

Acrosomal biogenesis in human globozoospermia: immunocytochemical, ultrastructural and proteomic studies

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BACKGROUND: Acrosome biogenesis is a key event in sperm differentiation that depends on the proper interaction between the Golgi complex and the nuclear envelope of early spermatids. We studied the development, structure and biochemical characteristics of human acrosomes in germ cells and spermatozoa from testicular biopsies and semen samples of fertile men and patients with acrosomeless spermatozoa (globozoospermia). A set of proteins collectively known as the perinuclear theca (PT), which has been related to acrosomal development in many mammalian species, were also investigated.

METHODS: We evaluated spermatozoa from five males with globozoospermia and six fertile men, and immature germ cells from testicular biopsies of one globozoospermic patient and three men with obstructive azoospermia. Samples were assessed by transmission electron microscopy, immunofluorescence microscopy, ultrastructural immunocytochemistry and proteomic analysis by western blot.

RESULTS: In normal spermiogenesis, the development of the acrosome depends on the correct formation of Golgi-derived proacrosomal vesicles and simultaneous modifications in the nuclear envelope. PT proteins are consistently found in proacrosomal vesicles, localize underneath the acrosome and expand over the nuclear surface along acrosome biogenesis. In fertile men, the PT is composed of six proteins, similar to those previously described for other mammals (16, 22, 29, 34, 50 and 68 kDa). In globozoospermia, abnormal proacrosomal vesicles and paranuclear multivesicular and multilamellar structures were observed that resulted in acrosomes insufficiently developed or detached from the nuclear envelope. PT proteins, dissociated from the acrosomes, were ectopically localized in the cytoplasm. Proteomic analysis showed a significant decrease in all six PT proteins.

CONCLUSIONS: The alterations observed during early acrosome biogenesis in globozoospermia are due to anomalous development of Golgi-derived proacrosomal vesicles, failure of PT proteins to properly associate with the nuclear surface and significant deficiencies in specific PT components that are necessary for proper acrosome formation, implantation and expansion over the spermatid nucleus.

Key words: acrosome biogenesis / perinuclear theca / globozoospermia

Introduction

Biogenesis of the acrosome starts with the generation of proacrosomal vesicles from the Golgi complex of early spermatids. These coalesce to form a single, dense-cored spherical acrosomal vesicle that approaches the nucleus, attaches to it and expands to cover the anterior two-thirds of the nuclear surface in mature spermatozoa (Bishop and Smiles, 1963; Christensen and Fawcett, 1966).

Acrosome morphogenesis is a dynamic process that involves a close interaction between the Golgi complex and the nuclear envelope of early spermatids and progresses through four different steps: Golgi, cap, acrosome and maturation phases (Clermont and Leblond, 1955; Escalier *et al.*, 1991; Moreno and Alvarado, 2006). At the beginning of the Golgi phase, there is intense synthesis, glycosylation and traffic of proteins heralded by the association of endoplasmic reticulum cisternae to the cis-Golgi surface (Fawcett, 1975; Moreno *et al.*, 2000a,b; Ramalho-Santos *et al.*, 2001). A group of proteins identified

as the perinuclear theca (PT) are essential for the interaction between Golgi-derived vesicles and the nuclear envelope (Okó and Sutovsky, 2009).

Abnormal acrosome formation results in absence or insufficient development of the acrosome and serious alterations in the shaping of sperm heads. This rare spermatogenic disorder is characterized by mature spermatozoa with round heads and no acrosomes (acrosome-less spermatozoa, globozoospermia). Round-headed spermatozoa also exhibit abnormalities in chromatin condensation. In the absence of acrosomal enzymes, they are unable to penetrate the oocytes surroundings. Escalier (1990) has indicated that a subacrosomal cytoskeletal protein (Calicin), which is part of the PT, is absent in globozoospermia.

Acrosome biogenesis depends on the presence and functionality of the PT that shields the nucleus during sperm development and contributes signaling molecules that may be important for oocyte activation (Okó, 1995; Sutovsky et al., 1997, 2003; Alvarez Sedó et al., 2009; Ito et al., 2009, 2010; Okó and Sutovsky, 2009). Fertilization failures have been reported after ICSI, and are related to insufficient oocyte activation (Chemes and Rawe, 2003; Dam et al., 2007). Activation and normal pregnancies may be achieved using stimulators of Ca^{++} influx such as Ca^{++} ionophore or strontium (Chemes and Alvarez Sedó, 2012). However, in these cases, some embryos may still be unable to grow or implant properly because abnormal chromatin organization has also been described in globozoospermic spermatozoa (Chemes, 2000; Banker et al., 2009; Barroso et al., 2009).

In the present study, we explore the subcellular mechanisms involved in this abnormal development by analyzing the morphogenesis of the acrosome and PT in testicular spermatogenic cells and spermatozoa from globozoospermic and fertile individuals using immunofluorescence (IF), transmission electron microscopy (TEM), ultrastructural immunocytochemistry and western blot analysis for components of the acrosome and PT.

Materials and Methods

Chemicals and antibodies

All chemicals were obtained from Sigma Chemical Co (St. Louis, MO), unless otherwise stated. Antibodies used to study ejaculated sperm and spermatogenic cells were mouse anti-acetylated tubulin (Sigma-Aldrich, USA), sheep anti- α - β tubulin (Cytoskeleton, Inc., USA), mouse anti-acrosin C5F10 (Biosonda, Chile) and rabbit anti-PT 427 (gently donated by Richard Okó and Peter Sutovsky; Okó and Maravei, 1994).

