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Ultrastructural immunocytochemical evidence for actin in the acrosomal complex during spermiogenesis of the lizard *Tropidurus itambere* (Rodrigues, 1987) (Reptilia: Tropiduridae)

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Abstract — During spermiogenesis of the lizard *Tropidurus itambere* early developmental stages of the acrosomal complex have been analysed and new suggestions made as to their origin. Actin has been detected in the acrosomal granule and pro-acrosomal vesicle of spermatid, using an antibody for α-smooth muscle actin (monoclonal α-SMA). Intense marking also identified actin in two regions of the acrosomal complex; the subacrosomal cone and the perforatorium. The acrosomal and subacrosomal granules appear to participate to the formation of the perforatorium, while the pro-acrosomal vesicle is responsible for the formation of the subacrosomal cone. The results therefore endorse the view that, in lizards, the perforatorium and subacrosomal cone are homologous, in composition and probably also in function.

Key words: acrosome, actin, immunocytochemistry, lizard, perforatorium, spermiogenesis, subacrosomal cone.

INTRODUCTION

Among the events that occur during spermiogenesis in lizards, the most relevant steps are nuclear elongation, chromatin condensation, acrosome formation, intensive cytoplasm elimination, and flagellar development. The acrosome of lizards consists of a set of structures known as the subacrosomal cone, the epinuclear clear zone, the perforatorium, the clear middle zone and the acrosome cap (Cruz-Höfling and Cruz Landim 1978; Teixeira *et al.* 1999; Vieira *et al.* 2001; Ferreira and Dolder 2003a). Herein we describe ultrastructural modifications of the head of spermatids and spermatozoa, and detect actin immunocytochemically in the acrosomal complex, of the neotropical lizard, *Tropidurus itambere*.

It is well known that the perforatorium presents a great amount of actin, evidenced by immunocytochemical techniques in spermatids and spermatozoa of the rabbit (Courtens *et al.* 1991), hamster (Fouquet *et al.* 1991), rat and mouse (Fouquet *et al.* 1992; Paranko *et al.* 1994). However, no information exists for the acrosomal complex in lizards.

Most lizards possess an acrosomal complex including perforatorium and an acrosomal cone (Butler and Gabri 1984; Al-Hajj *et al.* 1987; Teixeira *et al.* 1999). However little is known in relation to the functions to these organelles during the acrosomal reaction and penetration into the oocyte, as well as their differences in composition, and possible relations of homology (Garda *et al.* 2002).

MATERIAL AND METHODS

Adult lizards, *Tropidurus itambere* (Rodrigues 1987), were collected in their natural habitat, from the Atlantic forest in Valinhos region (23°00'S, 47°00'W), São Paulo state, Brazil, at monthly intervals between June 2001 and June 2002. The animals were killed by ethyl ether inhalation and testis removed by dissection.

Transmission electron microscopy - The organs were cut into fragments and fixed by immersion

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for 5 hours at 4º C in 2% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2. The tissue fragments were washed in buffer, dehydrated in acetone (30-90%), and embedded in L R White resin. The ultrathin sections were colleted on nickel grids and stained with uranyl acetate and lead citrate and observed with a Zeiss, Leo 906 Transmission Electron Microscope.

Ultrastructural immunocytochemistry - Ultrathin sections were collected on nickel grids, and preincubated in 0.05M Tris-HCl buffer pH7.6, containing 0.05% glycin and 1.0% bovine albumin for 10 minutes at room temperature. After several rinses in the same buffer, non-specific binding was blocked with 0.05M Tris-HCl containing 1% bovine albumin and 0.05% Tween 20 for 30 minutes. The sections were subsequently incubated for 1h with an antibody against α-smooth-muscle-actin (monoclonal α-SMA), diluted 1:100 (Novocastra Laboratories Ltd. - England). After washing with 0.05M Tris-HCl, containing 0.5% bovine albumin, the grids were incubated for 1h with the respective labeled secondary antibody - RAM (Rabbit Anti-mouse IgG-Au-conjugated, 10nm, Sigma-Aldrich Co. - USA) at a dilution of 1:50. After incubation, the grids were washed with 0.05M Tris-HCl and distilled water. The specimens were observed without staining.

