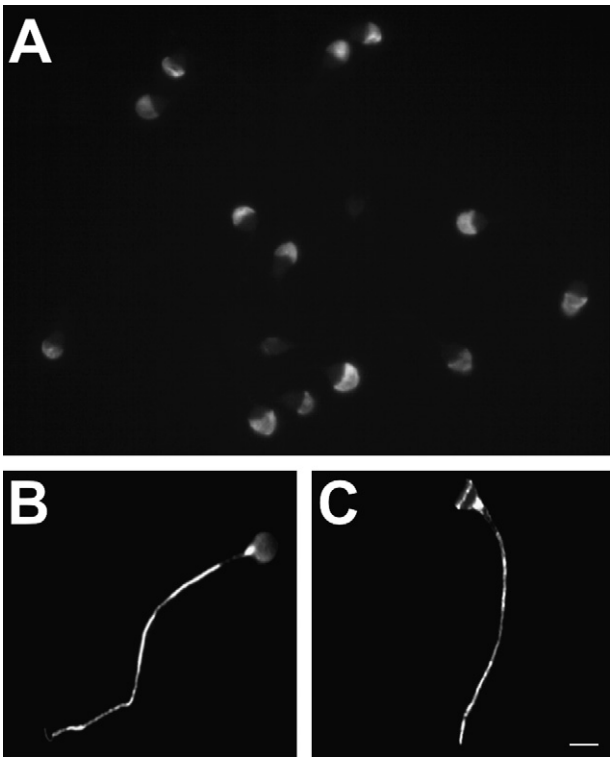
FIGURE 3

Immunofluorescence staining of human spermatozoa with a normal spermiogram using monoclonal antibodies Hs-14 (against acrosome proteins–acetone fixation) (**A**), TU-01 against α-tubulin (**B**), and TU-12 against β-tubulin (**C**).



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lane 3), 94.7%; and A-T (Fig. 2A, lane 4), 23.7%. In a similar way, we evaluated the amount of β-tubulin (sample normo, Fig. 2B, lane 1) as 100%. Pathological samples of sperm showed a lower amount of β-tubulin (sample astheno, Fig. 2B, lane 2, 70%; terato, Fig. 2B, lane 3, 79%; A-T, Fig. 2B, lane 4, 20%).

The major difference was observed in A-T samples, where both α- and β-tubulin were detected in a substantially lower amount when compared with the normo group.

Distribution of Intra-acrosomal Proteins

The quality of cells isolated from ejaculates of men with normal and pathological spermiograms was evaluated by immunostaining of tested spermatozoa (Table 1) using antibody against intra-acrosomal human sperm proteins that served as a marker of the state of the acrosome (Figs. 3A and 4A). Compared with the control, the percentage of cells with stained acrosome decreased in all pathological sperm groups (except for the oligo group). The astheno group showed a statistical difference compared with the normo group ($P<.05$), and the terato and A-T groups displayed a significant difference compared with the normo and oligo groups ($P<.01$) (Fig. 5). For colocalization (acrosome proteins and β-tubulin), additional fixation (methanol-acetone) was used, which was necessary to detect cytoskeletal proteins (Fig. 4A).

