

Cytoskeleton localization in the sperm head prior to fertilization

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Abstract

Three major cytoskeletal proteins, actin, tubulin and spectrin, are present in the head of mammalian spermatozoa. Although cytoskeletal proteins are implicated in the regulation of capacitation and the acrosome reaction (AR), their exact role remains poorly understood. The aim of this study was to compare the distribution of the sperm head cytoskeleton before and after the AR in spermatozoa representing a range of acrosome size and shape. Spermatozoa from the human and three rodents (rat, hamster and grey squirrel) were fixed before and after the AR in appropriate medium *in vitro*. Indirect immunofluorescent localization of cytoskeletal proteins was undertaken with antibodies recognizing actin, spectrin and α -tubulin. Preparations were counterstained with propidium iodide and examined by epifluorescent and confocal microscopy. Our results clearly demonstrated changes in localization of cytoskeleton during the AR, mainly in the apical acrosome with further changes to the equatorial segment and post-acrosomal regions. The pattern of cytoskeletal proteins in the sperm head of all the species was similar in respect to various sub-compartments. These observations indicated that the sperm head cortical cytoskeleton exhibits significant changes during the AR and, therefore, support the image of cytoskeletal proteins as highly dynamic structures participating actively in processes prior to fertilization.

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Introduction

The morphology of the head of the mammalian spermatozoon can vary significantly (see Yanagimachi 1981, Eddy & O'Brien 1994) and species display a wide variety of sperm head shapes and features. Under various selective pressures, spermatozoa evolve in order to benefit their biological functions in the male or female genital tracts and during fertilization. Co-evolution of the male and female gamete leads to further cellular and molecular diversification and reproductive isolation (Bedford 1991, Swanson & Vacquier 2002). This relatively rapid evolution means that, in most cases, the significance of the morphology of a particular sperm head of a species has remained obscure. Although the shape and relative size of the sperm head may vary considerably, basic organelles such as the apical acrosome, equatorial segment and post-acrosomal region are invariably present and these structures can be identified readily in spermatozoa of most mammals. This may indicate that critical constitutive aspects of the function of the sperm head have been conserved (e.g. capacitation, acrosome reaction (AR) and sperm–egg fusion). In contrast, other aspects of sperm

function such as motility and sperm transport through the female tract may be subject to greater evolutionary adaptation and might have resulted in the large morphological differences; for example, acrosome size (e.g. squirrel sperm) or some other bizarre feature (e.g. ventral sperm head hooks of plains rat or wood mouse (Breed *et al.* 2000, Moore *et al.* 2002)).

The cytoskeleton of the sperm head consists of the resistant structural proteins of the nucleus, and perinuclear theca, which are thought to be responsible, at least in part, for shaping the nucleus (Oko 1998), and the more dynamic proteins of the cortical cytoskeleton. As well as a morphological function, the latter is believed to be involved in cell signalling processes, where data suggest that actin polymerization may represent an important regulatory pathway which is associated with tyrosine phosphorylation in sperm (Brener *et al.* 2003, Seligman *et al.* 2004). In general, cytoskeletal proteins include actin, actin-binding proteins such as spectrin, various tubulins (e.g. α -, β -, γ -tubulin) and others. In somatic cells, actin filaments lying beneath the plasma membrane form a cortical network involved in functions such as

cell-to-cell contact and adhering to extracellular matrix, cell growth and migration, and exocytosis. In spermatozoa, a number of cytoskeletal proteins are implicated in key events such as capacitation and the AR (Yanagimachi 1981, Virtanen *et al.* 1984, Brucker & Lipford 1995, Brewis & Moore 1997, Howes *et al.* 2001, Moore 2001, Brener *et al.* 2003). For instance, actin may play a role in the AR (Vogl 1989, Palecek *et al.* 1999) and inhibition of actin polymerization by cytochalasin B and D blocked induction of the human AR by zona pellucida (Liu *et al.* 1999, Dvorakova *et al.* 2001). In contrast, phorbol myristate acetate was shown to induce a dramatic change in the ultrastructure of the human acrosome region, termed acrosomal ruffling, which is believed to involve actin polymerization (Liu *et al.* 2002, Brener *et al.* 2003). Recently, confocal and electron microscopic studies of boar spermatozoa confirmed that actin, spectrin and tubulin-containing structures are altered after the AR and that cytoskeletal inhibitors influence this event (Peknicova *et al.* 2001, Dvorakova *et al.* 2001, Brener *et al.* 2003).

