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Structural and ultrastructural characterization of zebu (*Bos indicus*) spermatozoa

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ABSTRACT: The ultrastructure of normal, ejaculated spermatozoa of *Bos indicus* was studied by means of electron microscopy, being evaluated in two principal parts, the head and the tail. The head is flat, oval or paddle-shaped with a square base, which provides a concave recess for the insertion of the tail. The acrosome tightly covers the anterior two thirds of the nucleus. A distinct unilateral acrosomal bulge was observed along the apical edge of the head. The equatorial region demarcates the acrosome from the post-equatorial region that covers the caudal one third of the nucleus. The classical 9+9+2 fiber pattern which composes the axoneme was observed along three segments of the tail, namely middle, principal and terminal pieces. The axoneme is anteriorly bound by the mitochondrial helix (middle piece) and posteriorly by the fibrous helix (principal piece), except at the terminal piece. The border between the middle piece and principal piece was well defined due to the termination of the thick mitochondrial helix and the presence of the *annulus*. Some of the spermatozoa presented cytoplasmatic droplets, which appeared as stalk-like appendages.

Introduction

The general ultrastructure of mammalian spermatozoa has been thoroughly investigated and this subject has been reviewed in a number of studies (Bradfield, 1954; Fawcett, 1958; 1975; Hancock, 1966; Schmehl and Graham, 1989; Juhász *et al.*, 2000). Concerning the biology of reproduction, structural studies of germinative cells of males and females have been of remarkable assistance not only on the development of new technologies, but also on the improvement of the existing ones. The review on the morphology of mammalian spermatozoon published by Fawcett in 1975 reinforced the important role of structural studies of spermatozoa on the determination of the reproductive capacity and potential of different animal species. The effective evaluation of semen has been constantly pointed as a key factor on the development of methodologies that warrant greater reproductive utilization of males of endangered and also economically important species (Baccetti *et al.*, 1997).

A diversity of reproductive technologies have been developed and intensely applied in domesticated animals, particularly in cattle. Such technologies have allowed efficient culling of subfertile males as well as the exploitation of the reproductive capacity of genetically superior sires (Aman and Schanbacher, 1983). Furthermore, successful techniques have been used to the extension and preservation of genetically superior bovine sperm.

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The bovine spermatozoon has also been a target of many studies (Bonadonna, 1958; Rahlman, 1961; Blom, 1963; Bahr and Zeitler, 1964; Saacke and Almquist, 1964a, b; Blom and Birch-Andersen, 1960, 1965; Wooding and O´Donnell, 1971; Bustos-Obregon and Fléchon, 1975; Oko *et al.*, 1976; Williams, 1987; Barth and Oko, 1989), but these studies generally concern European cattle, *Bos taurus* and not Zebu cattle, *Bos indicus*, which has great importance in the economy of tropical and subtropical countries (Crudeli *et al.*, 1991). Although the two species have many aspects in common, there are striking differences concerning physiological and anatomical features; moreover, particularities in the reproductive profile of each might be of great aid on the development of viable techniques. Since recent studies reveal that some differences can indeed occur in the morphology of germinative cells among females *Bos taurus* and *Bos indicus* (Chenoweth, 1994; Rocha *et al.*, 1998; Visintin *et al.*, 2002) it could be possible that this pattern extends to males as well. In fact, it has been shown recently that there are morphometric differences between the spermatozoon of *Bos taurus* and *Bos indicus*, with the latter being smaller and less elliptic (Beletti *et al*., 2004). The present study aims to comparatively describe the structure and ultrastructure of *Bos indicus* spermatozoa with that of *Bos taurus* by means of light and transmission electron microscopy.

Materials and Methods

Bovine spermatozoa for this study were obtained from three Curraleiro bulls by means of electroejaculation. Semen was diluted with PBS (phosphate buffer saline), pH 7.2. For light microscopy, spermatozoa were smeared on a glass slide and after air-drying, the preparations were stained with Congo red. For electron microscopy, spermatozoa were centrifuged from the diluent for 5 times at 4,000 rpm for 3 minutes at room temperature. After each time, the supernatant was decanted. The remaining pellets were divided into portions subjected to two distinct fixation protocols. One portion was fixed in 2% paraformaldehyde and 2% glutaraldehyde, while the other was fixed in 2% glutaraldehyde and 0.5% picric acid, both in sodium cacodylate buffer (pH 7.3) for 3 hours at room temperature. Next, they were post fixed in 1% osmium tetroxide, 0.8% potassium ferricianide in the same buffer; dehydrated in a graded series of acetone (30%-100%) and embedded in Spurr resin. Semi-thin sections were stained with toluidine blue. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined in a Jeol JEM 100C or 1011 transmission electron microscope.

