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SHORT COMMUNICATION

Capacitation and acrosome reaction changes α-tubulin immunodistribution in human spermatozoa

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Abstract Tubulin is a protein constituent of cytoskeletal microtubules, closely related to sperm motility. However, the changes in tubulin distribution following capacitation and acrosome reaction are poorly understood. This study immunolocalized and quantified the expression of α-tubulin in fresh, capacitated and acrosome-reacted samples. Immunocytochemical data showed that in capacitated and acrosome-reacted spermatozoa, α-tubulin is labelled throughout most of the flagellum (≥66.66%). However, the mean α-tubulin-labelled area in these samples was significantly lower than in fresh samples ($P < 0.05$). Thus, there are different sperm clusters distinguished by their α-tubulin immunoreactivity and this could be directly linked to structural changes following capacitation and acrosome reaction.

KEYWORDS: acrosome reaction, capacitation, cytoskeleton, immunocytochemistry, spermatozoa, tubulin

Introduction

The spermatozoon is the smallest cell in the human body and also one of the most differentiated. Its two main compartments are the head, which transports the genetic material, and the flagellum, which provides the motility necessary to propel the spermatozoa toward the oocyte (Turner, 2006; Ward and Coffey, 1991). The flagellum contains a microtubule-based cytoskeleton called the axoneme, a protein structure of nine pairs of microtubules surrounding a central pair (Amos and Klug, 1974). In turn, sperm microtubules consist of heterodimers of α- and β-tubulin (Luduena et al., 1992). Despite a limited number of genes, tubulin is highly diverse due to post-translational modifications (Luduena, 1998; Rosenbaum, 2000). The latter play an important role in sperm functionality because

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they are related to the regulation of motility (Garnham and Roll-Mecak, 2012). In this regard, it has been demonstrated that expression of tubulin decreases in asthenozoospermic samples (Peknicova et al., 2007).

When the spermatozoon is ejaculated, it does not immediately have the ability to fertilize the egg, but rather acquires this capacity *in vivo* as it crosses the female genital tract, in a process known as capacitation (Abou-haila and Tulsiani, 2009; Tulsiani and Abou-Haila, 2011). This process can also be induced *in vitro* by incubating spermatozoa in specific culture media (Mahadevan and Baker, 1984). Capacitation produces biochemical and molecular changes that lead to hyperactivated sperm motility (Suarez and Ho, 2003). When a capacitated spermatozoon reaches the oocyte and binds to the zona pellucida, the acrosome reaction is triggered (Brucker and Lipford, 1995). This exocytosis of hydrolytic enzymes allows the spermatozoon to penetrate the zona pellucida and fuse its membrane with that of the oocyte membrane (Wassarman, 1999). While capacitation and the acrosome reaction are crucial to the fertilization process, the changes that occur during these events have not yet been fully elucidated. For example, changes in the immunolocalization of cytoskeletal proteins following capacitation and acrosome reaction in human spermatozoa are poorly understood. In mammalian spermatozoa, it has been shown that *in vitro* capacitation causes an increase in actin polymerization and a redistribution of actin epitopes (Brener et al., 2003). Thus, the aim of this study was to quantify and determine the cellular distribution of α-tubulin in fresh, capacitated and acrosome-reacted subsamples of human spermatozoa.

Materials and methods

Semen samples were collected from healthy donors with proven fertility ($n = 7$) after 4 days of sexual abstinence. Samples were ejaculated into sterile containers and allowed to liquefy at 37°C for 30 min before processing. Sperm concentration, semen volume, liquefaction, pH, motility, viability and morphology assessment validated samples as normozoospermic, in accordance with World Health Organization criteria (2010). The samples were divided into three and processed so that there were subsamples of fresh, capacitated and acrosome-reacted spermatozoa.

Semen capacitation was performed using the swim-up method: semen samples were washed in F10 Nutrient Mixture Medium (HAM; Invitrogen, Carlsbad, CA, USA) supplemented with HSA (1% human serum albumin, 0.003% sodium pyruvate, 0.36% sodium lactate, 0.2% sodium bicarbonate) at 37°C and centrifuged at 400***g*** for 10 min. Sperm pellets were then incubated with fresh HAM supplemented with HSA in a 5% CO_2 atmosphere at 37°C for 60 min. Highly motile spermatozoa were selected by carefully collecting the top supernatant layer. The efficiency of the swim-up technique was evaluated with phase-contrast microscopy (DMRB; Leica), discarding those samples with <95% of progressive motile spermatozoa. Individual aliquots of swim-up selected spermatozoa were incubated in calcium ionophore A23187 (Sigma–Aldrich) for induction of the acrosome reaction (Aitken et al., 1993). Acrosomal status was evaluated by fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin labelling (FITC-PSA, dilution 1:20; Sigma–Aldrich; Cross and Meizel, 1989), in a total of 200 sperm cells ($n = 7$ independent samples).