Patients

Ejaculates from five adult males with primary sterility due to globozoospermia and six normal men were analyzed. Three testicular samples from patients with obstructive azoospermia and complete spermatogenesis and one from a globozoospermic patient (Patient no. 1, Table 1) were also studied. Complete clinical and laboratory work-up was undertaken in all cases.

Semen and testicular samples

Semen samples were donated by consenting men (normal and globozoospermic patients) and collected after 3 days of sexual abstinence. After semen liquefaction at room temperature, semen parameters were evaluated according to the World Health Organization (WHO, 2010).

Table 1 Summary of semen parameters (globozoospermic patients) according to the WHO (2010).

	Patient				
	1 ^a	2	3	4	5
Semen volume (ml)	1.8	5	2.5	2.2	3.1
Sperm count ($10^6/\text{ml}$)	15	70	155	77	125
Progressive motility (%)	30	5	44	55	48
Round-headed sperm (%)	86	89	98	87	99
Viability (%)	92	90	89	90	86

^aA biopsy sample was also taken from this patient.

Samples from testicular biopsies of obstructive or globozoospermic patients were donated by consenting men at CEGyR (Centro de Estudios en Ginecología y Reproducción).

Isolation of spermatogenic cells

Human spermatogenic cells were isolated from small pieces of testicular biopsies that were first dissected in a Petri dish with H-HTF (modified human tubal fluid; Irvine Scientific, CA, USA), placed in a conical centrifuge tube containing H-HTF supplemented with 0.5 mg/ml collagenase and incubated for 45 min at 37°C with gentle stirring. Once the seminiferous tubules had dispersed, they were allowed to settle at the bottom of the tube and the medium was discarded. The tubules were placed in fresh H-HTF containing DNase I (1 mg/ml) and trypsin (0.25 mg/ml) and incubated for 45 min at 37°C with stirring and gentle pipetting. Enzyme digestion was stopped by adding trypsin inhibitor (0.25 mg/ml), the cells were pelleted, washed twice in H-HTF and subsequently fixed for IF and TEM as described below.

Immunocytochemistry for light microscopy

Coverslips to which human spermatogenic cells and ejaculated sperm were attached were placed in PBS (0.1 M phosphate-buffered saline with 0.9% NaCl, pH 7.4) containing 2% formaldehyde and fixed for 1 h. Following fixation, the samples were permeabilized for 60 min in PBS containing 1% Triton X-100 and non-specific reactions were blocked by further incubation in PBS containing 3% bovine serum albumin (BSA) and 1% fetal bovine serum. For immunolabeling, primary antibodies were solubilized in blocking solution and incubated overnight at 4°C (anti-acrosin, C5F10, 1:100; anti-PT, PT 427, 1:200; anti- α - β tubulin, 1:200). After incubation, samples were washed in PBS containing 0.1% Triton X-100 and labeled with fluorescence conjugated secondary antibodies (Alexa-488 or Alexa-594; Molecular Probes, USA) 1:200 in blocking solution for 1 h at 37°C. DNA was identified by staining with Hoechst 33342 (0.5 $\mu\text{g}/\text{ml}$) and the coverslips were mounted onto glass slides with VectaShield mounting medium (Vector Laboratories, USA) and sealed. Samples were examined with an Olympus BX40 epifluorescence-equipped microscope operated with a DS-5Mc Nikon camera and ACT-2U software. Negative controls were performed by omitting the first antibody or by incubating with rabbit immunoglobulin G (IgG; Sigma Chemical Co) at the same protein concentration as the first antibody. Image acquisition times were similar in control and labeled samples.

Immunocytochemistry for electron microscopy

Isolated spermatogenic cells from testicular biopsies were fixed in 4% paraformaldehyde, 0.25% glutaraldehyde in phosphate buffer (0.1 mol/l, pH 7.4) and centrifuged for 5 min at 500g. The pellet was resuspended and rinsed gently with washing buffer (5% sucrose and 0.25% sodium azide in phosphate buffer) and cells were permeabilized with 0.5% Triton X-100 for 45 min at 4°C. Non-specific reactions were blocked using PBS containing 3% BSA. Cells in suspension were incubated overnight in primary antibodies (anti-acrosin, C5F10, 1:500 or anti-PT, PT 427, 1:250) diluted in 1% BSA and 3% normal goat serum in phosphate buffer followed by biotinylated secondary antibodies and peroxidase-labeled streptavidin and were finally developed with diaminobenzidine + H₂O₂ (LSAB System-HRP + DAB kit, Dako, Denmark). After centrifugation, the pellet was post-fixed in 1.3% osmium tetroxide, rinsed in buffer and processed for TEM following the protocol detailed below.

