Spermiogenesis in scorpion mud turtle, *Kinosternon scorpioides**

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ABSTRACT. Viana D.C., Santos A.C. & Assis Neto A.C. **Spermiogenesis in scorpion mud turtle, *Kinosternon scorpioide*.** [Espermatogênese em tartaruga da lama *Kinosternon scorpioides*.] *Revista Brasileira de Medicina Veterinária, 37(4):389-396, 2015*. Department of Surgery, Sector Anatomy, School of Veterinary Medicine and Animal Science, University of Sao Paulo, Avenida Prof. Dr. Orlando Marques de Paiva, 87, Sao Paulo, SP 05508-270, Brazil. E-mail: diego_carvalho_@hotmail.com

The spermatogenesis events have been commonly studied in mammals. On the other hands, there are few studies in reptiles, especially in turtles. In this group there are unique structural variations for sperm formation, determined by reproductive adaptive divergence. The aim of present study was to deepen the understanding about the subcellular process of the spermiogenesis by transmission electron micrographic analysis in turtles *Kinosternon scorpioides*. The result showed the cytoplasmatic elongation of the spermatid in order to removing the content excess and allows the meeting between the spermatozoa and ova. Such morphological aspect could be necessarily to move efficiently in direction to the ova in female reproductive tract. In addition mitochondrial structure and shape was poorly defined with ridges and dense nuclei surrounded by concentric lipid bilayers.

KEY WORDS. Electron microscopy, manchette, spermatozoa, ultrastructure, reptile.

RESUMO. Os eventos da espermiogênese foram bem estudados em mamíferos, entretanto, poucas investigações foram feitas em répteis e principalmente em tartarugas. Nesste grupo estão presentes animais que possuem estruturas únicas na formação dos seus espermatozoides definidos por divergentes adaptações reprodutivas. O objetivo do presente estudo foi aprofundar a compreensão dos eventos subcelulares da espermiogênese utilizando análises minuciosas de microscopia eletrônica de transmissão. Os resultados demonstraram um alongamento citoplasmático de espermátides que podem estar relacionadas a eliminação de conteúdos citoplasmáticos. Esta disposição morfológica pode ser necessária para permitir que os espermatozoides se locomovam de forma mais eficiente até os óvulos no trato reprodutivo feminino. Outra observação, foi a presença de mitocôndrias com estrutura e forma mal definidas, cristas e núcleos densos, rodeada por bicamadas concêntrica de lipídios.

PALAVRAS-CHAVE. Microscopia eletrônica, espermatozoide, manchete, ultraestrutura.

INTRODUCTION

Ultrastructural studies about spermiogenesis were performed in reptiles such as lizards and snakes (Ferreira & Dolder 2003, Liu et al. 2005). Another comparative studies on spermatozoa of turtles (Hess et al. 1991, Scheltinga et al. 2001), lizards (Gribbins et al. 2011) and alligator (Gribbins et al. 2011) revealed that each species have unique structures.

In the turtle *Kinosternon scorpioides* were investigated the structure and function of the testis. Particularly the aspects related to spermatogenic cycle and the entire process cycle length were 12 and 55 days, respectively (Sousa et al. 2014.

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The flesh turtles *K. scorpioides* are very appreciated as food in fine restaurants and on popular feed. Thus all the turtle's restaurants will need maintain farms for captivity this specie for be licensed by official organism of preservation in its activity. However, due to predatory hunting and deforestation, the number of individuals has decreased systematically in the last decade (Pereira et al. 2007, Carvalho et al. 2010, Viana et al. 2014b). The contribution for the study of phylogenetic relationships justifies the detailed comparisons between diverse reptiles. On the other hand, this study will contribute with the little literature about spermiogenesis in turtles (Dehlawi et al. 1992, Zhang 2007).

In temperate zone regions, studies have shown that male turtles are not synchronized with the female reproductive cycle seasonal. This fact results in the production of gametes at different times of year (Licht 1984, Viana et al. 2013). The prolonged survival of spermatozoa in both reproductive systems, male or female suggests that the spermatozoa of turtles may have morphological or functional specializations contributing to their longevity (Bian et al. 2013). Thus, there is need to examine the cytology of spermatogenesis in most species of reptiles.

The understanding of spermatogenesis can support the development of appropriate plans for reproduction, conservation and management by reproductive breeding programs in natural and artificial environment, besides being useful for comparison between spermatozoa of different reptiles and other vertebrates. Therefore, the aim of this research was to provide data about the ultra spermatic differentiation on the spermiogenesis of *K. scorpioides*.