Four main sperm head types can be readily distinguished in mammals (Yanagimachi 1981). These are a spatulate shape (e.g. boar, bull and rabbit), an ovoid shape (e.g. human and other primates), a falciform shape (e.g. most rodents) and spermatozoa with large acrosomal regions (e.g. guinea pig and grey squirrel). Cross-species comparisons may provide important clues as to the function of cytoskeletal proteins during the fertilization processes and in shaping the sperm head. However, the relationship between sperm head morphology, cytoskeletal elements and events, such as capacitation and the AR, have not been investigated in detail with the exception of spatulate-shaped boar spermatozoa (Yagi & Paranko 1995, Dvorakova *et al.* 2001, Peknicova *et al.* 2001). We report here on the localization of cytoskeletal proteins before and after induction of the AR *in vitro* in representative sperm types, namely spermatozoa of the human, the laboratory rat, the hamster and the grey squirrel.

Materials and Methods

Animals

The following male animals were used for our experiments: rat (Wistar), golden hamster (*Mesocricetus auratus*) and grey squirrel (*Sciurus carolinensis*). Golden hamsters and rats were maintained and housed at the Field Laboratories, University of Sheffield. Grey squirrel spermatozoa were kindly provided by Mr Mark Ferryman, The Forestry Commission, Wreclsham, Surrey, UK.

Ethics of experimentation

Ethical permission to use donated human sperm for research was given by the South Sheffield Research Ethics Committee and consent was also obtained from patients.

All animal procedures were carried out in strict accordance with the Animal (Scientific Procedure) Act 1986, and subjected to review by the local ethics committee. Animals were killed by lethal injection (2 µl/g) of sodium pentobarbitone.

Reagents

All chemicals were purchased from Sigma Chemicals Ltd, UK unless otherwise stated.

Collection and preparation of spermatozoa

Human sperm ejaculates were obtained from donors of proven fertility attending the Andrology Laboratory, Jessop Hospital, Sheffield, UK. All samples were in the normal range for sperm concentration, motility and morphology as determined by standard protocols (WHO 1992). Spermatozoa of the rat, golden hamster and grey squirrel were collected from the cauda epididymis of adult males.

Spermatozoa were recovered from the cauda epididymis by puncturing epididymal tubules and releasing spermatozoa in phosphate-buffered saline (PBS) for 10 min. The suspension of both epididymal and human ejaculated sperm was washed twice in PBS. Sperm motility was examined by an inverted microscope with a thermostatically controlled stage at 37°C. The AR was induced after the incubation of spermatozoa by the addition of the calcium ionophore A-23187 at a final concentration of 2 µM, followed by a 30-min incubation at 37°C in 5% CO₂ in air (Moos *et al.* 1993). In order to evaluate whether sperm had undergone the AR, lectin PNA conjugate Alexa Fluor 488 (L-21409; Molecular Probes, Prague, Czech Republic) was used. Research has shown PNA to be selective for acrosomes in the sperm of rat, human, hamster and others (Calvo *et al.* 1995, Fierro *et al.* 1996, Aviles *et al.* 1997). PNA solution of a final concentration of 1 µl/ml was added to the sperm suspension and the status of the acrosome in 200 sperm was examined under the epifluorescent microscopy. Drops of spermatozoa from the stock were aspirated gently by glass pipette onto a glass slide, smeared, air-dried and fixed for immunocytochemistry. For spectrin and α-tubulin immunolocalization, spermatozoa were fixed with 100% methanol for 8 min and 100% acetone for 6 min at -20°C. For actin immunolocalization, spermatozoa were fixed with 4% formaldehyde in PBS at 22°C (45 min) then washed in PBS, rinsed with distilled H₂O and air-dried. Adequate control slides were run simultaneously through the whole experiment.

Indirect immunofluorescent localization

Slides with fixed spermatozoa were rinsed with PBS and, after 1-h blocking with 3% bovine serum albumin (BSA) in PBS, incubated for 1 h at 37°C with primary antibodies against G- and F-actin (A2668), spectrin (S1515), α-tubulin (T9026), or without BSA blocking, and at room

temperature with fungal toxin Phalloidin-Fluorescein Iso-cyanate (FITC; P5282). Antibodies were diluted in 3% BSA. After washing in PBS, slides were incubated for a further hour with secondary antibody FITC conjugate sheep anti-mouse IgG (F5387) or goat anti-rabbit IgG (F7512). Alexa Fluor 594 donkey anti-rabbit IgG (A21207; Molecular Probes) was used to detect spectrin in spectrin-actin co-localization experiments. After repeated washing in PBS, slides were mounted in Vectashield mounting medium with or without propidium iodide (Vector Laboratories, Prague, Czech Republic) for counterstaining the nucleus. Slides were routinely examined by epifluorescent microscope. The photomicrographs shown were taken by confocal microscope Leica TCS 4D, with a tunable 457–675 nm laser excitation and digital image capture (Fig. 2) or Leica TCS SP2 RS high-speed confocal/two photon system for live cell imaging and dynamics (Figs. 3 and 4).