Immunogold labeling was assessed modifying a protocol previously reported by Musse and Chemes (2005). In brief, spermatozoa were washed free of seminal plasma with phosphate buffer (0.1 mol/l, pH 7.4), pelleted by centrifugation (10 min at 500g) and fixed for 1 h at 4°C in 4% paraformaldehyde, 0.25% glutaraldehyde in phosphate buffer, rinsed in buffer, dehydrated in an increasing series of ethanol, infiltrated in LR-white resin, medium grade (London Resin Co, England) and polymerized at 60°C for 24 h. Thin sections were mounted on 300 mesh nickel grids and dried at room temperature. Grids were hydrated on distilled water drops by floatation. Antigen retrieval was attempted by immersing the grids in 10 mM Na citrate buffer, pH 6.0 and subjecting them to 10–15 min of microwave irradiation at 800 W. The grids were washed for several times and incubated for 45 min at room temperature in blocking buffer [Tris-buffered saline (TBS) 50 mM, pH 7.5 + 3% normal goat serum] and then floated on drops of primary antibody on a humid chamber overnight at 4°C. Primary antibodies employed were anti-PT, PT 427 (1:25), anti-acrosin, C5F10 (1:50) and anti-acetylated tubulin, 6-11B-1 (1:50). After six washes in TBS, the grids were incubated for 1 h at 4°C with colloidal gold labeled anti-mouse (10 nm) or anti-rabbit (20 nm) IgG (Pelco International, Redding, CA, USA) at 1:50 dilutions in blocking buffer. Grids were rinsed three times in TBS and lightly stained with uranyl acetate and lead citrate.

Transmission electron microscopy

A fresh semen sample from five globozoospermic patients was processed for TEM within 30 min of ejaculation, according to methods previously described (Chemes *et al.*, 1987). In brief, spermatozoa were washed free of seminal plasma with phosphate buffer (0.1 mol/l, pH 7.4), pelleted by centrifugation (10 min at 500g) and fixed in 3% glutaraldehyde followed by 1.3% osmium tetroxide. Pellets were embedded in Eponate 12; Araldite 502 kit (Pelco) and thin sections displaying pale golden-silver interference colors were examined and photographed in a Zeiss 109 electron microscope (Zeiss Oberkochen, Germany) after double staining with uranyl acetate and lead citrate.

Sperm sample preparation for SDS-polyacrylamide gel electrophoresis and western blotting

Normal and globozoospermic semen samples were collected by masturbation. Semen was washed twice in H-HTF containing 3% synthetic serum substitute (Irvine Scientific). After separation of motile sperm by swim-up, sperm suspensions were divided into aliquots containing 15×10^6 /ml and pelleted by centrifugation (500g) for 15 min at 4°C in 1.5 ml microfuge tubes. Each sperm aliquot was washed by suspension in 1 ml

of TBS with protease inhibitors (pH 7.4) and centrifuged at 500g at 4°C. The supernatant was discarded and the washing procedure was repeated three times. The final sperm concentration was adjusted to 1.5×10^6 /ml. Pellets were then stored at –80°C until use.

SDS-polyacrylamide gel electrophoresis and western blotting

Samples from three fertile men and three globozoospermic patients were boiled for 10 min in Laemmli sample buffer 2× (Bio-Rad Laboratories, CA, USA) and loaded in pairs. Equal amounts of each protein sample (from 1.5×10^6 spermatozoa) were separated by electrophoresis on a 10% SDS-polyacrylamide gel at 120 V and transferred to a polyvinylidene difluoride (PVDF) blotting membrane (Millipore, Bedford, MA, USA). Equal loading was verified by Ponceau S staining. The resulting membranes were immunoblotted with PT427 (1:250) and acetylated tubulin (1:250) antibody followed by anti-rabbit and mouse horse-radish peroxidase-conjugated antibody (1:500), respectively (Santa Cruz Biotech, CA, USA) and enhanced by chemiluminescent (LuminGLO; Cell Signaling Technology, Inc., MA, USA). Chemiluminescence images were recorded using the UMAX Magic Scan V4.4 imaging system (Techville, Inc., USA). Densitometric analysis of the images was performed by NIS-Elements BR-Imaging Software v. 3.00 (Nikon, NY, USA) and expressed as the mean relative intensity (the mean of intensity of the normal or globozoospermic sperm band/mean intensity of control bands) after adjustment for sperm concentration. Ponceau S staining of membranes was used to verify equal loading. Molecular weights of detected sperm proteins were derived by comparison with prestained recombinant molecular weight standards (Amersham Full-Range Rainbow Molecular Weight Markers; GE Healthcare, USA).

Statistical analysis

Statistical analysis of western blot data was performed by univariate ANOVA using MedCalc 11.6.1 (Belgium) software. A value of $P < 0.01$ was considered statistically significant.

Results

Table 1 documents sperm features of five patients characterized by the presence of round-headed spermatozoa with no visible acrosome. The proportion of spermatozoa that lacked acrosomes ranged from 86 to 99%.

PT and acrosin IF

In testicular samples with complete spermatogenesis, all stages of spermiogenic differentiation could be identified by IF using anti-acrosin (green), together with anti-PT427 (red) or anti-tubulin (red; Fig. 1A–D). In normal conditions, early proacrosomic granules develop inside Golgi vesicles that fuse together, approach the nucleus and attach to it (Fig. 1A and B). In more advanced stages, the acrosome spreads over the nucleus forming the acrosomal cap. At this stage, microtubules are seen in the narrow rim of the surrounding cytoplasm. In elongated spermatids, the acrosomes further develop while microtubules assemble the manchette that grows caudally towards the residual cytoplasm (Fig. 1C). In mature spermatids, the acrosome covers the anterior two-thirds of the condensed head while microtubules are still seen in the vanishing manchette and along the axoneme (Fig. 1D). Serious abnormalities of acrosome development are observed during spermiogenesis in globozoospermia (Fig. 1E–H). In developing spermatids, the PT