MATERIAL AND METHODS

Nine adult turtles males *K. scorpioides* were used. The specimens were from *ex-situ* capture in São Bento, MA, Brazil, as authorized by Chico Mendes Institute for Biodiversity Conservation - ICMBio / MMA, No. 33021-4 and with the approval of Ethics and Animal Experimentation Committee of Veterinary Medicine, State University of Maranhão, MA, Brazil (EAEC/UEMA).

The animals were anesthetized with xylazine 2% (40mg/kg/IM) and ketamine hydrochloride 1% (60mg/kg/IM) and euthanized with thiopental sodium 2.5% (60mg/kg/EV) by catheterization of the cervical venous sinus, according to technique of Hernandez-Divers et al. (2005). The coelomic cavity was opened with a steel handsaw to disarticulate the bone bridge that joins the carapace to the plastron. The gonads were removed and the testes isolated and cut in blocks (1 mm3). Then, tissue was fixed in 2.5% de glutaraldehyde washed in 0.1 M phosphate buffer and post-fixed in osmium tetroxide 1%. The samples were dehydrated by ethanol in increasing concentrations (from 50 to 100%), in propylene oxide and resin. The mixture was replaced by pure resin and placed in molds. The ultrathin sections were collected on copper screens and contrasted with uranyl acetate solution at 2% and 0.5% lead citrate. The samples were analyzed by transmission electron microscopy Morgagni 268d, FEI Company, Tokyo, Japan.

RESULTS

Morphological differences were not observed between the phases for all specimens. At early of development, the spermatids had a rounded nucleus with granular chromatin. There are some mitochondria in the cytoplasm surrounding the nucleus. The Golgi apparatus and the endoplasmic reticulum were well developed. For comparison, the spermatic differentiation was classified by five consecutive stages in according to the terminology of Gribbins et al. (2003) and based on the classification of Vieira et al. (2001) and Zhang et al. (2007).

Stage I

A proacrosomal vesicle develops from the Golgi apparatus near the nucleus (Figure 1). The proacrosomal vesicle is pressed against the nuclear wall, in which rise a shallow depression (Figure 2). As proacrosomal vesicle develops, a dense granule appears inside. This granule is initially connected to the vesicle. Between the nucleus and the vesicle, a dense fibrous layer, known as the manchette was observed. Then, a subacrosomal granule is formed in the middle of the fibrous layer under the acrosomal gra-

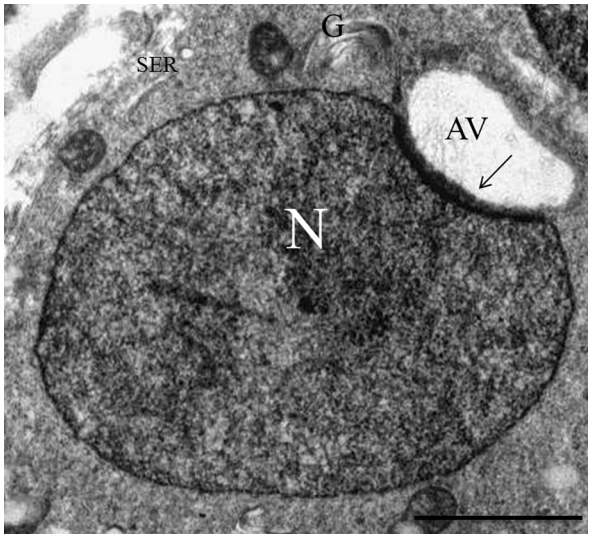

Figure 1. The proacrosomal vesicle (AV) is pressed against the nuclear (N) wall to form a wide shallow depression (arrows). Note the well developed Golgi body (G) in close proximity to the acrosomal vesicle. Note also the well developed smooth endoplasmic reticulum (SER). Scale bar = 2µm.