Fluorescence immunocytochemistry was carried out on spermatozoa fixed in methanol at −20°C. Samples were blocked with bovine serum albumin and incubated with monoclonal anti-α-tubulin antibody (1:500; Sigma–Aldrich). Samples were washed in phosphate-buffered saline and then incubated for 1 h at room temperature with DyLight 488 donkey anti-mouse IgG antibody (1:300; Jackson Immuno Laboratories; Fouquet et al., 1998). Negative controls were performed by omitting either primary or secondary antibody. Immunofluorescence was analysed using a Leica TCS SP2 confocal laser scanning microscope. The whole flagellum area and the α-tubulin-labelled area were measured using Adobe Photoshop CS4 (Adobe Systems, Mountain View, CA, USA).

The percentage of α-tubulin-labelled area in relation to flagellum total area was quantified in 200 sperm cells. This percentage was then used to conduct a two-way hierarchical clustering analysis performed on each sample using the Ward Method from the JMP 8 statistical software package (SAS Institute, Cary, NC, USA) (De Juan et al., 1992; Ward, 1963).

To examine differences between the means, multiple comparison tests were performed using one- and two-way analysis of variance followed by the Bonferroni post-hoc test with α 0.05 (95% confidence interval). Data were expressed as the mean ± SEM and $P < 0.05$ was considered statistically significant.

This study used retrospective data from anonymous medical records and was approved by the Institutional Review Board (2012JAN01, approved 10 January 2012).

Results

Immunocytochemical data showed that α-tubulin was not distributed uniformly throughout the flagellum; thus, in some spermatozoa, only a small percentage of the flagellum was labelled for α-tubulin (**Figure 1A–C**) while in others, this protein was located throughout most of the flagellum (**Figure 1D–F**). α-Tubulin immunofluorescence was absent in negative controls (data not shown).

On the basis of the α-tubulin-labelled area, the spermatozoa were classified into three clusters: cluster A (area ≥66.66%), cluster B (area 33.33–66.66%) and cluster C (area ≤33.33%). Then, based on the areas, a heat map and dendrograms were obtained, providing a visual representation of sperm distribution in fresh, capacitated and acrosome-reacted samples (**Figure 2**).

In cluster A, the sperm percentage increased in capacitated and acrosome-reacted samples (cluster A: fresh 22.95%, capacitated 31.65%, acrosome-reacted 46.97%). However, in clusters B and C, the sperm percentage decreased in capacitated and acrosome-reacted samples (cluster B: fresh 29.51%, capacitated 26.61%, acrosome-reacted 17.42%; cluster C: fresh 47.54%, capacitated 41.74%, acrosome-reacted 35.61%; **Figure 3**).

On the other hand, when the mean for the α-tubulin-labelled area in cluster A was statistically

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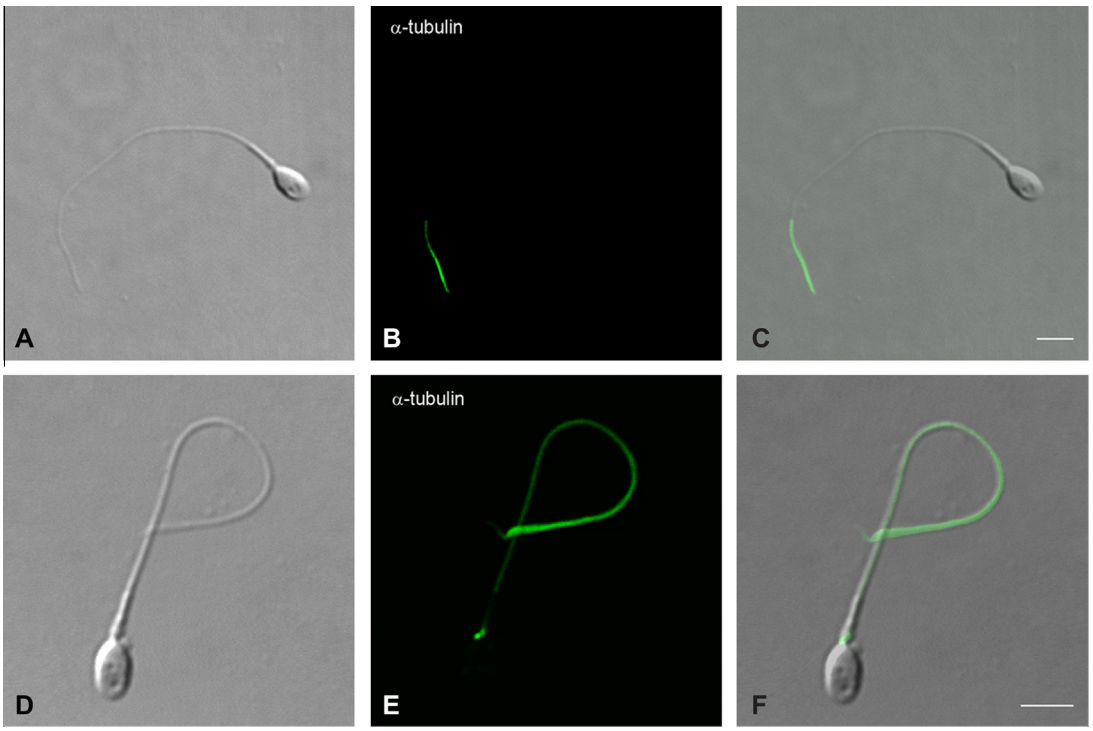