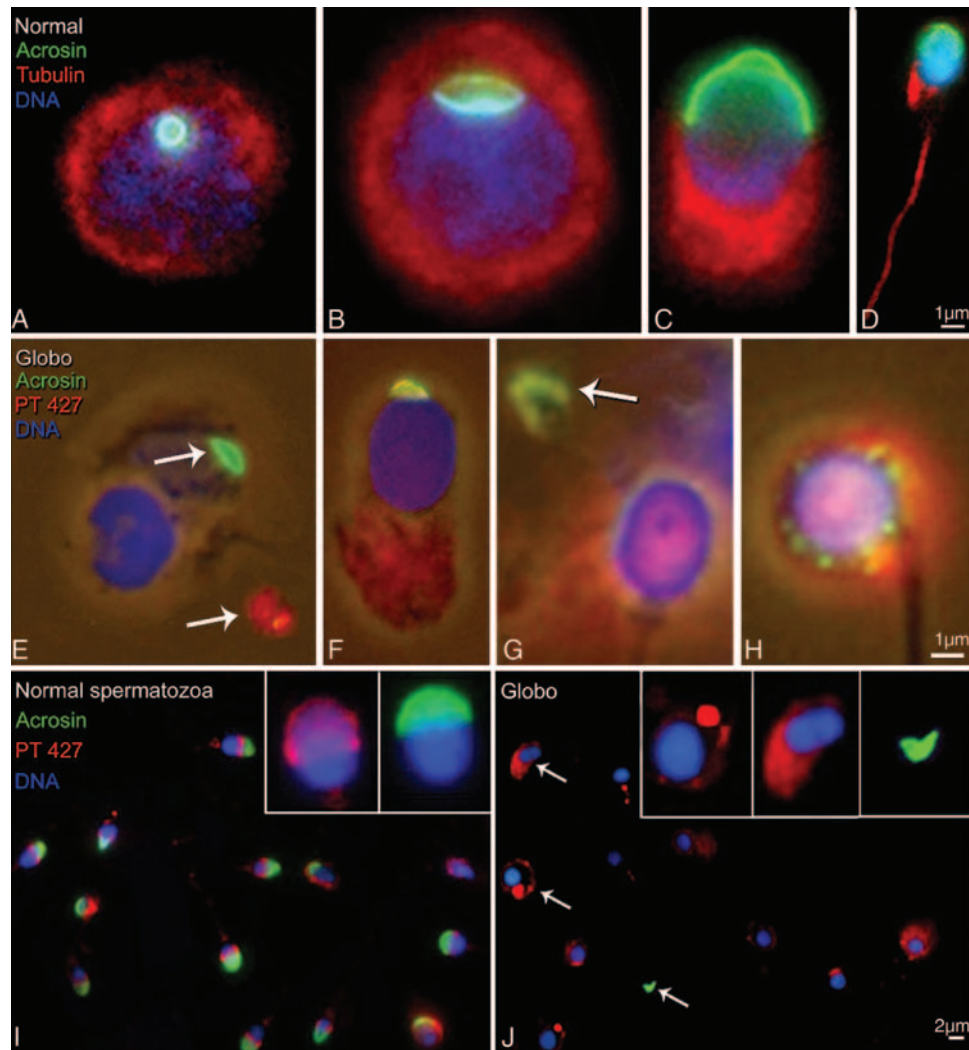


Figure 1 Distribution of PT and acrosin during spermiogenesis in normal and globozoospermic biopsies and semen samples. (A–D) Normal acrosome formation (acrosin) during spermiogenesis. (E–H) PT and acrosin distribution in globozoospermic cells from testicular biopsies. (E) PT and acrosin ectopic location (arrows) in spermatid with round nucleus. (F) Elongated spermatid depicting acrosome hypoplasia and cytoplasmic PT localization. (G) Mature spermatozoon with complete acrosome dissociation (arrow). (H) Round-headed spermatozoon with multiple perinuclear acrosin containing vesicles and perinuclear abnormal PT reactivity. (I and J) Sperm semen samples from normal donors and globozoospermic patient. (I) PT and acrosin co-localize on the acrosomal cap. PT proteins lie on the nuclear surface, underlying the acrosome (inset). (J) In globozoospermia, PT and acrosin are dissociated from each other and the nucleus (arrows and inset details). In this case, the biopsy sample belongs to the Patient no. 1.

and the acrosome were found in the cytoplasm, dissociated from each other and from the nucleus. In some cells, while keeping a normal location over the nucleus, the acrosomes were hypoplastic. Sperm heads were characteristically round in mature testicular spermatids where acrosomes and PT remained dissociated. The panoramic distribution of acrosomes and PT in normal and globozoospermic semen samples is depicted in the two bottom panels (Fig. 1I and J). In normal spermatozoa, PT and acrosin co-localize on the acrosomal cap covering the anterior two-thirds of the sperm nucleus. PT proteins lie directly over the nucleus, under the acrosome, and extend caudally towards the equatorial segment (inset, Fig. 1I). In globozoospermia, PT and acrosin are dissociated from each other and translocated to the cytoplasm away from the apical area of the nucleus (inset, Fig. 1J).