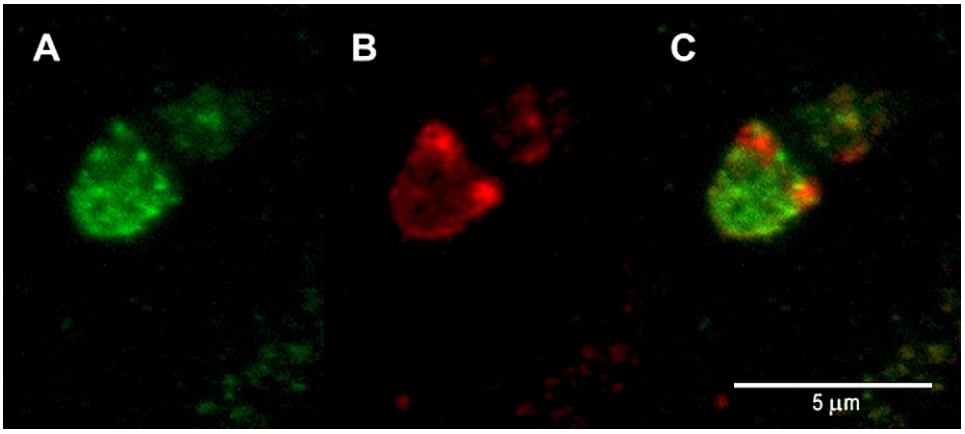
Distribution of Tubulin Epitopes

The typical distribution of the TU-01 epitope in a fixed sample of normal human sperm is shown in Figure 3B. The antibody brightly stained the neck, principal piece, and end piece of the tail, while the head was unstained. On the other hand, antibody TU-12 clearly labeled the equatorial segment of the head and tail (Fig. 3C). Negative control antibodies of IgG and IgM classes and the conjugate itself displayed no staining. When the antibody TU-12 was preabsorbed with tubulin, no staining of spermatozoa was observed. On the other hand, absorption of the antibody with control protein caused no reduction of immunofluorescence staining. The same results were obtained regardless of whether the molar ratio of antibody and tubulin was 1:10 or 1:50.

A double-label experiment revealed that TU-12 epitope was also detectable (Fig. 4B) when sperm heads were stained

FIGURE 4

Double staining in the sperm head. Immunofluorescence with monoclonal antibodies Hs-14 against acrosome proteins (green) (**A**), TU-12 against β-tubulin (red) (**B**), and colocalization with both antibodies (**C**).



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with Hs-14 antibody (Fig. 4A). Although the distribution of both epitopes was different, in some regions of the sperm head partial codistribution could be observed (Fig. 4C).

When immunofluorescence experiments were performed on samples from pathological groups (Table 1), differences in the staining of the equatorial band with the TU-12 antibody were clearly discernible. Typical staining with the TU-12 antibody was identical for sperm of all categories; the differences were in the percentage of positively stained cells. The percentage of stained equatorial band in the normal and pathological groups is summarized in Figure 5. No statistical difference was found between the normo and oligo groups; on the other hand, the other pathological groups significantly differed compared with these two groups ($P<.01$) (Fig. 5).

Small differences between normal and pathological groups were also observed in the staining of the tail region with the TU-12 antibody. The percentage of cells with a stained sperm

FIGURE 5

Statistical evaluation of immunofluorescence staining (%) of the human sperm head in human normal (normo) and pathological sperms obtained from patients with oligozoospermia (oligo), asthenozoospermia (astheno), teratozoospermia (terato), and astheno-teratozoospermia (A-T), using monoclonal antibody Hs-14 against acrosomal proteins (acrosome labeling) and antibody TU-12 against β-tubulin (equatorial band labeling). *$P<.05$; **$P<.01$.

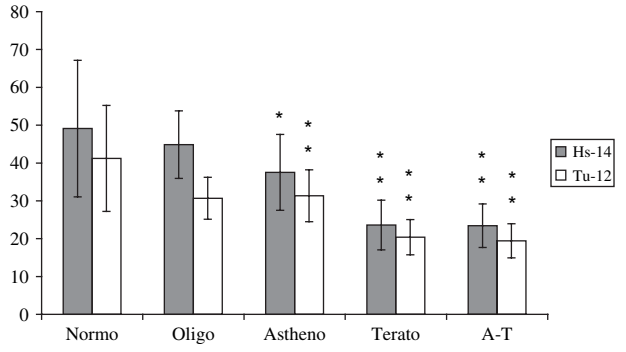

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FIGURE 6

Statistical evaluation of immunofluorescence staining (%) of the human sperm tail in human normal (normo) and pathological sperms obtained from patients with oligozoospermia (oligo), asthenozoospermia (astheno), teratozoospermia (terato), and astheno-teratozoospermia (A-T), using antibody TU-01 against α-tubulin and antibody TU-12 against β-tubulin. *$P<.05$; **$P<.01$.