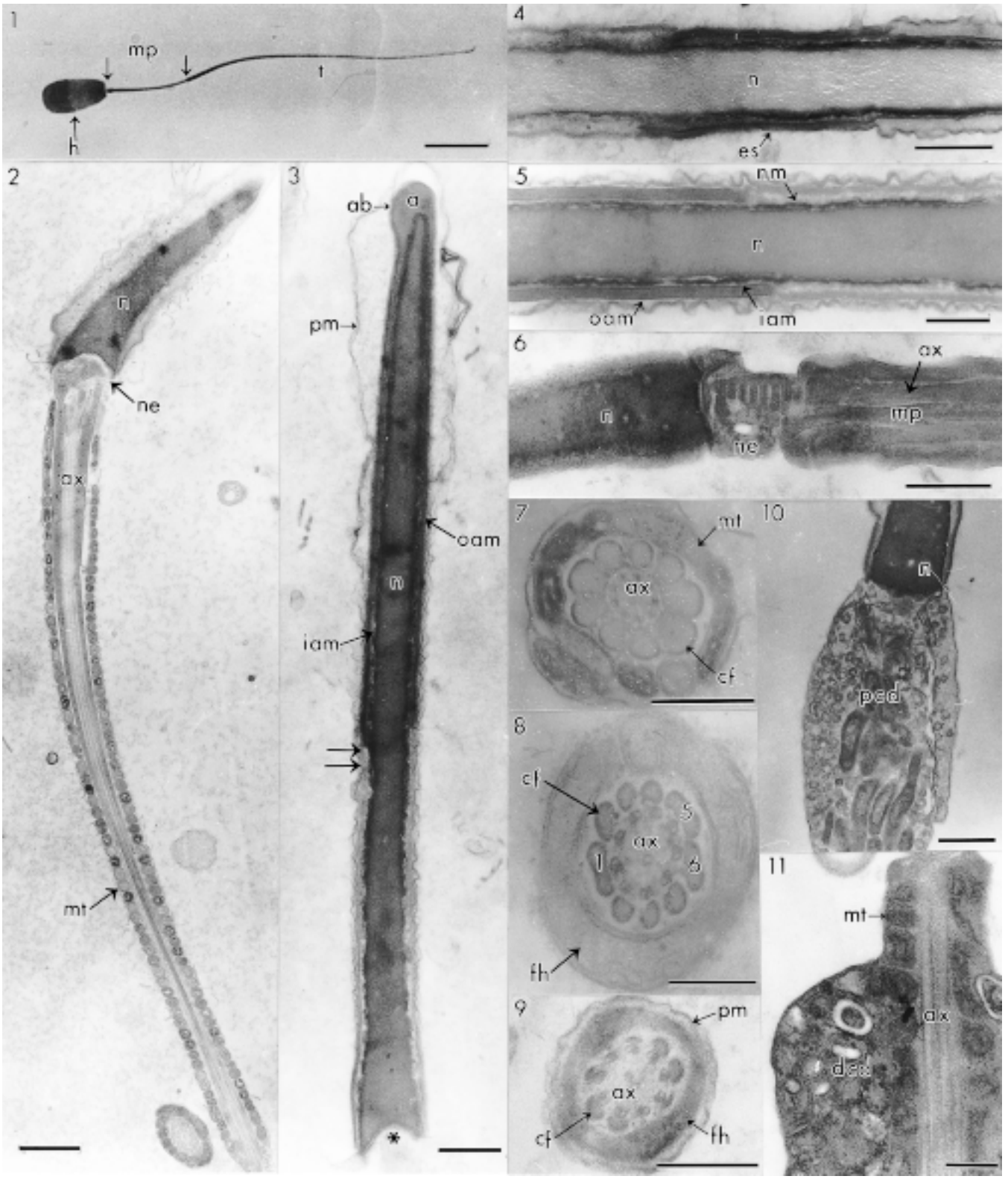
Results

The structure and ultrastructure of the spermatozoa was evaluated in two principal parts, namely head and tail (Fig. 1), and the latter was subdivided into four distinct parts: the neck, the middle piece, principal piece and terminal or end piece. The head presented externally a division between the acrosomal and post-acrosomal areas (Fig. 1), which was clearly observed with the Congo red stain. The mean total length of bovine sperm was approximately 40.3 μm.

The head

The head measured ±5.3μm in length and ±2.8μm in greatest width. The major characteristic is its flat,

FIGURE 1. Light micrograph of *Bos indicus* spermatozoon, head (h) and tail (t). The arrow indicates the external division between the acrosomal and post-acrosomal regions. The middle piece (mp) is limited by two arrows.
FIGURES 2-11. Electron micrographs of *Bos indicus* spermatozoon. **(2)** Longitudinal section of the head, neck (ne) and middle piece. (ax) axoneme; (mt) mitochondria; (n) nucleus. **(3)** Sagittal section of the head. The beginning of the post-acrosomal region and the equatorial segment is marked with two arrows. Note the concave recess at the base of the head (*). (a) acrosome; (ab) apical body; (iam) inner acrosome membrane; (oam) outer acrosome membrane; (n) nucleus; (pm) plasmatic membrane. **(4-5)** Longitudinal sections of the head. Note the closer contact of the membranes at the equatorial segment (es). (oam) outer acrosome membrane; (iam) inner acrosome membrane; (nm) nuclear membrane; (n) nucleus. **(6)** The neck (ne) is the connecting piece between the head ad the tail. (ax) axoneme; (mp) middle piece; (n) nucleus. **(7-9)** Transverse sections of different regions of the tail. **(7)** Middle piece. In the middle piece, the axoneme (ax) is surrounded externally by nine coarse longitudinal fibers (cf) and the mitochondrial helix (mt). **(8-9)** Principal piece, anterior and posterior regions, respectively. (ax) axoneme; (cf) coarse