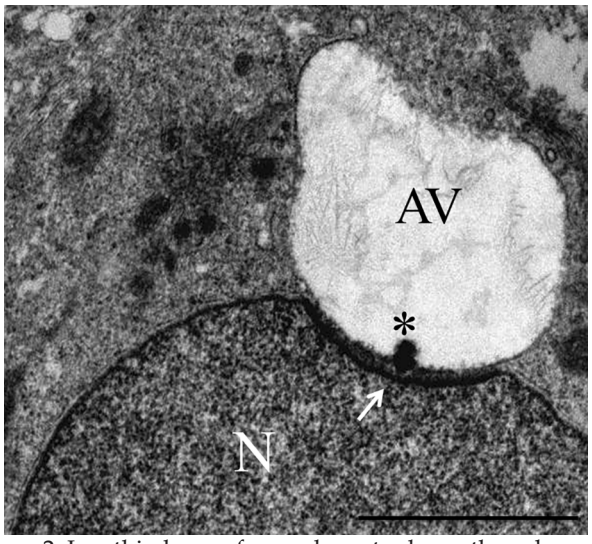

Figure 2. In a thin layer of granular cytoplasm, the subacrosomal granule (arrows) is formed in the middle of the fibrous layer under the acrosomal granule (*). Scale bar = 1µm.

nule. The subacrosomal granule disappears (Figure 3) within the nucleus. In this moment, the spermatid has a round nucleus, which is slowly lengthening.

Stage II

A salience against acrosomal vesicle is formed in the anterior region of the nucleus, which becomes progressively concave. Dense acrosomal granule propagates within the vesicle, as well as the fibrous layer became in the subacrosomal cone. The acrosomal complex involves the acrosomal extremity and subacrosomal cone, which covers the narrow portion of the initial nucleus (Figure 4). The nucleus elongates and chromatin continues to condense into compacted granules. Due to condensation of the nucleus, the nuclear volume decreases substantially. The specialized structure called circular manchette surrounds the spermatid cell nucleus during the period of nuclear condensation and elongation. In the longitudinal direction of the head, the manchette is formed by small vesicles (Figure 5). The cytoplasm rearrangement is consequence of the eccentric movement of the nucleus.

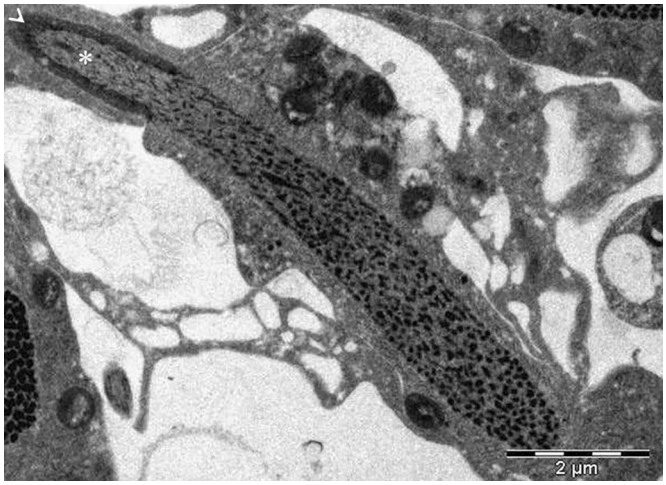

Figure 4. The anterior region of the nucleus forms a protrusion (*) that presses against the acrosomal vesicle. The acrosomal granule diffuses throughout the acrosomal cap (arrowhead). Scale bar = 2µm.

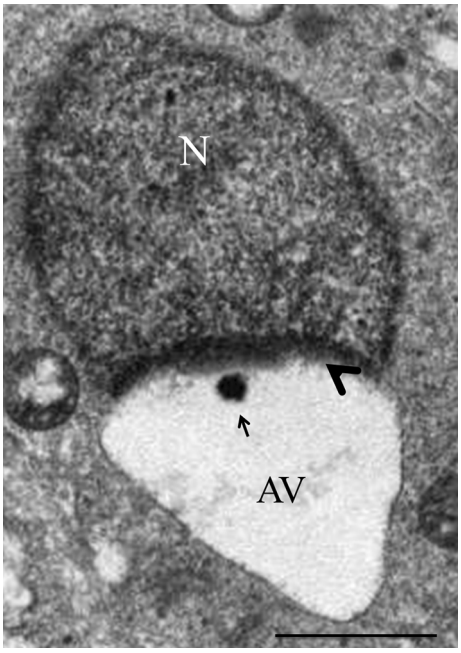

Figure 3**.** The nucleus becomes more eccentric within the spermatid and the acrosome flattens slightly. As the proacrosomal vesicle develops, a dense fibrous layer (arrowhead) and an acrosomal granule (arrows) appear in its interior. Scale bar = 1µm.