RESULTS

In early spermatids, the Golgi complex produces several vesicles; that merge together, forming a larger structure, called the pro-acrosomal vesicle that fits into a nuclear invagination (figs. 1a, 1b). In the region where the vesicle is annexed onto the nucleus, other structures are formed, such as an electron dense granule consisting of two parts, the acrosomal-granule inside the acrosomal vesicle and a small subacrosomal-granule just outside the acrosomal vesicle membrane (fig. 1a). At their nuclear cistern an electron-dense layer develops below the pro-acrosomal vesicle, which has been called the "subacrosomal material" or "layer" (fig. 1a). This layer is separated from the nuclear envelope by a clear layer (fig. 1b). During chromatin condensation and nuclear elongation, it is possible to observe the formation of thick filaments arranged longitudinally and undergoing a light twist around the central axis (fig. 1c). In cross section, the chromatin filaments fuse to make a network arrangement of interconnecting filaments (fig. 1d).

Intermediate spermatids can be identified by the more closely compacted chromatin (figs. 1e, 1f). The acrosomal complex surrounds the nuclear tip in layers in which the epinuclear clear zone, the perforatorium, the subacrosomal cone, the clear middle zone and the acrosome cap can be identified (fig. 1e, 1f). Since the head of these spermatids is sickle-shaped and the nucleus in figure 1e is tangentially sectioned, the subacrosomal cone, which covers only the nuclear tip, is not shown. These cells enter into an intimate association with the Sertoli cell, and membranes of this cell appear parallel to the acrosomal complex (fig. 1e). Also marking the limit of this complex are the electron-dense expansions of the nucleus, called the nuclear shoulders (fig. 1f).

Late spermatids are elongated, as is the acrosomal complex, that here presents clearly defined regions, such as the subacrosomal cone, the less dense perforatorium, located at the nuclear apex, and more externally, the acrosomal cap. These structures are separated by clear regions, such as the epinuclear electron-translucent zone, between the subacrosomal cone and the perforatorium, and by the clear middle zone, located between the perforatorium and the acrosomal cap (fig. 2a, 2b, 2c). Chromatin is totally compacted (fig. 2d), and this extreme condensation is associated with a layer of microtubules, called the manchette, that surrounds the nucleus (fig. 2e).

Intense immunocytochemical marking identified actin in two regions of the acrosomal complex, the subacrosomal cone and the perforatorium (figs. 2f, 2g).

DISCUSSION

The spermiogenesis process of *Tropidurus itambere* involves, among other events, the formation of an acrosomal complex located at the nuclear apex. The acrosomal complex surrounds the nuclear tip in layers where the epinuclear electron-translucent zone, the perforatorium, the subacrosomal cone, the clear middle zone and the acrosome cap can be identified (FERREIRA and DOLDER 2003a).

FAWCETT *et al.* (1971) furnished the classical description, elucidating the factors that influence the establishment of the spermatozoon head shape, among which the manchette appears to exert the strongest influence. Moreover a nuclear ring was observed, at the contact of the nuclear membrane with the acrosomal complex, similar to an annulus. In our study, this structure has been