fibers; (fh) fibrous helix; (pm) plasmatic membrane. **(10-11)** Cytoplasmatic droplets. **(10)** At the neck region, proximal cytoplasmatic droplets (pcd). (n) nucleus. **(11)** At the middle piece, distal cytoplasmatic droplets (dcd). (ax) axoneme; (mt) mitochondria. **Scale bars: 1:** 10μm;. **2:** 1.0μm; **3-4,6-7,10:** 0.5μm; **5,8-9,11:** 0.25μm.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oval or paddle-shaped form with a square base, which provides a concave recess for the insertion of the tail (Fig. 2). The plasmatic membrane did not show any particular differentiation, being smooth in all its extension. The tapered nucleus was composed of a compact mass of homogeneously distributed electron dense chromatin in which some small vacuoles could be randomly seen. It was covered by a double nuclear membrane. The head cap tightly covered the anterior two thirds of the nucleus (Fig. 3); it was divided in three major layers: the inner and outer membranes of the acrosome and the cell membrane (Fig. 5), and was considerably thicker across the anterior margin of the head, reaching maximum thickness at the head apex. The acrosome is composed of fine homogeneous material of moderate electron density (Figs. 3 and 5). A distinct unilateral acrosomal bulge, the apical body (Fig. 3), was observed along the apical edge of the head, and it gradually decreased in size along the edge of the head until it ended in the vicinity of the equatorial region. The apical body has been previously described as a region where the head cap bends over itself.

The equatorial region demarcated the acrosome from the post-equatorial dense lamina that covered the caudal one third of the nucleus. It emerged from caudal extremity of the acrosome, and formed a posterior ring due to the closer contact of the plasmalemma and acrosome membranes (Fig. 4). The post-acrosomal region (Fig. 3) is constituted of the nucleus and its membranes and the cell membrane.