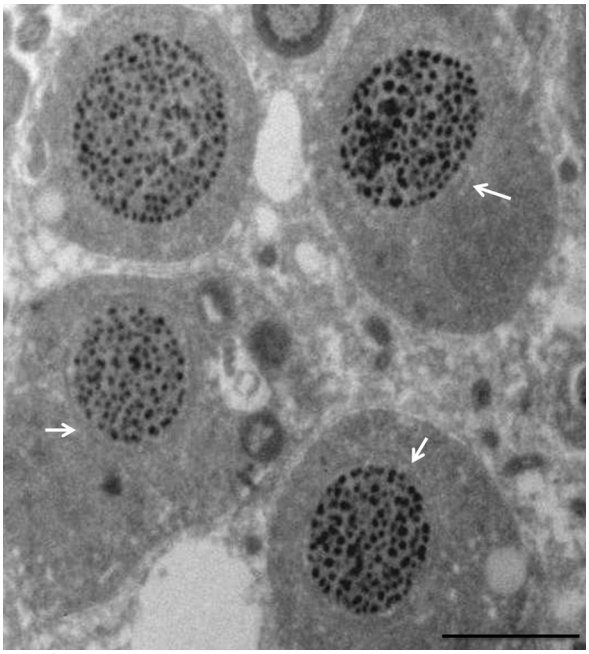

Figure 5. Cross-section of the spermatid nucleus (N). The loose chromatin continues to condense into coarse granules. The circular manchette (arrows) still consists essentially of a single coil of microtubules. Scale bar = 2µm.

Stage III

The base of the flagellum shows a depression at the caudal extremity of the nucleus (Figure 6). The cytoplasm of the spermatids shows flanking part of nucleus of spermatids and the proximal flagellum (Figure 7). In the posterior pole of the nucleus, the mitochondria moves and accumulates around the centrioles (Figure 8). Around these mitochondria seven or eight concentric membranes and a ring,

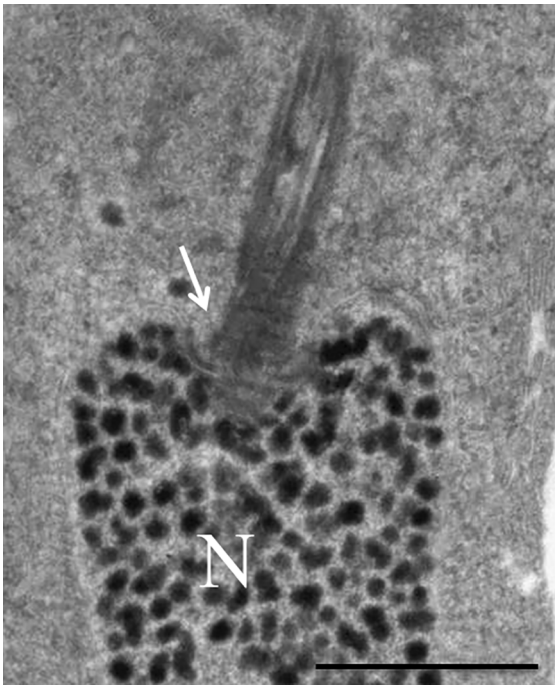

Figure 6. The caudal end of the nucleus is now lodged into a depression (arrow). The nucleus (N) is filled with dense granular material. Scale bar = 0.5µm.

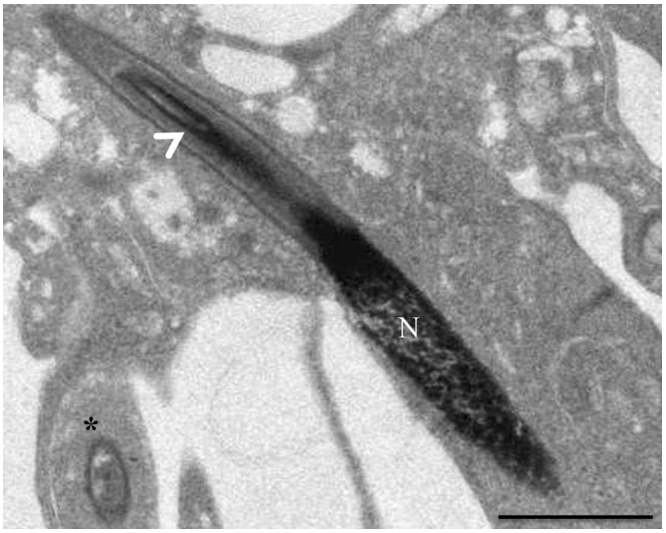

Figure 7. The nucleus gradually becomes compact. The trailing cytoplasm, containing clustered mitochondria (*), flanks the nucleus at the caudal region of the cell. The circular manchette is formed of small vesicles (arrowhead). Scale bar = 2µm.