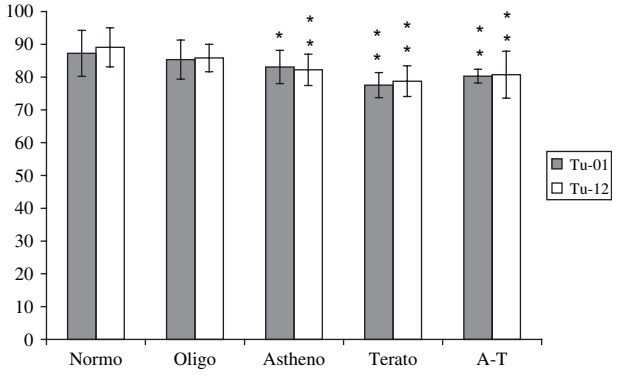

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TABLE 2

The immunofluorescent analysis of variance for all groups of spermastozoa (normo, oligo, astheno, terato, and A-T) tested by monoclonal antibodies (Hs-14, TU-01, TU-12).

Antibody	Normal	Pathological spermatozoa				*F*-value	*P*
	Normo (n = 66)	Oligo (n = 6)	Astheno (n = 19)	Terato (n = 15)	A-T (n = 7)		
Hs-14	49.106 ± 18.185	44.833 ± 9.786	37.526 ± 10.281	23.600 ± 6.780	23.429 ± 6.214	12.28	.001
TU-01	87.242 ± 7.047	85.333 ± 6.501	83.105 ± 5.195	77.533 ± 3.962	80.286 ± 2.289	8.95	.001
TU-12 equat.	41.212 ± 14.106	30.667 ± 6.088	31.368 ± 7.041	20.400 ± 4.808	19.429 ± 4.860	14.55	.001
TU-12 tail	89.091 ± 6.015	85.833 ± 4.579	82.211 ± 4.906	78.733 ± 4.847	80.714 ± 7.740	14.26	.001

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tail was evaluated between the normo and oligo groups without a statistical difference, while the astheno and A-T groups displayed a statistical difference compared with the normo group ($P<.05$ and $P<.01$, respectively) (Fig. 6).

Differences among all tested groups were evaluated by one-way analysis of variance. The strong statistical significance was found for all parameters (Table 2). The Dunnet test, comparing two groups (pathological and control), was therefore used. No statistical difference was found between the normo and oligo groups for all tested parameters (Hs-14 antigen, $\alpha\beta$-tubulins). On the contrary, the other pathological groups showed a statistical difference compared with the normo and oligo groups (Table 3).

DISCUSSION

Previously we have shown that monoclonal antibodies against tubulin can be used to discriminate between different structural regions on human (9) and boar spermatozoa (20). Interestingly, TU-12, an antibody recognizing an epitope in the C-terminal structural domain of β-tubulin, stained the equatorial band in human sperm and the triangular segment of the postacrosome part. The combination of proteolytic digestion of porcine brain β-tubulin and peptide scan of the immobilized overlapping peptides disclosed that the antibody recognized an epitope located in the phylogenetically conserved β-tubulin region β426–435. The present work demonstrates changes in the localization of this β-tubulin epitope in the sperm head in the samples with normal and pathological spermiograms. In contrast to Dvorakova et al. (11), we did not observe staining of α-tubulin in the sperm head. This could be explained by the use in these studies of different monoclonal antibodies, which recognize epitopes located in different structural domains of tubulin. Reactivity with the equatorial band was not detected with anti-β-tubulin antibodies TU-06 and TUB2.1 or with anti-α-tubulin antibody.

The restricted reactivity of the TU-12 antibody in the equatorial band of pathological spermatozoa cannot be attributed to the steric hindrance of the large IgM molecule as the antibody stained sperm tails in the same cell. The TU-12 staining could be prevented by preabsorption of the antibody with purified porcine brain tubulin; this proves that the staining of the equatorial band was due to interaction with tubulin. We therefore believe that the observed immunostaining reflects the actual distribution of the epitope and is not the result of artifacts caused by sample preparation or antibody penetration. The TU-12 staining could thus reflect the exposition of this unique epitope on tubulin dimers that are sequestered at the membranous structures of the sperm head. As the other antibody against intracrosomal proteins showed similar quantitative loss of immunolabeling in pathological samples, we believe that the observed changes in distribution most probably reflect changes in sperm head membranous components.

Several reports indicate that post-translationally modified tubulins might be nonuniformly distributed within the cells. This could lead to speculation that there might be a specific

TABLE 3

Statistical evaluation of the immunofluorescence staining of all pathological groups compares to the normozoospermia (normo) group.