The tail

The mean total length of the tail was ±35µm, being the middle piece ±7.5µm long. The classical 9+9+2 fiber pattern was observed along three segments of the tail, namely the middle piece, principal piece and terminal piece. It consists of nine double fibers spaced equally in a ring around the central pair, composing the axoneme (Figs. 7-9). The axoneme is anteriorly bound to the mitochondrial helix (middle piece) and posteriorly to the fibrous helix (principal piece), except at the terminal piece.

- *Neck and middle piece*

The middle piece is joined with the head by a short segment of structure not clearly discerned referred to as neck or implantation region (Fig. 6). The neck is characterized by the lack of mitochondria and the presence of laminated coarse fibers entering the concave recess at the base of the head (Figs. 2 and 6). A homogeneous matrix across the top of the laminated fibers forms a base for the insertion of the fibers into the recess (Fig. 6).

The presence of the mitochondrial helix defines distinctively the middle piece, which is located between the neck and the *annulus*. It is the thickest and most anterior portion of the tail. Several strands of variable length form the mitochondrial helix, and they begin and terminate at different levels along the middle piece. Each strand is composed by an undetermined number of elongated mitochondria disposed end to end against one another. In longitudinal sections, the strands appeared as columns of mitochondria exterior to the axial fiber bundle (Fig. 2). Both longitudinal and transverse sections of the middle piece demonstrated that the mitochondria presented double membrane and cristae (Figs. 2 and 7).

In the middle piece, the axoneme is surrounded externally by nine coarse longitudinal fibers, which is clearly evident in cross sections of this region (Fig. 7). In cross sections, the coarse fibers appeared as electron dense rods of variable size and morphology and were always oriented in relationship to the central pair. The coarse fibers tapered as they passed down the middle piece, terminating at different levels within the principal piece (Figs. 7-9). Considering the already accepted numbering system for the coarse fibers (the single large fiber is number 1, proceeding in a clockwise direction), it could be observed large (numbers 1, 5 and 6), intermediate (9) and thin (2, 3, 4, 7 and 8) fibers. Nevertheless, if the coarse fibers are examined in cross sections obtained from the most anterior portion of the middle piece, they appear nearly equal in area (Fig. 7). The border between the middle piece and principal piece was well defined due to the termination of the thick mitochondrial helix and the presence of the *annulus*. This is an electron dense structure resembling a flat cone-shaped annular ring that surrounds the axial fiber bundle at the junction of the middle piece and the principal piece.

- *Principal piece*

The principal piece initiates at the *annulus* and tapers gradually towards the terminal piece. A fibrous sheath or helix begins at the termination of the mitochondrial helix, involving the axoneme externally. It is composed of a continuous dorsal and ventral longitudinal column connected by circumferential bands. The fibrous helix becomes thinner as it moves posteriorly along the axial fiber bundle, and moves closer to the axial filament complex as the coarse fibers disappear,

at the taper of the principal piece (Figs. 8 and 9). The two longitudinal elements (dorsal and ventral) of the fibrous sheath were evident in cross sections of the principal piece, and appeared as two thickenings on opposite sides of the fibrous helix (Fig. 9). These thickenings lay in line with the coarse fibers number 3 and 8 and became less and less evident at the posterior extremity of the principal piece.

- *Terminal piece*

The anterior portion of the principal piece tapered posteriorly to the end piece. The terminal piece formed the very short and thin termination of the tail and consisted only of the axoneme covered by the cell membrane. In general, the organized structure of the axoneme became disrupted towards the end of the tail (data not shown).

- *Other findings*

Some of the spermatozoa presented cytoplasmatic droplets, which are not considered to be a normal component of ejaculated sperm. They could only be observed in two major locations: at the neck region, where they are denominated proximal cytoplasmatic droplets (Fig. 10), and at the middle piece, where they are denominated distal cytoplasmatic droplets (Fig. 11).

Discussion

Mammalian spermatozoa exhibit considerable species differences in their size and shape, yet they all possess the same set of cellular organelles assembled on a common architectural pattern (Olson and Winfrey, 1991). This is also true for the ultrastructure of *Bos indicus* spermatozoa. Preliminary studies on the spermatozoa of *Bos taurus* (Bonadonna, 1958; Rahlman, 1961; Blom, 1963; Saacke and Almquist, 1964a, b; Blom and Birch-Andersen, 1965; Wooding and O´Donnell, 1971; Bustos-Obregon and Fléchon, 1975; Oko *et al.*, 1976; Williams, 1987; Barth and Oko, 1989) provide information on the ultrastructure of this species. Most of the ultrastructural features observed in this study were similar between the two species, which is not surprising since they are both members of the family Bovidae (Skinner and Smithers, 1990).