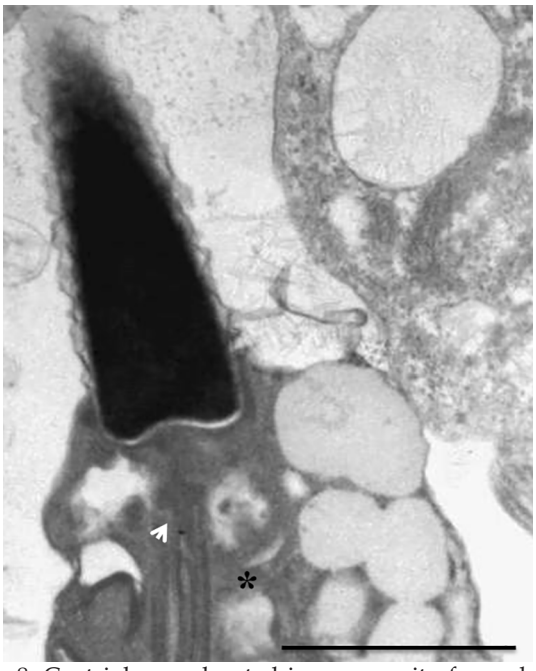

Figure 8. Centrioles are located in a concavity formed at the posterior pole of the nucleus. The centriole complex comprises the proximal centriole and the distal centriole (arrowhead). The early arrangement of mitochondria (*) surrounds the centriole complex. Scale bar = 1µm.

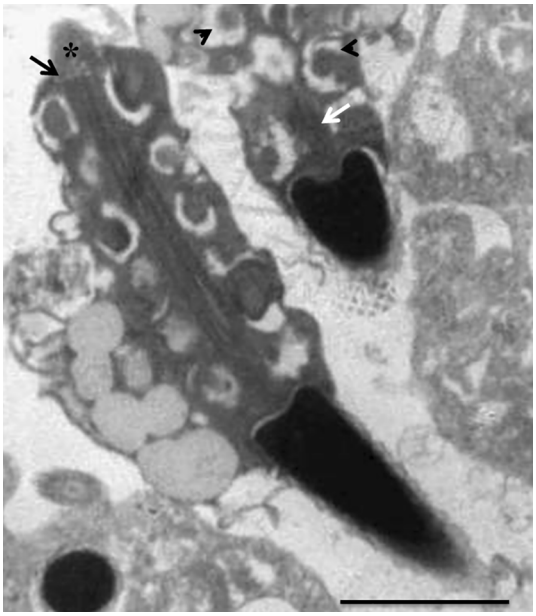

Figure 9. The annulus (small arrow) and the proximal flagella (*). The mitochondria have changed their shapes (arrowhead) and start to gather around the distal centriole (white arrow). Scale bar = 2µm.

which separates the last mitochondria of the tail was observed (Figure. 9).

Stage IV

The nucleus becomes curved and its anterior portion becomes conical. During the phases of nuclear elongation and condensation, the granules form in a homogeneous compact mass, typical of mature