Antibodies	Oligo (n = 6)	Astheno (n = 19)	Terato (n = 15)	A-T (n = 7)
Hs-14	NSD	$P \leq .05$	$P \leq .01$	$P \leq .01$
TU-01	NSD	$P \leq .05$	$P \leq .01$	$P \leq .05$
TU-12 equator.	NSD	$P \leq .01$	$P \leq .01$	$P \leq .01$
TU-12 tail	NSD	$P \leq .01$	$P \leq .01$	$P \leq .01$

Note: NSD = No significantly different.

Peknicova. Tubulin in pathological human sperm. Fertil Steril 2007.

masking of the corresponding epitope caused by post-translational modifications that occur in pathological sperm in this region. It was shown that various post-translational modifications occur in the C-terminal domain of β-tubulin, including polyglutamylation, polyglycylation, and phosphorylation (for review, see 4). However, up to now, reported post-translational modifications were only found in spermatozoa in the axoneme. Therefore, it seems improbable that a differential staining can be assigned to mask corresponding epitope in pathological sperms by post-translational modification(s) of β-tubulin.

The immunofluorescence proved a decreasing sperm quality, which was evaluated by the state of the acrosome in all groups when compared with the normo group. In the group with normozoospermia (n = 66), more than half of samples (n = 37) had about 50% of labeled sperm (Fig. 3A and Fig. 4). This divergence therefore showed that the state of the acrosome in the normo group was not at a similar level. However, in the end, the state of the acrosome in this group was better when compared with all tested groups (except for the oligo group). Nevertheless, it should be stressed that in this study we used ejaculates of patients from centers for assisted reproduction. These samples therefore did not represent random samples of men in the population. This could explain why the labeling for both acrosomal protein and TU-12 epitope was not observed in normo samples in all cells. As the state of the acrosome and acrosomal proteins is important for secondary sperm–zona pellucida binding (36), intra-acrosomal proteins can be used as markers for testing the sperm quality in human spermatozoa (17, 18, 37).

Tubulin molecules in the equatorial band could fulfill a structural role in the formation and sustenance of the sperm head cytoskeleton. On the other hand, as the head participates in interaction with the oocyte (38), it is possible that tubulin with other cytoskeletal proteins could also play an active role in fertilization (8). Tubulin in the sperm head might be important for the first stages of fertilization. Except for this, we have demonstrated changes in the distribution of the acrosome protein and β-tubulin in the sperm heads under the pathological conditions. The typical staining pattern observed with antibodies TU-01 and TU-12 was identical for sperm in all categories; however, there were differences in the percentage of positively stained cells. Normozoospermia and oligozoospermia, which differ quantitatively in sperm amount but not in other sperm characteristics, were without statistical difference in all tested parameters (intra-acrosomal protein, α- and β-tubulins). Contrary to this, astheno- and asthenoteratozoospermia showed higher morphological variability in different parts of the sperm.

The cytoskeletal proteins in the sperm tail play a key role in motility (19, 39). It was reported that the C-terminal sequence of β-tubulin Asp427-Glu432 is important for flagellar motility and that post-translational modification of α-tubulin regulates this process (7). Quantitative biochemical analysis in the normo and other pathological groups revealed that the total amount of α-tubulin decreased in the astheno group on average by 15% (Fig. 2A) and that the total amount of β-tubulin decreased by 30%. This effect was manifested more strongly in the A-T group, where α-tubulin decreased approximately by 77% and β-tubulin by 46% (Fig. 2B). Our analysis showed that the lowest amount of tubulin was observed in the patients with asthenoteratozoospermia (Fig. 2A and 2B). It means that this pathological group had a lower level of tubulin detectible by immunofluorescence as well as a lower total amount of synthesized proteins.

The difference in tubulin content in the sperm axoneme together with the acrosomal status (evaluated by hyalurinidase content) and the DNA strand break between normal and pathological spermiograms were reported (40). Our results similarly indicated a difference in the amount of tubulin in the pathological spermatozoa but more strictly demonstrated changes in the exposition of a well-characterized β-tubulin epitope in the head of normal and pathological spermatozoa. A striking difference was observed mainly in the staining of the equatorial segment in the head. In conclusion, we suggest that the monitoring of tubulin epitopes could help in the evaluation of pathological sperm and therefore be applied in assisted reproduction.

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