The head of the spermatozoon was the region that presented most differences, even though they were subtle ones. The shape was similar to that already described for other mammals (Fawcett, 1958, 1975; Bradfield, 1954; Hancock, 1966; Schmehl and Graham, 1989; Juhász *et al.*, 2000). The apical body described by Blom (1963) was also seen in all spermatozoa observed, with differentiation in size and shape. Such differences were already observed by Saacke and Almquist (1964a) and explained as a considerable individuality that spermatozoa may express with respect to the morphology of the apical body. A perforatorium was observed in the study of Barth and Oko (1989) as cone-shaped accumulation of partially electron dense material between the acrosome and the nucleus; this feature could not be confirmed by Saacke and Almquist (1964a) neither by the present study. The more electron dense and posterior region of the acrosome forms the equatorial segment, and corresponds to the thinning of the inner and outer acrosomal membranes (Wooding and O´Donnell, 1971). This structure remains intact when the anterior parts have disintegrated (Saacke and Almquist, 1964a). A basal plate located lining the implantation recess formed by the nucleus (Nicander and Bane, 1962) was not observed in the present study, but could be seen in previous studies of *Bos taurus* (Saacke and Almquist, 1964a). The nucleus presented rare and random small vacuoles, perhaps less in number than the ones observed by Saacke and Almquist (1964a). An intermittent nuclear cover observed outside of the nucleus in the post-acrosome region of the head by Nicander and Bane (1962) was not seen in the present study. The membrane involving the post-acrosome region was continuous and not porous as previously described (Rahlman, 1961). Furthermore, the head is smaller than that of *Bos taurus* (Saacke and Almquist, 1964a).

The tail, in general, showed even less variations than the head. Actually, the bovine sperm tail conforms quite closely to the general pattern described for other mammalian sperm (Saacke and Almquist, 1964b). The axoneme follows the classical 9+2 fiber arrangement, already observed in bull (Bradfield, 1955; Blom and Birch-Andersen, 1960; Rahlman, 1961) and other mammalian (Bradfield, 1954, 1955; Fawcett, 1958; Telkka *et al.*, 1961). The mitochondrial helix in the middle piece and the fibrous helix in the principal piece, both involving the more internal axoneme, are common features as well. The proximal centriole located in the recess at the base of the head could be rarely seen in longitudinal sections, and were always oblique (data not shown). In general, the morphology of the proximal centriole remains speculative (Saacke and Almquist, 1964b). In the middle piece, Telkka *et al.* (1961) observed in cross sections of the axoneme small arms ex-

tending laterally and connecting each doublet toward the adjacent doublet. Such arms could not be seen in the spermatozoa of *Bos indicus*. Saacke and Almquist (1964b) observed in cross sections of the middle piece that the coarse fibers had a core of moderate electron density with a border of greater density. This differentiation in electron density in the coarse fibers could not be confirmed. The *annulus* observed in the present study resembles the one already described by Barth and Oko (1989); it is a ring-like, dense annular structure, accompanied by a typical plasmalemma invagination. The ultrastructure of the principal and terminal piece is in agreement with general descriptions of mammalian spermatozoa (Fawcett, 1975).

The cytoplasmatic droplets are a common feature of bovine sperm (Rahlman, 1961; Blom, 1963; Saacke and Almquist, 1964b; Blom and Birch-Andersen, 1965; Wooding and O´Donnell, 1971; Barth and Oko, 1989). They could be observed in some spermatozoa and are defined as cytoplasmatic appendages that were not efficiently removed during the process of epididymal maturation, remaining associated with the sperm cell during spermiation. Furthermore, they could represent the remnants of the cytoplasmatic bridges, which are a common feature of mammalian spermatogenesis.

The present work shows that there are indeed particularities between the ultrastructure of the two species. Nevertheless, the significance of such particularities on the development and efficiency of different reproductive technologies can only be evaluated with additional research.

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