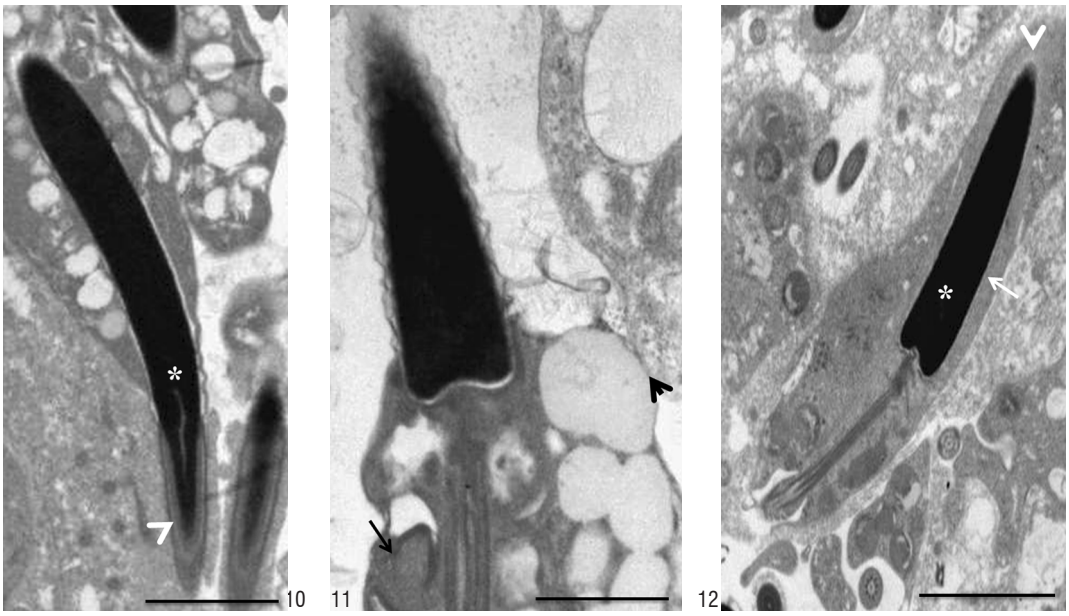

Figure 10. The anterior region of the nucleus becomes narrow and extends through formed nuclear protrusion (*), to which the acrosomal cap (arrowhead) corresponds. Scale bar = 2µm.

Figure 11. A cytoplasmic lobe (arrowhead) forms from the caudal spermatid head and mitochondria sheath (arrow) and extends toward the lumen of the seminiferous tubules. Scale bar = 2µm.

Figure 12. The shapes of the acrosomal cap (arrowhead) and acrosomal cone match the nuclear protrusion (*). Nuclear shoulders mark the posterior limit of the acrosome (small arrow). Scale bar = 2µm.

sperm chromatin. In the longitudinal section of the spermatozoid head, the manchette assumes an oblique orientation (Figure 10). The residual cytoplasmic bodies are derivatives of cytoplasmic lobes during elongated action on the spermatid and it is separated of spermatid before the spermiation (Figure 11).

Estágio V

In the last stage, the longitudinal manchette dissolves and spermatids become mature spermatozoids. The anterior region of the nucleus becomes narrow and long, as result of the formation of a nuclear salience and its outside is connected directly with the subacrosomal cone. The transition region, known as nuclear adjacent is the mark abrupt of the limit acrosomal (Figure 12). The head of the spermatozoids acquires conical shape, and the mitochondrial sheath shows two rows (Figure 13).

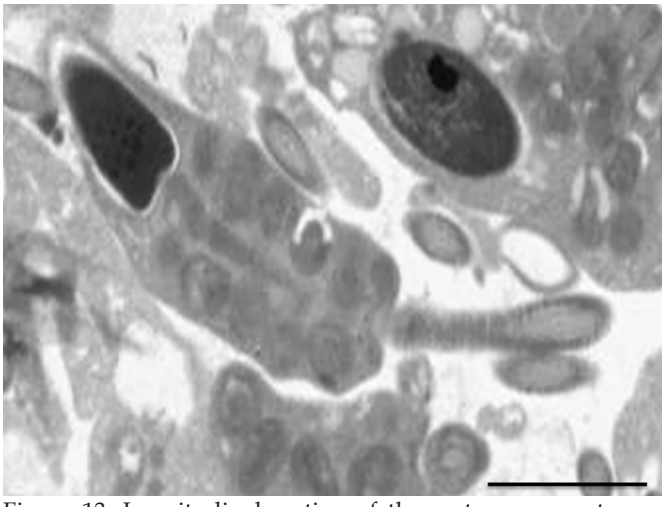

Figure 13. Longitudinal section of the mature spermatozoa, showing part of the head and the mitochondria sheath of the middle piece. Scale bar = 2µm.

DISCUSSION

The form of the spermatozoa of *K. scorpioides* is similar to domestic birds, amphibians and some reptiles. However, some structures are uniquely different those observed in mammalian spermatozoa and another reptiles (Wing et al. 1986, Philips & Wing 1989, Liu et al. 2005, Zhang et al. 2007).

The nuclear morphology, which involves elongating and compaction of chromatin, is one of the key events of spermiogenesis. This particular manchette formation suggests a prominent role for nuclear formation in some animals. Asa and Phillips (1988) investigated this nuclear formation in spermatids of Thai leaf frog, *Megophrys Montana*, wherein the nuclear conformation was independent of microtubular influence. Another example is the spermatogenesis in tree frog *Hyla chinensis* (Lin & You 2000), with nuclear formation without microtubules.