Figure 1 Immunolocaization of α-tubulin in human spermatozoa. Confocal micrographs shows that in some spermatozoa, α-tubulin was located only in the flagellum end part (**A**–**C**), while in others it was detected all over the flagellum (**D**–**F**). Bars = 5 μm.

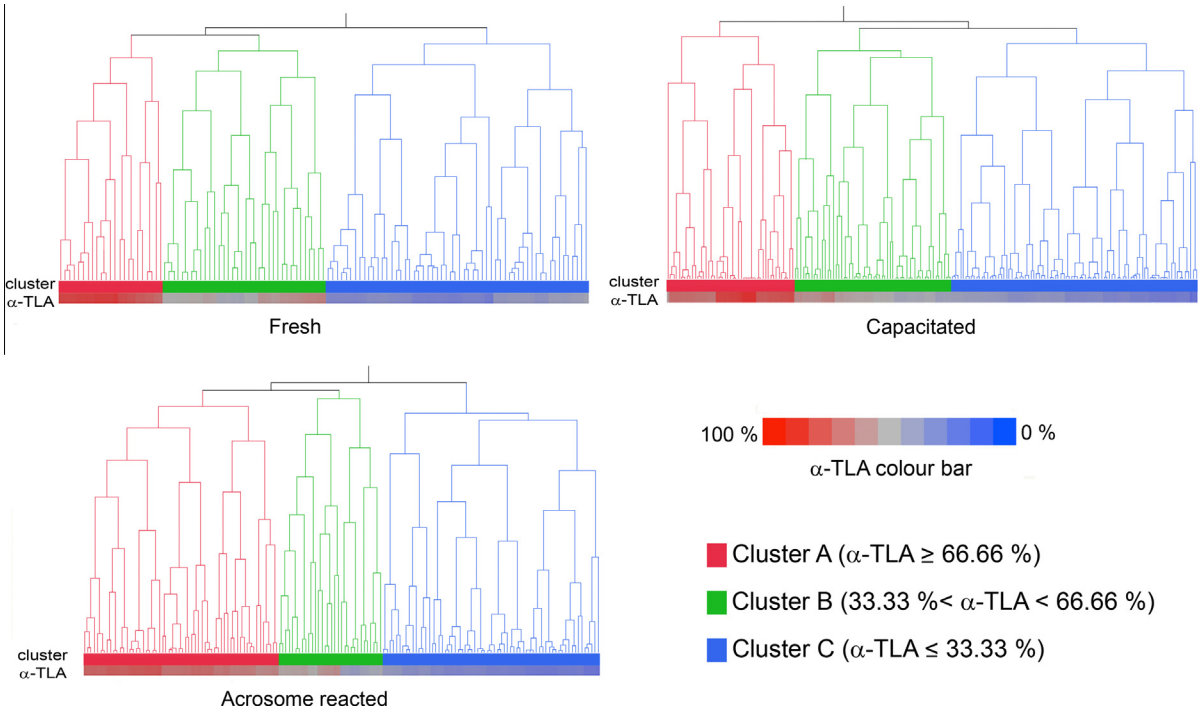

Figure 2 Cluster sperm distribution. Heat maps and dendrograms provided visual representations of the distribution of spermatozoa in fresh, capacitated and acrosome-reacted samples according to the α-tubulin-labelled area (α-TLA). (For interpretation to colors in this figure, the reader is referred to the web version of this paper.)

analysed, there were significantly lower values for capacitated and acrosome-reacted samples in relation to fresh samples ($P < 0.05$). However, the α-tubulin-labelled area mean was homogeneous in clusters B and C, with no significant difference between fresh, capacitated and acrosome-reacted samples (Table 1).