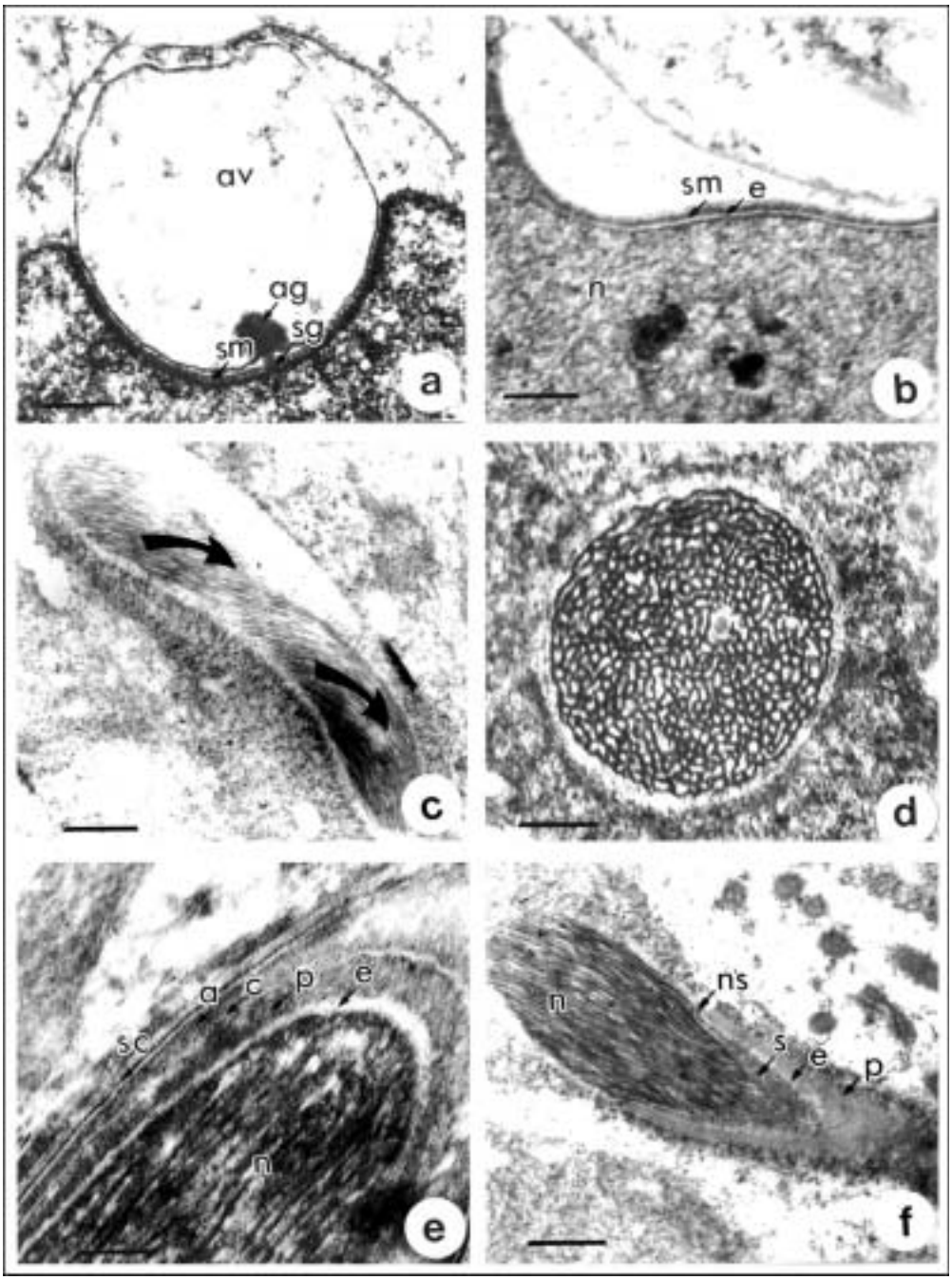

Fig. 1 — Early spermatids. **a.** - Pro-acrosomal vesicle (av) in close contact with the nucleus. Observe the acrosomal granule (ag) inside the vesicle and the subacrosomal granule (sg) outside this vesicle. Notice also the electron dense layer called the "subacrosomal material" (sm) below the pro-acrosomal vesicle, and in contact with the nuclear membrane. Bar = 0,46μm. **b.** - Pro-acrosomal vesicle, showing the "subacrosomal material" (sm) and the initial formation of one of the clear layers of the acrosomal complex, apparently the epinuclear clear zone (e) in the contact with the nucleus (n). Bar = 0,11μm. **c.-f.** - Intermediate spermatids. **c.** - A longitudinal section shows the compacting of chromatin in thick filaments that are twisted (curved arrows), in a process of molding and elongation of the nucleus. Bar = 0,64μm. **d.** - Transverse section of an intermediate spermatid. Notice the network of condensing chromatin filaments. Bar = 0,16μm. **e.** and **f.** - Longitudinal or slightly tangential views of the acrosomal complex in formation. It is possible to distinguish the different layers, such as, acrosome cap (a), clear middle zone (c), perforatorium (p), epinuclear clear zone (e) and subacrosomal cone (s). External to the acrosomal complex, membranes of the Sertoli cell (SC) can be seen. At the limit between the acrosomal complex and the nucleus, observe the nuclear shoulders (ns). **e.** Bar= 0,12μm. **f.** Bar= 0,46μm.