Immunoblot analysis

The biochemical specificity of primary antibodies was examined by SDS-electrophoresis and immunoblotting technique. SDS-PAGE was performed according to the method of Laemmli (1970), Western blot was done according to Towbin *et al.* (1979). Briefly, sperm samples were washed twice with PBS, resuspended in non-reducing lysis buffer and heated to 100 °C for 5 min. A protein equivalent of 10⁶ cells was loaded into wells of an SDS-PAGE gel. Precision Plus Protein All Blue Standards (BioRad, Nad ostrovem, Prague, Czech Republic) were used as molecular weight markers. Separated proteins were transferred electrophoretically onto nitrocellulose membrane and detected by incubation with a primary antibody, followed by a secondary antibody, either anti-mouse IgG (A0168) or anti-rabbit (A0545) IgG peroxidase conjugate. Non-specific sites were blocked with PBS blocking solution (5% skim milk and 0.05% Tween 20). Protein bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Amersham, Bucks, UK).

Results

For clarity, the comparative localization of cytoskeleton changes in the sperm head in control and after the AR induction is described for each protein investigated. The fluorescent staining of comparative localization is shown in Table 1. For each analysed group, after optimizing working conditions, 200 cells were examined in three parallel experiments. Results given represent approximately 90% of the population. The status of the acrosome after the calcium ionophore-induced AR was examined with PNA lectin in 200 cells for each group and experiment. If a minimum of 95% of sperm had undergone the AR, then the population of sperm was accepted for further experiments. Parallel controls with untreated sperm were run for each experiment.

Table 1 Localization of cytoskeletal proteins in sperm head control and after the AR. In the case of actin, data show summarized results, obtained by both antibody anti-G- and F-actin and phalloidin-FITC conjugate.

Protein	Species	Acrosome	ES	PA
Actin	Human	+/ \pm	\pm /+	+/+
	Rat	+/ \pm	\pm /+	+/+
	Hamster	+/ \pm	\pm /+	+/+
	Squirrel	+/ \pm	+/+	+/+
Spectrin	Human	-/-	+/+	+/+
	Rat	+/-	+/+	+/+
	Hamster	+/-	+/+	+/+
	Squirrel	+/-	+/+	+/+
α -tubulin	Human	-/ \pm	+/+	+/+
	Rat	-/-	\pm /+	+/+
	Hamster	+/-	\pm /+	+/+
	Squirrel	+/-	+/+	+/+

Fluorescence signal: positive (+); absent (-) and weak (\pm). ES, equatorial segment; PA, post-acrosomal region.

Specificity of antibodies

Immunofluorescence was not detected on controls without primary antibody, which were run throughout the whole experiment (not shown). The specificity of antibodies was also proved by pre-incubation of antibodies with authentic antigens, anti-actin, spectrin and α -tubulin, which blocked the immunostaining. Immunoblotting of proteins extracted from human and rodent sperm samples showed clear bands at the relative molecular weights for the corresponding antigen (actin, 42 kDa; α - and β -spectrin, 240 and 220 kDa; α -tubulin, 55 kDa) (Fig. 1, rat sperm extract). Equivalent bands were detected for all species indicating similar cross-species specificity. The specificity of the polyclonal antibody for G- and F-actin was stressed by results obtained with Phalloidin-FITC or Alexa Fluor conjugate, which binds specifically to F-actin only.

Localization of cytoskeletal proteins in the sperm head

Representative micrographs of studied cytoskeletal proteins in control spermatozoa and after the calcium ionophore-induced AR are presented in Figs. 2 and 3, and summarized in Table 1.

Actin

There were similarities in the localization of actin in the sperm head for each selected species before treatment (Figs. 2a and 3a and 3c). Bright immunofluorescent staining was seen in the entire region of the apical acrosome, covering the cortical cytoskeleton between the plasma membrane and the outer acrosomal membrane, and the sub-acrosomal cytoskeleton between the inner acrosome membrane and the nuclear envelope. In spermatozoa of the grey squirrel, the cortical cytoskeleton of apical acrosome displayed a spotty localization and, besides the staining in equatorial segment, there was another actin

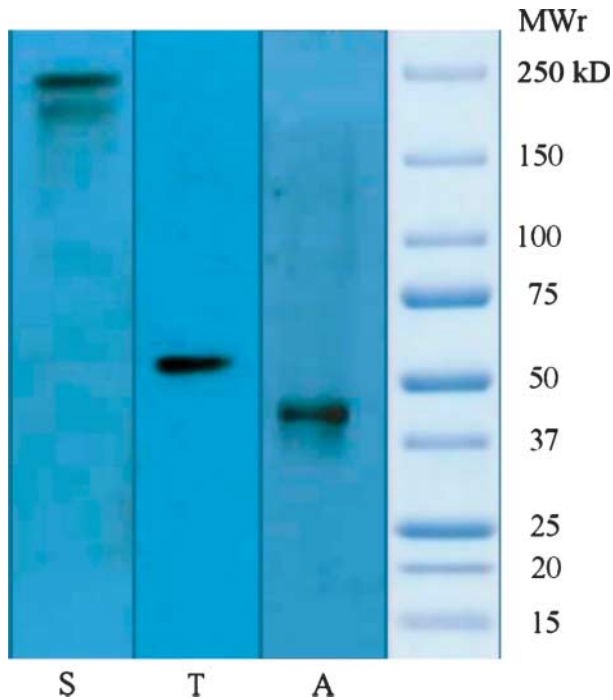


Figure 1 Immunoblotting analysis of rat spermatozoa extract for (S) spectrin (α and β), 240 and 220 kDa, (T) α -tubulin, 55 kDa and (A) actin, 42 kDa. Analysis for the remaining rodent and human sperm was the same as for the rat (not shown). MWr; Molecular weight marker.