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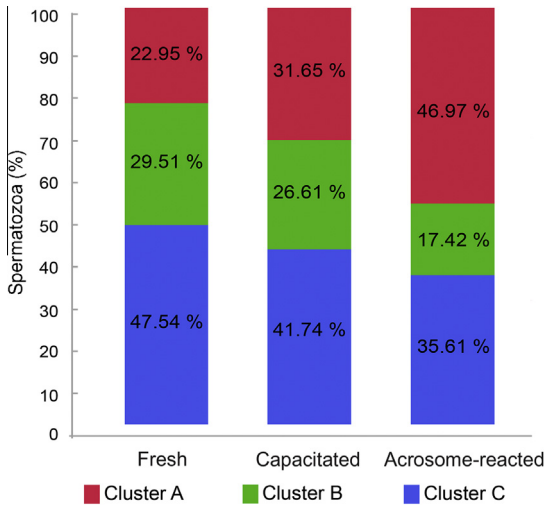

Figure 3 Quantification of cluster sperm distribution. In cluster A (α-tubulin-labelled area ≥66.66%), the sperm percentage increased in capacitated and acrosome-reacted versus fresh samples, while in clusters B (α-tubulin-labelled area 33.33–66.66%) and C (α-tubulin-labelled area ≤33.33%), this percentage decreased in capacitated and acrosome-reacted versus fresh samples.

Table 1 Quantification of α-tubulin-labelled area.

	α-Tubulin-labelled area (%)		
Cluster A	*Cluster B*	*Cluster C*	
Fresh	90.40 ± 1.63	48.44 ± 1.51	19.00 ± 1.01
Capacitated	82.80 ± 1.61[a]	46.73 ± 1.21	20.79 ± 0.80
Acrosome-reacted	85.35 ± 1.20[a]	50.18 ± 2.04	18.09 ± 1.28

Values are mean ± SEM.
[a]Significant difference versus fresh ($P < 0.05$).

Discussion

These data show that α-tubulin is not uniformly distributed throughout the flagellum. It is well known that tubulin undergoes different post-translational modifications at the C-terminus, such as polyglutamylation (Garnham and Roll-Mecak, 2012), and polyglutamylated tubulin is present in certain areas of the flagellum. For example, in mouse spermatozoa polyglutamylated tubulin was mainly found in the middle and terminal piece (Fouquet et al., 1996). Thus, post-translational modifications could mask the α-tubulin epitope, resulting in the different immunocytochemical patterns observed in the samples.

Moreover, this study has demonstrated that, in enriched motile sperm samples (capacitated and acrosome-reacted), the percentage of spermatozoa increased where most of the flagellum is immunoreactive for α-tubulin. In this sense, tubulin is a structural protein of the flagellum and it has been proven that tubulin expression decreases in low-motility samples (asthenozoospermia) (Peknicova et al., 2007). Consequently, the presence of α-tubulin throughout most of the flagellum could be associated with the structural functionality required for sperm motility.

However, although there was a higher percentage of spermatozoa in cluster A capacitated and acrosome-reacted samples, the mean labelled area was significantly lower compared with fresh samples. Capacitation is a process that involves various changes, among them are an increase in intracellular calcium (Baldi et al., 1991), hyperactivated motility (Tulsiani and Abou-Haila, 2011) and an increase in protein phosphorylation (Urner and Sakkas, 2003; Visconti et al., 2011).The latter is the main post-translational modification involved in the capacitation of many mammalian species (Galantino-Homer et al., 1997; Kalab et al., 1998; Osheroff et al., 1999). Most proteins in human spermatozoa that undergo phosphorylation are found in the flagellum (Liu et al., 2006; Sakkas et al., 2003) and require the presence of albumin and bicarbonate in the extracellular medium (Wolf et al., 1986). Albumin produces cholesterol sequestration changing membrane fluidity, while bicarbonate is related to the synthesis of cAMP (Visconti et al., 1999). Increased cAMP activates protein kinase A, which in turn activates other kinase enzymes that are responsible for protein phosphorylation (Visconti et al., 1999). Among the target proteins of these kinases include glutathione S-transferase, NADH dehydrogenase and tubulin (Arcelay et al., 2008; Ficarro et al., 2003). In this regard, the decrease in α-tubulin-labelled area in capacitated spermatozoa could be related to protein phosphorylation produced in these proteins during the capacitation process.

In summary, this work has demonstrated that there are different sperm clusters distinguished by their α-tubulin immunoreactivity and that the number of members of each cluster is modified in capacitated and acrosome-reacted samples. These findings suggest that α-tubulin could be directly related to structural changes during capacitation and acrosome reaction.

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