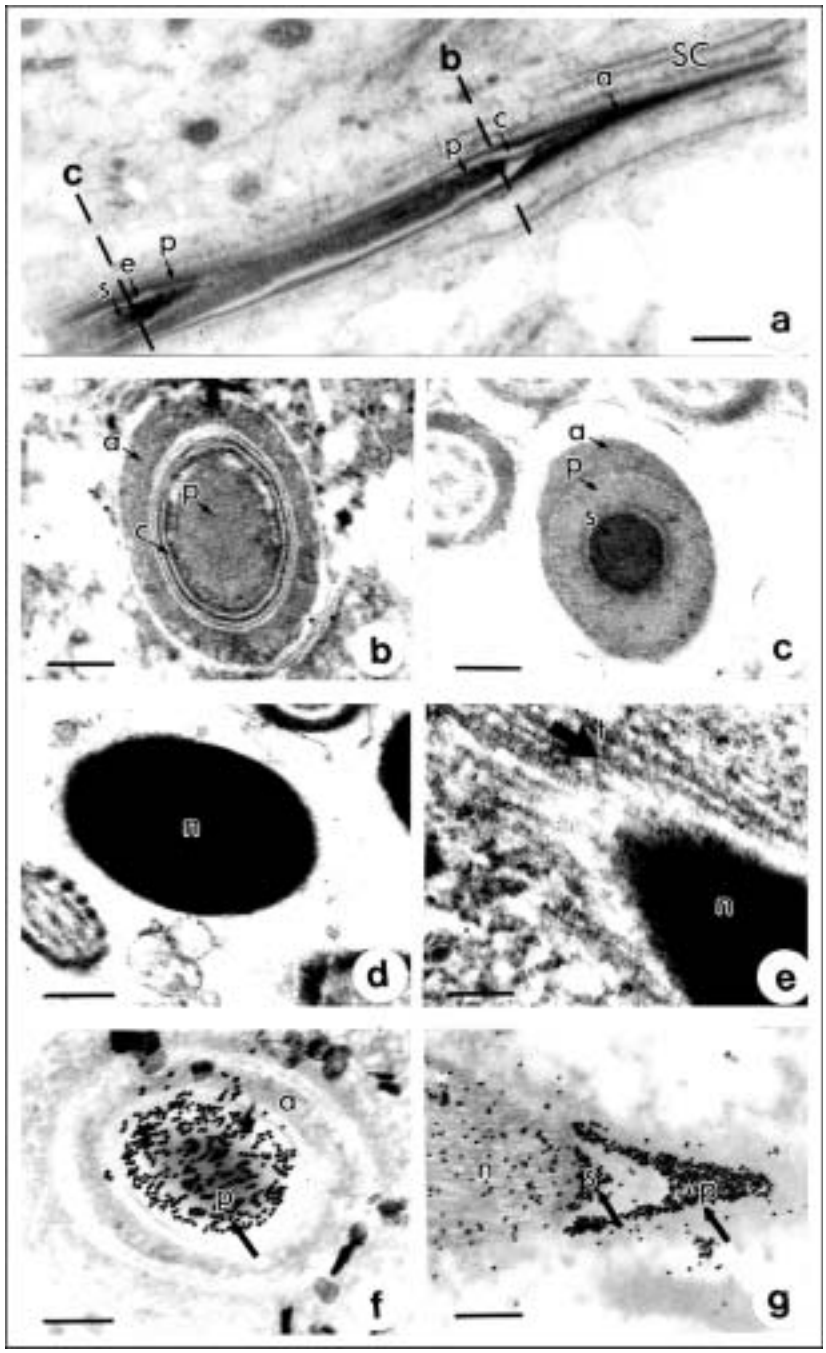

Fig. 2 — Late spermatids. **a. -** Longitudinal section of the acrosomal complex. This structure is divided into compartments: the subacrosomal cone (s), epinuclear clear zone (e) perforatorium (p), clear middle zone (c) and acrosomal cap (a). Observe the membranes of the surrounding Sertoli cell (SC). The hatched lines indicate the probable regions for the transverse sections in figures **b.** and **c.** Bar = 0,49μm. **b. -** The apical region of the acrosomal complex. Perforatorium (p), clear middle zone (c) and acrosome cap (a). Bar = 0,14μm. **c. -** Transverse section of the basal region of the acrosomal complex. Subacrosomal cone (s), perforatorium (p) and acrosome cap (a). Bar = 0,18μm. **d. -** Transverse section of the nucleus (n) showing complete chromatin condensation. Bar = 0,27μm. **e. -** Longitudinal section. Observe the longitudinally (arrow) arranged microtubules (t) around the nucleus (n). Bar = 0,13μm. **f. -** Transverse section of the apical region of the acrosomal complex, similar to the section in figure 2b. Observe immunogold labeling of the perforatorium (p) and its absence in the acrosome (a). Bar = 0,18μm. **g. -** Longitudinal section of an intermediate spermatid. The acrosomal complex is sectioned near the region shown in figure 1f. Observe intense immunogold labeling in the region of the perforatorium (p) and the subacrosomal cone (s) and its absence in the intermediate (epinulear clear zone) and the external region (acrosome cap), nucleus (n). Bar = 0,23μm.

described as the nuclear shoulders, in accordance with more recent publications of TEIXEIRA *et al.* (1999) and VIEIRA *et al.* (2001). This would be the attachment point of the manchette, for the establishment and maintenance of shape of the spermatid head in formation. Another external factor involved in the molding of the nucleus is the influence exerted by the Sertoli cell (FAWCETT *et al.* 1971).

It is impossible to attribute the shape of the nucleus entirely to external forces applied by the microtubules. Intrinsic forces must also determine nuclear configuration. The factors determining acrosome shape appear to be generated from within rather than occurring as a consequence of externally applied forces. FAWCETT *et al.