Transmission electron microscopy

Spermiogenesis was analyzed by TEM in biopsies of normal or globozoospermic men, in spermatogenic cells obtained after enzymatic digestion of testicular biopsies and in semen from two globozoospermic patients. In normal conditions, early acrosome organization derives from the development and fusion of various dense-cored proacrosomic vesicles from Golgi cisternae. At this stage, the nuclear envelope displays various modifications at the site of incoming acrosomic vesicle attachment (Fig. 2A). The acrosomic vesicle spreads over the nucleus forming the acrosomal cap (Fig. 2B). In maturing spermatids, the acrosome has a dense content and covers the anterior two-thirds of the condensing nucleus (Fig. 2C). Acrosomes develop abnormally in globozoospermia. The first stages are frequently characterized by the

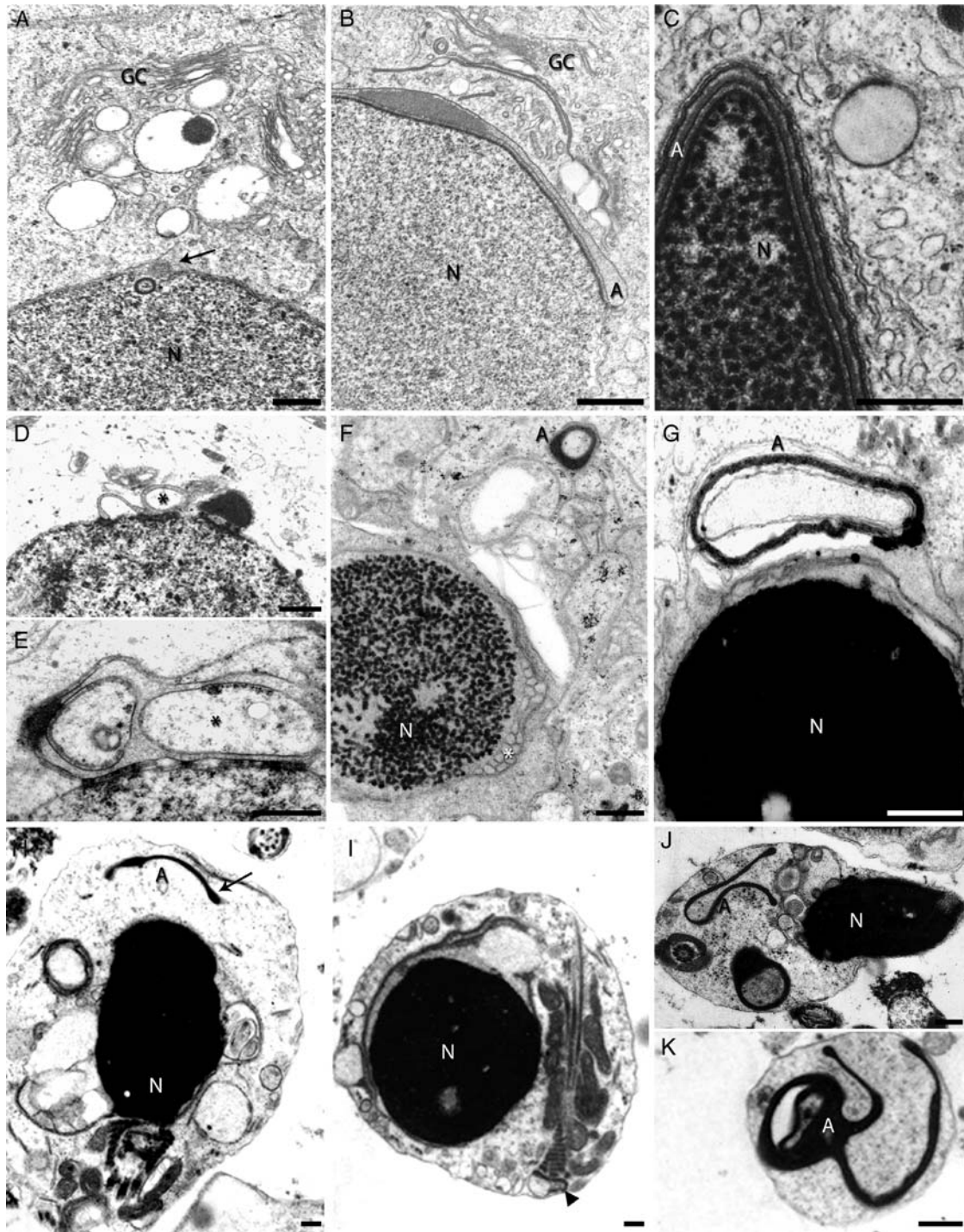


Figure 2 TEM of normal and globozoospermic germ cells and spermatozoa. (A–C) Normal spermiogenesis. (A) Nuclear membrane modifications were visualized at the site of incoming acrosomic vesicle attachment (arrow). A typical arrangement of Golgi cisternae and acrosomic vesicle is observed. (B) The acrosomic vesicle spreads over the nuclear surface in a cap phase spermatid. (C) Full acrosome development over a condensing nucleus. (D–G) Abnormal acrosome formation in globozoospermia (D–E). At initial stages, atypical proacrosomic granules with multivesicular and multilamellar structures were observed (arrows). (F–G) In later stages, mature acrosomes fail to attach and remain separated from the nucleus. (H–K) Ejaculated spermatozoa in globozoospermia. (H, J) Oval spermatozoa with detached acrosomes (hypoplastic in H, arrow). (I) Round sperm with irregular acrosome formation and tail alignment defect (arrow head). (K) Residual body with detached acrosome. Bar = 1 μ m. N, nucleus; GC, Golgi cisternae; A, acrosome.