ring seen in the post-acrosomal region. Several changes were observed after the AR. Generally, the most prominent became the equatorial segment that was clearly stained in all species studied (Figs. 2b and 3b and 3d). In squirrel sperm after the AR, the inner acrosome membrane had a punctuate appearance compared with untreated sperm (Fig. 2b). The pattern given by staining with Phalloidin-FITC (Fig. 3) was quite different in control spermatozoa. The anti-actin antibody detected intense staining in the entire region of the apical acrosome, even between the plasma membrane and the acrosomal membrane. In contrast, Phalloidin detected F-actin enriched in the acrosomal region, clearly defining the shape of the acrosome vesicle (Fig. 3c and 3e). Generally in human sperm, the distribution of actin cytoskeleton in the post-acrosomal region was weaker in control sperm than in those after AR which is best seen in the pictures without propidium iodide nuclear staining (Fig. 3). In rodent spermatozoa after the AR, actin localization became prominent in the very distal apical region, which contains para-acrosomal cytoskeleton (Figs. 2b and 3d and 3f). However, the labelling was present in control sperm as well (Fig. 2a and 3c and 3e).

In order to obtain the sharpest picture of the sperm head, which was our main interest, we sometimes merged only selected sections obtained by confocal microscopy. These sections did not necessarily have to cover the plane of the tail; therefore the staining of the tail; could sometimes be seen as weak or not present.

Spectrin

The staining pattern for spectrin was similar to the one for actin in certain aspects. This protein was present in the acrosome domain of rodent sperm head (Figs. 2c and 4b). In human spermatozoa, there was discrete bright immunofluorescence of the apical equatorial segment without a strong labelling of cortical cytoskeleton lying between the plasma and outer acrosomal membrane. Localization in the equatorial segment similar to the one seen in actin was apparent again in squirrel spermatozoa (Fig. 2c). After the AR, the pattern in human spermatozoa in the equatorial segment remained mainly unchanged; however, a slight increase in labelling over the post-acrosomal region was seen (Fig. 2d). Localization of spectrin in rodent spermatozoa was similar to the one for actin in the equatorial segment, with a stronger immunostaining in the post-acrosomal region (Fig. 2d). Para-acrosomal cytoskeleton in rat and hamster sperm head was strongly labelled both before (Figs. 2c and 4b) and after the AR (Fig. 2d).

The double immunofluorescence staining technique confirmed that spectrin and F-actin co-localize with each other (Fig. 4, control rat spermatozoa only shown). In human spermatozoa before the AR, spectrin and F-actin co-localized only in the equatorial segment and the apical peri-nuclear theca, due to the fact that in control human spermatozoa spectrin was not detected in the acrosome. In addition we ran fluorescent resonance energy transfer analysis, which confirmed the co-localization of these two proteins in the above-mentioned organelles of control and acrosome-reacted sperm (K Dvorakova & F Difato, unpublished results).

α -Tubulin

Prior to the AR, the apical acrosome cytoskeleton and equatorial segment in human and all rodent species displayed some fluorescence (Fig. 2e). In human sperm, a ring in the middle of the apical acrosome was prominent. Fluorescence was also seen in the post-acrosomal region, especially in hamster sperm where strong well-defined dorsal and ventral localization was observed. After the AR (Fig. 2f), the equatorial segment became more crisply defined but in the sub-acrosomal layer the faint fluorescence was seen only in the grey squirrel sperm. The para-acrosomal and post-acrosomal regions remained unchanged essentially in all falciform head sperm types. (Fig. 2e and 2f).

Discussion

The objective of this study was to examine the microfilament and microtubular cytoskeleton of the sperm head in relation to the ionophore-induced AR. The relocation of cytoskeletal proteins following the AR is likely to be important for subsequent events of fertilization, such as sperm-zona binding, sperm penetration of the zona