* (1971) concluded that there is a redistribution of cytoplasm that takes place during spermatid elongation, but this is probably not directly involved in nuclear shaping. The different patterns, which can be observed in condensing spermatid nuclei of various species, probably depend on the synthesis of specific arginine-rich histones that control the shape and aggregation mode of the DNA-histone complexes formed during nuclear condensation. Therefore, the condensation would be the result of the elimination of certain nuclear components, including water and non-histonic proteins. This is, at least, partially in agreement with CRUZ-HÖFLING and CRUZ LANDIM (1978) who assumed that the process of chromatin condensation would be a result of the dehydration of the nucleoprotein as the nucleoplasm is eliminated.

Synchronous with the nuclear changes, the acrosomal vesicle and granule, originating from the Golgi complex, make up the anterior acrosomal cap. The perforatorium, according to HUMPHREYS (1975), is derived from a subacrosomal granule interposed between the acrosomal vesicle and the nucleus, in birds. This was also accepted by DEL CONTE (1976) for reptiles, and even for mammals this concept has been generally accepted. DEL CONTE (1976) suggested that the granule is a product of acrosome and nucleus interaction. The subacrosomal space becomes dilated and its material diffuses, originating the subacrosomal cone, according to VIEIRA *et al.* (2001).

Our results have lead us to disagree with this hypothesis. The acrosomal granule appears to be the responsible for the formation of the perforatorium, together with the subacrosomal granule located just below the acrosomal granule. However, beside it is the pro-acrosomal vesicle, which is responsible for the formation of the subacrosomal cone. This conclusion, is based as much on the location of the structures during spermiogenesis, as well as on the density observed with electron microscopy, and cytochemical properties such as the amount of basic protein, which is very similar in the acrosomal and subacrosomal granules, perforatorium and subacrosomal cone (FERREIRA and DOLDER 2003b). This location also coincides with what was ultrastructurally demonstrated by BUTLER and GABRI (1984) in the lizard *Podarcis taurica*.

The formation of the spermatozoon head of *Tropidurus itambere* occurs in a manner very similar to the lacertilian. It also presents many similarities with the spermiogenesis of birds, according to the description of SOLEY (1997) in *Struthio camelus*, but with the difference that in lizards, chromatin condensation occurs in tufts and not in filaments, and the manchette is not so well defined.