presence of Golgi-derived multivesicular and multilamellar structures containing small and atypical proacrosomic granules (Fig. 2D and E). In later stages, mature acrosomes either fail to develop (Fig. 2F), remain unattached to the nucleus in the spermatid cytoplasm or are shed in residual bodies (Fig. 2G–K). Even though most mature spermatids are round-headed (Fig. 2F, G and I), oval acrosomeless spermatozoa are also observed (Fig. 2H and J). In coincidence with abnormal acrosomes, the neck region showed defective head–mid piece attachments that resulted in abnormal tail alignment or independent development of heads and tails (Figs 1H and 2I and J).

Ultrastructural immunocytochemistry

Ultrastructural localization and relationships between acrosin and proteins of the PT were studied in spermatogenic cells isolated from a normal testicular biopsy either by the pre-embedding immunoperoxidase method or by on-grid immunogold cytochemistry (Fig. 3). The subacrosomal localization of the PT is clearly depicted at various stages of acrosome development (Fig. 3A, D and E). Acrosin is restricted to the acrosome content (Fig. 3B and F). No immunostaining is observed in control samples in which the first antibody was omitted (Fig. 3C). Similar results were obtained by immunogold on-grid labeling. The PT was labeled by 20 nm colloidal gold particles that were concentrated in the subacrosomal space (Fig. 3G) while 10 nm particles localized acrosin between the inner and outer acrosomal membranes (Fig. 3H). Immunodetection of tubulin in axonemal microtubules was used as a positive marker of immunostaining specificity (Fig. 3I).

Western blot analysis

Equal protein loading was ascertained by α - β tubulin bands (50 kDa) that showed similar densities in fertile and globozoospermic samples (Fig. 4). Six protein bands (16, 22, 29, 34, 50 and 68 kDa) can be clearly distinguished in western blots with positive labeling for the PT427 antibody that identifies the six protein components of the PT. Their location and relative intensity are depicted in the accompanying histogram. The six bands are present in fertile and globozoospermic samples, but the latter are significantly weaker than the control ones (30–60% diminution, $P < 0.001$).

Discussion

In the present study, we show that lack of acrosomes or incomplete acrosomal development originates in the inability of Golgi-derived vesicles to attach and spread over the spermatid nuclear envelope. The PT is a cytoskeletal structure that covers the sperm nucleus in mammals and has been shown to play an essential role in normal acrosome formation (Okó 1995; Okó and Maravei 1995; Okó and Sutovsky, 2009). PT has been classically divided into two regions, the subacrosomal PT (SAR-PT) and the post-acrosomal sheath PT (PAS-PT). Various proteins have been identified as PT constituents, including a murine 15 kDa protein with high homology with the lipid protein binding family, a 60 kDa actin binding protein (Calicin), three other murine and bovine proteins of 25–36 kDa and SubH2Bv (17 kDa), a variant of 2HB histone (Olson and Winfrey, 1988; Longo and Cook, 1991; Fouquet and Kann, 1994; Okó and Morales, 1994; Korley et al., 1997; Lécuyer et al., 2000; Aul and

Okó, 2001). Proteins of the PT-like SubH2Bv play a role in migration and attachment of proacrosomic vesicles to the nucleus (Görllich et al., 1996; Cingolani et al., 1999; Okó and Sutovsky, 2009). In later stages (acrosomal cap and elongation) PT proteins relocate to the subacrosomal region, as previously reported in bovines by Okó and Maravei (1995) and Okó (1998).

Using a PT427 antibody with exclusive SAR-PT localization, we have previously reported in humans a close topographical relationship between PT development and acrosome biogenesis (Alvarez Sedó et al., 2009). The present is the first report of the SAR-PT protein composition in normal and globozoospermic human spermatozoa. Consistent with reports by Okó and Maravei (1994) in bulls, we confirm the existence of six major PT proteins in normal human spermatozoa and document serious deficiencies in globozoospermia possibly responsible for abnormal acrosome generation, implantation and expansion over the nucleus. Lacking or hypoplastic acrosomes may derive not only from proacrosomal vesicle and nuclear envelope disarrangements but also from insufficiencies in specific PT components.

Interactions between the nuclear envelope and the Golgi at early steps of acrosome generation that evidence an active nuclear role in early acrosome morphogenesis have previously been described in humans and rodents (Chemes et al. 1978; Fawcett and Chemes, 1978). More recent reports have shown that the absence of the surface adhesion molecule JAM-C over the apical pole of the spermatid nucleus results in lack of acrosome attachment in JAM-C^{-/-} mice (Ebnet et al., 2004; Glikli et al., 2004).

The study of testicular biopsies and sperm semen samples of globozoospermic patients provides interesting material to understand the different steps of abnormal acrosome development. Globozoospermia was described by Schirren et al. (1971) and revisited by Dam et al. (2007). In the five patients here reported, the vast majority of spermatozoa (86–99%) depicted round heads without acrosomes and may be considered 'partial globozoospermia'. However, the distinction between complete and partial globozoospermia may be difficult, and... 'whether classic/total globozoospermia and partial globozoospermia are part of the same syndrome remains to be elucidated' (Dam et al., 2007). Since the percentage of acrosomeless spermatozoa in our patients is so close to 100%, the distinction is probably unimportant. We propose that the abnormal membranous structures and ectopic acrosomes observed during spermiogenesis represent failed Golgi attempts of acrosome development. This interpretation is at variance with that of other authors that described these features as regressive changes of the acrosome (Holstein et al., 1973; Anton-Lamprecht et al., 1976; Baccetti et al., 1977, 1991; Nistal and Paniagua, 1978; Holstein and Schirren, 1979).