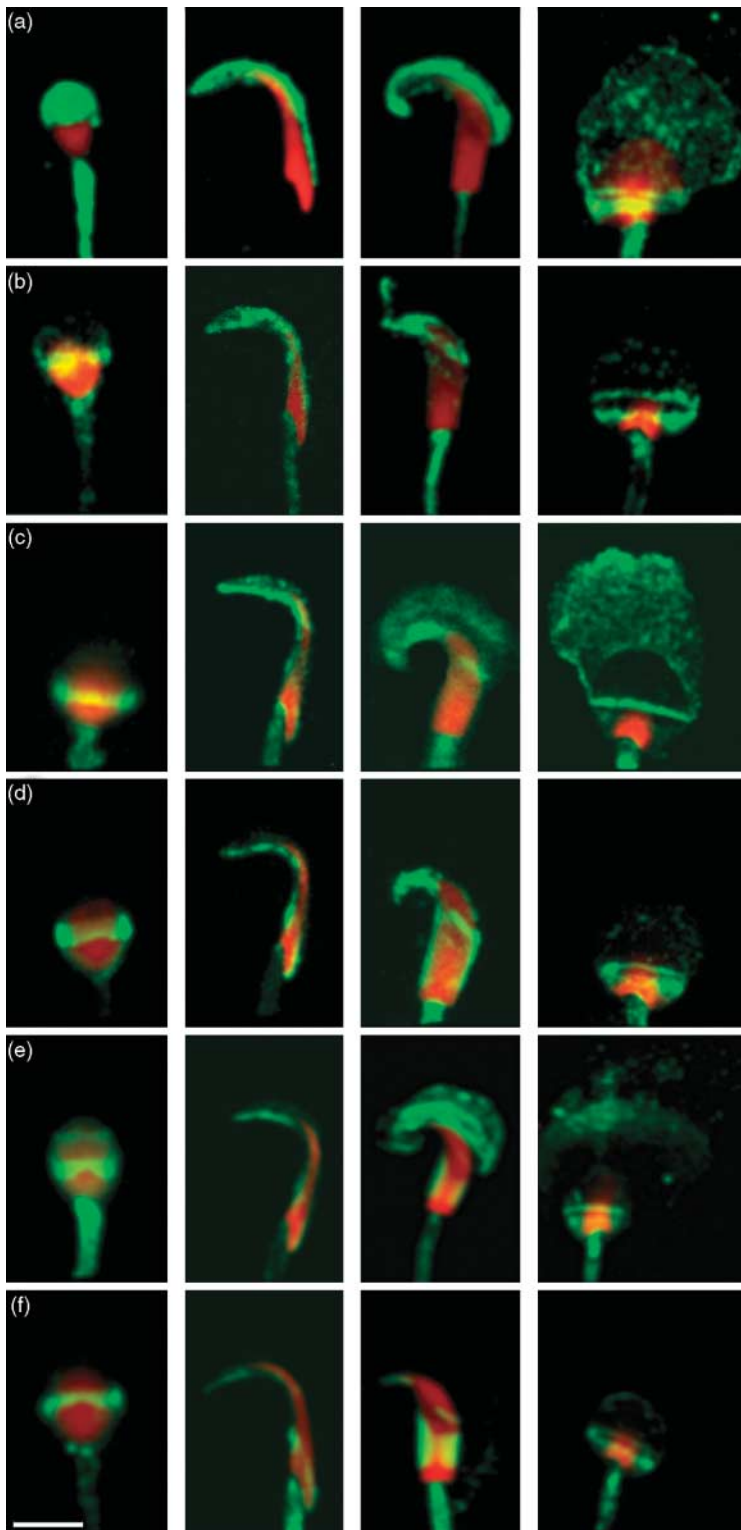


Figure 2 Immunofluorescent localization of cytoskeletal proteins in the sperm head of human, rat, hamster and grey squirrel spermatozoa (left to right). Actin (a) control and (b) after AR, spectrin (c) control and (d) after AR and α -tubulin (e) control and (f) after AR. Red is propidium iodide staining of the nucleus; green corresponds to the cytoskeletal proteins. Scale bar = 5 μ m.

pellucida or sperm–egg fusion (Brewis & Moore 1997, Pri-makoff & Myles 2002). Here, we were interested in comparing generic aspects of cytoskeletal localization in mammalian spermatozoa with the more specific features related to morphology, since it is clear that the kinetics of

the AR in terms of vesiculation of membrane and loss of contents varies considerably between species (Yanagima-chi 1981). It was surmised that by carefully comparing spermatozoa with different head shape one might ascertain whether antigen localization patterns reflected primarily