Some relations of homology between structures of the acrosomal complex have already been observed. In descriptions of spermatozoon ultrastructure of some Anura, the subacrosomal cone was not considered homologous to the conical perforatorium (BURGOS and FAWCETT 1956; JAMIESON *et al.* 1993) mainly based on differences in electron density. However, more recent work (JAMIESON 1999; GARDA *et al.* 2002) defines these organelles as homologous. If a comparison is made of this data related to the subacrosomal cone and perforatorium of Anura with lizard *Tropidurus itambere*, it can be noticed that these structures have differences in electron density, but immunocytochemical methods demonstrate that, at least in regard to actin composition, and therefore possibly to function, these organelles can be considered homologous.

AL-HAJJ *et al.* (1987) when studying the lizard *Agama stellio* found other differences in the initial stages of the acrosomal complex, such as the formation of two pro-acrosomal vesicles, which in *Tropidurus itambere* does not occur. Also, they believed that the subacrosomal cone, located between the pro-acrosomal vesicle and the nucleus of spermatids would be responsible for the future perforatorium.

During differentiation, the subacrosomal material does not diffuse homogeneously, leading to the formation of a lateral electron-lucent layer between the acrosomal vesicle and the subacrosomal cone (or unilateral ridge) and a slender electron-lucent channel above the nucleus, the epinuclear clear zone. The subacrosomal granule has been reported in other lizard species (DEL CONTE 1976; BUTLER and GABRI 1984; AL-HAJJ *et al.* 1987) and

has been considered responsible for perforatorium formation.

According to BACCETTI *et al.* (1980) the perforatorium is made up of an oriented bundle of actin filaments in vertebrates, which would maintain the sperm shape and is conserved in the ejaculated sperm until penetration of the egg; the same role cannot be attributed to the developing perforatorium during spermiogenesis, because the organelle is too small to sustain nuclear shape. Therefore, according to these authors, this organelle in birds and reptiles seems to be a residual one, destined to be lost during evolution.

Actin was detected in the subacrosomal space of spermatids during the greater part of spermiogenesis in the rat, hamster, monkey and man (FOUQUET *et al.* 1989). We also identified immunocytochemically the presence of actin in the perforatorium and in the subacrosomal cone of the lizard *Tropidurus itambere*.

The role of actin from late spermatids onwards remains an unsolved question, subacrosomal actin is a constant feature during spermiogenesis verified so far, which suggests that this cytoskeletal protein may have a role in spermatid differentiation (FOUQUET *et al.* 1989).

The subacrosomal layer of spermatids in hamster contains filamentous actin during the greater part of spermiogenesis (FOUQUET *et al.* 1992). However, in early spermatids of *Tropidurus itambere*, markings with actin antibody were not sufficiently specific to determine the presence of this molecule in the region of the subacrosomal material, nor in the subacrosomal and acrosomal granules.

According to FOUQUET *et al.* (1992) the filamentous actin of the subacrosomal layer appears as transitory scaffolding that might be involved in the assembly of other proteins at the perinuclear cytoskeleton and indirectly in nuclear shaping.

One of the explanations for the presence of actin in the structures that compose the acrosomal complex is the fact that during spermatozoon approach to the egg a sequence of events takes place. First, the acrosomal reaction occurs when the spermatozoon is in the vicinity of the egg envelope, then the perforatorium suffers an enlargement and becomes associated to the oolema. Finally, the perforatorium material contracts to bring the whole spermatozoon in contact with the egg (GUERRA *et al.* 1994). This has been described for spermatozoa of mammals, however in lizards, the subacrosomal cone has been suggested to possess the same function as that of the perforatorium (JAMIESON 1995).

Another interesting explanation is that actin filaments may be involved in Golgi vesicle transport during acrosome formation; however, there is no indication that subacrosomal actin is of the contraction variety (FOUQUET *et al.* 1989). Subacrosomal actin also may have a structural and binding function and may serve to anchor the acrosome to the nucleus in order to harmonize their shaping through mutual interactions during the elongation phase of spermatids. In addition, actin of the subacrosomal space may give a certain structural rigidity to the acrosome of the spermatid, and even of the spermatozoon, if a modified actin remains present at this location (FOUQUET *et al.* 1989).

We present in this study some suggestions for the origin of the structures that compose the acrosomal complex. Intense immunocytochemical marking identified actin in two regions: the subacrosomal cone and the perforatorium. Many similarities exist between the localization of actin in the different animal groups, and the purpose of these structures, which may be relevant to fertilization process in vertebrates.

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