Teratozoospermia has been reported as an important cause of deficient fertility in men. As proposed by Chemes (2000) when introducing the concept of sperm pathology, two main kinds of sperm anomalies can be distinguished. Teratozoospermic phenotypes of proven or suspected genetic origin are characterized by a homogeneous microscopic pattern. Globozoospermia and acrosomal hypoplasia (Zamboni, 1987; Baccetti et al., 1991) belong to this group of alterations that may be called *systematic* because there is a common phenotype that predominates in all patients suffering from the same condition. The homogeneous phenotype in globozoospermia, a genetically determined condition, represents the full range of acrosome

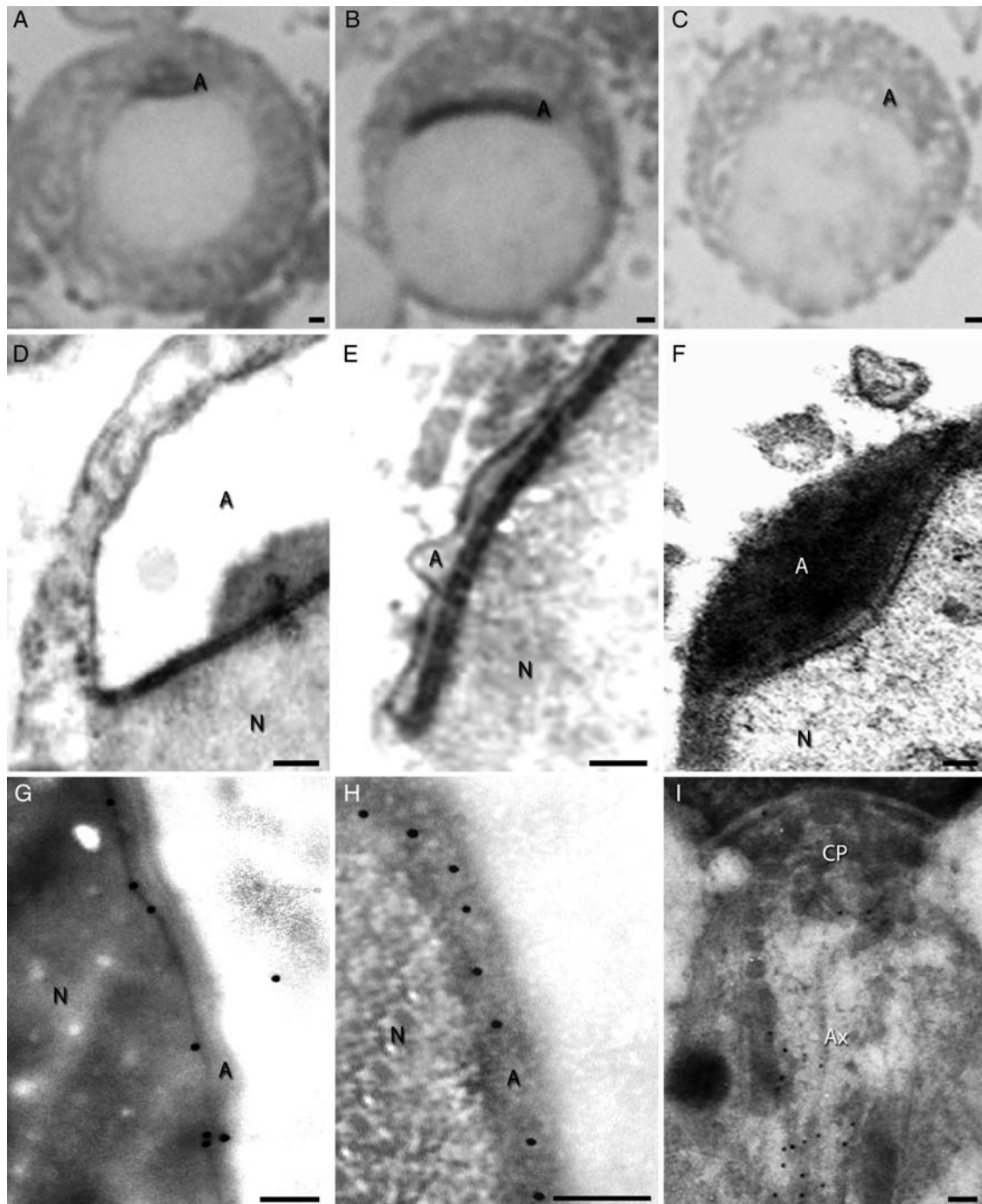


Figure 3 Ultrastructural immunocytochemistry with anti-acrosin and anti-PT antibodies in normal spermiogenesis. (**A–C**) Pre-embedding immunoperoxidase method in a bright field. (A) PT immunodetection, showing subacrosomal labeling. (B) Acrosin localization. (C) Control samples (without first antibody; **D–F**) TEM with pre-embedding immunoperoxidase method. (D–E) Subacrosomal immunolocalization of PT. (F) Acrosin immunodetection is restricted to the acrosome content. (**G–I**) Immunogold localization. (G) Subacrosomal location of PT labeled by 20 nm colloidal gold particles. (H) Acrosin-positive localization within the acrosome, labeling with 10 nm colloidal gold particles. (I) Positive control for immunostaining specificity (tubulin positive over axonemal microtubules). Bars = 1 μm. N, nucleus; A, acrosome; Ax, axoneme; CP, connecting piece.

impaired development, while the mixture of spermatozoa with hypoplastic or absent acrosomes with other non-related sperm pathologies exemplifies the heterogeneous phenotype most probably acquired nature of severe (non systematic) teratozoospermia. There is a very

recent report on partial globozoospermia in humans (Dam et al., 2011).