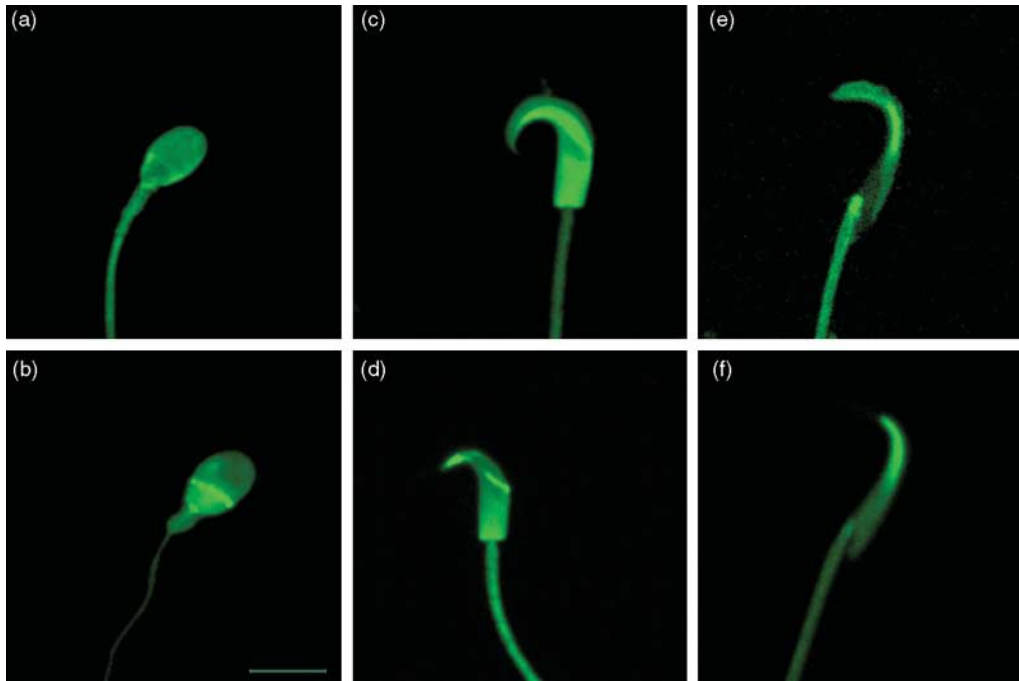


Figure 3 Immunofluorescent localization of F-actin by Phalloidin-FITC in the sperm head of human (a) control and (b) after AR, hamster (c) control and (d) after AR and rat (e) control and (f) after AR. Scale bar = 5 μm .

morphological or functional features of the cell. The cytoskeletal proteins selected were the same as those used in a previous study with boar spermatozoa (representing the spatulate morphology), where it was shown that these

proteins were redistributed during and after the AR (Dvorakova *et al.* 2001). The AR was induced with calcium ionophore to produce a consistent population of cells which had undergone the completed AR (Jaiswal *et al.* 1999).

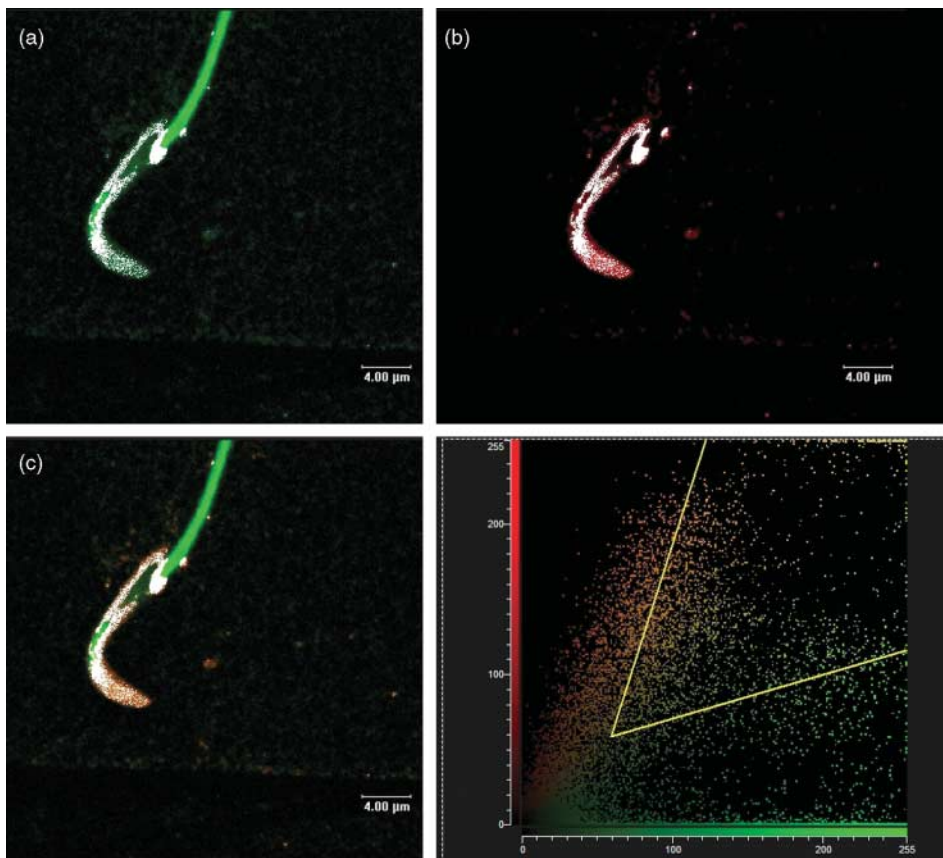


Figure 4 Co-localization of F-actin and spectrin in control spermatozoa; the white marking represents the co-localization of both proteins, which is defined by the graph; (a) F-actin, (b) spectrin and (c) merged. Green corresponds to F-actin; red corresponds to spectrin. In the graph, the co-localization of two proteins is defined by the trapezoid lying between the x and y axes. Scale bars = 4 μm .