The sites of acrosomal and flagellar implantation define the bipolarity of round spermatids nuclei and the longitudinal sperm axis. As

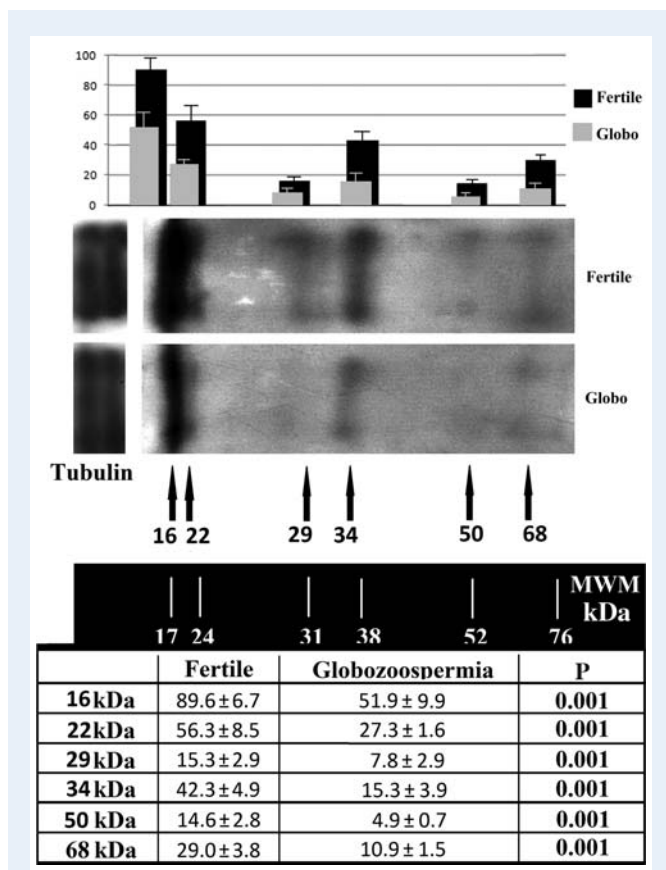


Figure 4 Representative immunoblotting analysis of PT427-reactive protein extracts from spermatozoa of a fertile man and a globozoospermic patient. Protein bands of 16, 22, 29, 34, 50 and 68 kDa, labeled by the anti-PT antibody, were present in normal spermatozoa and globozoospermia. A significant reduction (30–60%) was showed in all protein bands in globozoospermia. Control of equal loading was performed with tubulin antibodies (similar values in both samples). A broad range prestained SDS-polyacrylamide gel electrophoresis standard was used to estimate molecular weights. Densitometric values are expressed as a mean relative intensity.

previously discussed, acrosomeless spermatozoa arise as a failure of acrosome attachment and development, while defects of the head–tail implantation derive from an abnormal positioning of the flagellar anlage on the opposite (caudal) nuclear pole. The coexistence of abnormal tail implantations and lack of acrosomes here documented in some spermatozoa have been previously noticed and indicates that these two mechanisms may combine, probably as a result of an abnormal differentiation of the bipolar nature of the spermatid nucleus (Chemes et al., 1999; Chemes and Rawe, 2003; Suzuki-Toyota et al., 2004; Dam et al., 2007).

No mutations have been found in globozoospermic patients in genes such as Hrb, GOPC and Csnk2a2 that are known to be associated with globozoospermic-phenotypes in mutant mice (Pirrello et al., 2005; Christensen et al., 2006). GOPC^{−/−} mice that share most of the characteristics of the human phenotype have confirmed the main role played by the peri-nuclear matrix for acrosome formation and oocyte activation (Yao et al., 2002; Ito et al., 2004, 2009,

2010). There have been recent studies on mutations in genes such as PICK1 (protein interacting with C kinase 1), DPY19L2E and PLCzeta in human globozoospermia (Heytens et al., 2009; Liu et al., 2010; Harbuz et al., 2011; Kosciński et al., 2011).

In summary, we have demonstrated that the alterations observed during early acrosome biogenesis in globozoospermia are due to anomalous development of Golgi-derived proacrosomic vesicles, failure of PT proteins to properly associate with the nuclear surface and significant deficiencies in specific PT components that are necessary for proper acrosome formation, implantation and expansion over the spermatid nucleus.

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Authors' roles

H.E.C. and V.Y.R. were involved in the conception and design of the study. C.A.S. and V.Y.R. were involved in the acquisition of data. H.E.C., C.A.S. and V.Y.R. contributed to analysis and interpretation of data, drafting the article and final approval.

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Conflict of interest

None declared.

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