The presence of actin within the head of the selected spermatozoa has been confirmed previously in several studies, although the exact distribution has been shown to vary, possibly because of the methods of fixation, antibody specificity, sensitivity of technique and membrane permeabilization (Fouquet *et al.* 1990, Fouquet & Kann 1992). The reasons for choosing anti-actin and spectrin polyclonal antibodies for this study was their convenient cross-reactivity and therefore consistency in immunohistochemical testing across all selected species, and also because of obtaining consistent results with previous testing of boar spermatozoa representing spatulate sperm head shape (Dvorakova *et al.* 2001). We also used fungal toxin Phalloidin conjugated with FITC for the exclusive staining of polymerized F-actin microfilaments. The results obtained by Phalloidin-FITC in control sperm were quite different from those obtained by polyclonal anti-actin antibody. In control sperm, anti-actin antibody detected the entire apical acrosome, even between the plasma and the outer acrosomal membrane; however, in contrast, F-actin, detected by Phalloidin, was clearly confined to the acrosome region. This difference could be explained by the pool of G-actin localized between the plasma membrane and the acrosomal membrane, which would only be detected by the anti-actin antibody. These results contrast somewhat with the investigation of Howes *et al.* (2001), where actin and actin-binding proteins were localized to just the margin of the anterior acrosomal and post-acrosomal region of mature epididymal bovine spermatozoa and relocated to a post-acrosomal region after the AR. However, the relocalization of F-actin to the post-acrosomal region in boar ejaculated sperm after the AR described by Howes *et al.* (2001) correlates with our results on human sperm after the AR; however, the weak labelling in this region was seen in control sperm as well. This clear redistribution of F-actin in the post-acrosomal region was not seen in rodent sperm, and may be due to the fact that samples of rodent sperm were obtained from the epididymis. Nevertheless, in agreement with the studies of Brener *et al.* (2003) and Paranko *et al.* (1994), the post-acrosomal region in control sperm showed the presence of F-actin, and this was in contrast to Howes *et al.* (2001). The polymeric form of actin (F-actin) is thought to be particularly important as its depolymerization between the outer acrosomal and plasma membrane is correlated with initiation of the AR in studies on guinea pig (Breitbart & Spungin 1997) and on bull, ram, mouse and human sperm (Brener *et al.* 2003). In this respect, the spermatozoa of the grey squirrel represents the closest comparison as they have a massive acrosome, and actin was clearly localized mainly to the cortical cytoskeleton between the plasma and outer acrosomal membrane in control spermatozoa. A punctuate appearance over the acrosomal region suggested that there were focal points of actin along the membrane. The wider filamentous network might have been observed in spermatozoa which were capacitated (Brener *et al.* 2003). This would be in correlation with our findings on the localization of a

monomeric form of actin between the plasma membrane and the outer acrosomal membrane in control spermatozoa. This pool of G-actin could therefore be polymerized during the capacitation; however, this was not the aim in this study. These observations suggested that actin does not serve only as a structural cytoskeletal protein to maintain shape but may also be involved in the events prior to fertilization, such as the AR.

After induction of the AR, the relative distribution of actin seemed to be related to the type of reaction. In both the squirrel and the hamster, the AR is characterized by rapid divestment of the entire acrosomal cap. In these species, actin remained localized under the inner acrosomal membrane and was more prevalent at the apical margin of the equatorial segment. Moreno-Fierros *et al.* (1992) showed a similar redistribution of F-actin in guinea pig spermatozoa. For human and rat spermatozoa there was noticeably more actin localized on vesiculated acrosomal membrane. This may correspond to actin playing a role in maintaining the presence of vesiculated membranes after the AR to aid in the tethering of the fertilizing spermatozoa to the zona pellucida. Generally, the most prominent became the equatorial segment after the AR induction in all species studied, and the apical acrosome showed a decrease of actin labelling, which is in agreement with the findings of both Howes *et al.* (2001) and Brener *et al.* (2003). Moreover Yagi & Paranko (1995) suggested that lateral prongs may be a merger zone for actin and two other actin-binding proteins such as α -actinin and spectrin.

The role of actin-binding proteins has also been investigated previously by Virtanen *et al.* (1984) who suggested that spectrin and vimentin can be in correlation with distinct surface specializations of the sperm cells. These findings are in agreement with our results which demonstrated the co-localization of spectrin with actin in both control and acrosome-reacted sperm, with the exception of human spermatozoa before the AR, where spectrin co-localized only in the equatorial segment and the apical peri-nuclear theca. In addition, perforatorium, containing a para-acrosomal cytoskeleton, and post-acrosomal regions stained for spectrin, in the case of rat and hamster sperm. Its presence in the acrosomal plasma membrane has also been confirmed by results from electron microscopy observations (Hernandez-Gonzalez *et al.* 2000). Interestingly, even though squirrel sperm exhibited actin at the proximal and distal margin of the equatorial segment in both control sperm and acrosome-reacted sperm, in the case of spectrin only one narrow band across the equatorial segment was seen in control sperm. However, after AR induction the second band in the post-acrosomal region was revealed. This may suggest the relocalization of spectrin during the AR due to its structural role in the stabilization of the equatorial segment as suggested by Yagi & Paranko (1995). Besides spectrin possibly having a structural role, there is a possibility that it also plays an active role during sperm-egg fusion (Yagi

& Paranko 1995) or even during capacitation and the AR by its ability to form the spectrin/calmodulin complex that involves Ca^{2+} as a mediator (Virtanen *et al.* 1984).

There is plenty of evidence for the presence of tubulins in spermatocytes and spermatids during mammalian spermatogenesis (Moreno & Schatten 2000, Kierszenbaum 2002, Kato *et al.* 2004) and also in mature spermatozoa (Draber *et al.* 1991, Dvorakova *et al.* 2001, Peknicova *et al.* 2001). However, because of the large diversity in the tubulin superfamily there is a gap in the field covering spermatogenesis and mature sperm undergoing changes prior to fertilization. Most eukaryotic cells can express multiple isoforms of α - and β -tubulin and this diversity is further expanded by a number of post-translational modifications (Luduena 1998). Although the functional significance of tubulin diversity is still difficult to associate with consistent biological functions, there is increasing evidence that different α - and β -tubulin isoforms and post-translational modification can impact microtubule structure and function (Kierszenbaum 2002).

We have here reported the obvious differences in the localization of staining of the α -tubulin suggesting that these proteins may be related to morphology as well as function. In squirrel, and hamster control sperm, α -tubulin was clearly localized within the cortical cytoskeleton of the apical acrosome region. These cortical microtubules might be a network which was originally distributed in the cortex of spermatocytes and later helped with forming the acrosomal vesicle by being attached to Golgi apparatus (Moreno & Schatten 2000). In human spermatozoa, α -tubulin was displayed as a distinct band within the apical acrosome, suggesting a restricted distribution of this protein in the apical peri-nuclear theca between the inner acrosomal and nuclear membranes. This might be in correlation with the previously reported localization of α -tubulin by Moreno & Schatten (2000), forming a bundle running around the nucleus in round spermatids, as it seems that tubulins persist in the sperm head of mature spermatozoa. However, in rat spermatozoa, the para-acrosomal cytoskeleton was mainly stained. Certainly, in the squirrel, the localization of α -tubulin represented adjacent regions of the apical acrosome. Interestingly, this region in boar spermatozoa exhibited distinct changes in the presence of inhibitors such as vinblastine (Dvorakova *et al.* 2001). After the AR, α -tubulin was restricted mainly to the apical equatorial margin and post-acrosomal region, which was especially interesting in the case of hamster sperm, where its ventral and dorsal part became distinct compared with the control. Similar findings on human and boar sperm on the redistribution of β -tubulin in the sperm head due to the AR were published by Draber *et al.* (1991) and Peknicova *et al.* (2001); however, Virtanen *et al.* (1984) reported no staining with anti-tubulin antibodies in the head of human sperm, only in the neck region and entire tail. Nevertheless, Whyard *et al.* (2000) found that the regulatory subunit (RS) of the RNA binding protein (RBP) which is 20 kDa microtubule-associated protein, was

present besides the tail localization in human and rat sperm and also in the rat sperm head. Moreover, its distribution co-localized with β -tubulin. The comparison between our study on α -tubulin and others on β -tubulin (Draber *et al.* 1991, Peknicova *et al.* 2001) is appropriate as the tubulin is a heterodimer consisting of α - and β -tubulin subunits, which is nearly ubiquitous among eukaryotic cells. It is relevant to assume that the presence of α -tubulin is accompanied by the presence of a β -tubulin subunit at the same time. There are different forms of tubulin but they are very similar, and will generally polymerize into mixed microtubules *in vitro* (Chakraborty *et al.* 1999).

In summary, the selected cytoskeletal proteins were mostly localized to the apical and the equatorial acrosomal region of the sperm head. While the intensity of staining differed between the sperm types, there was a general similarity in the localization patterns in control sperm and after the AR induction. Hence, these results suggested that cytoskeletal proteins in mature spermatozoa, at least, may play more of a role in the development of the AR and priming the spermatozoa for other fertilization events than in maintaining distinctive head morphologies. The modified localization of cytoskeletal proteins within the equatorial segment of the acrosome after the AR may be significant. This region is altered in response to the AR and the overlying plasma membrane becomes capable of fusing with the egg membrane at gamete fusion. It is likely that cytoskeletal proteins would be important at this time to reorganize membrane domains into fusogenic regions.

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