In some species of birds, the appearance of manchette in the spermiogenesis has generated consi-

derable controversy in different studies. In chicken (*Gallus domesticus*) (Sprando & Russell 1988) was observed that the development of a perinuclear region surrounding the array of microtubules. In addition, in *Rhea americans albisceus*, the manchette surrounds the spermatids nucleus, and subsequently the microtubules becomes rearranged in a longitudinal manchette.

At the times when the manchette is circular and longitudinal, the microtubules are arranged in oblique shape (Asa & Phillips 1988). The spermatids of ostrich have the manchette in circular shape. Also is more developed than when in longitudinal shape, similar that observed in the spermatids of chicken (Nagano 1962, McIntosh & Porter 1967, Nishiyama & Okamura 1976, Xia et al. 1986).

Some species of reptiles have organized perinuclear microtubules. For example, in lizards (*Tropidurus itambere)* (Hofling 1978) the manchette coating has helicoidal shape surrounding the nucleus at the beginning. After acquires a longitudinal arrangement condensing the nucleus. On the other hand, spermatids no show the microtubules in lizard of specie *Natrix piscator* (Gao et al. 2001). Present study revealed circular and longitudinal manchette during spermiogenesis, which is very similar to many species of birds. Furthermore, we observed that manchette may apply a mechanical force against the nucleus of spermatids.

During spermiogenesis in amphibians as the frog *H. chinensis* (Lin & You 2000), the chromatin fibers become thicker and are arranged in cylindrical shape, which is wound within nuclear envelope. Subsequently, the thickness of the chromatin fiber and winding structure increases. The process of chromatin condensation in *Rhea americans* (Asa & Phillips 1988) and domestic birds (Sprando & Russell, 1988) is different from frog *H. chinensis*. They do not exhibit cylindrical structure or remodeling of the nuclear envelope.

These chromatin fibers are reoriented along its longitudinal axis. In *K. scorpioides*, the pattern of condensation of chromatin is slightly different because chromatin begins to condense into small granules rather small fibers while the nucleus is in spherical shape. The progress of condensation promoted spaces between chromatin granules decrease and, finally the chromatin aggregates in a homogeneous electro-dense mass. In spermatids of lizard *Takydromus septentrionalis* (Liu et al. 2005) chromatin condenses into fibers as short filaments and the nucleus increases.

Due to condensation of chromatin, the nuclear volume decreases gradually, and do not observe continuous dissolution and regeneration of the envelope in this study. Comparative studies of spermiogenesis in related species of amphibians and reptiles as turtle *Pelodiscus sinensis* (Zhang et al. 2007) have shown that each species has a unique chromatin compaction process.

Most of the acrosome of spermatozoa of turtles consists of small cone on the surface of the nucleus, similar to that observed for birds and reptiles (Nishiyama & Okamura 1976, Gunawardana & Scott 1977, Jones & Lin 1993). However, as is typical mammalian spermatozoids, the acrosome extends caudally and is significantly higher than in reptiles and birds. In the lizards *Agama stellio* (Al-Hajj et al. 1987) two pro-acrosomal vesicles appear in the cytoplasm near of the nuclear depression, and then, they merge to form a large acrosomal vesicle. The subacrosomal granule appears in the middle of a fibrous layer at a stage in which nuclear chromatin does not seem condensed, and then disappears.

In birds, a small amount of cytoplasm is found in the elongation; however, most of the residual cytoplasm in the chicken is lost as a residual body near the nuclear extremity of sperm (Sprando & Russell 1988). In the *K. scorpioides* is observed what the cytoplasm of the spermatid during the elongation is displaced along the nuclear body and in the proximal flagellum. This elimination of cytoplasm excess is required to allow the sperm movement and become efficient manner for reach of ova in the female reproductive tract. On the other hand, in mammals, the mitochondria are longitudinal elongated columnar structures, but the mitochondria in *K. scorpioides* have structure and shape poorly defined with ridges and dense nucleus surrounded by concentric layers.

The mitochondria of spermatozoa form a columnar arrangement around of distal centrioles, rather than a helix as found in mammalian (Fawcett 1975), in birds (Hess & Thurston 1987) and in turtles (*Pelodiscus sinensis*) (Bian et al., 2013). The mitochondria are merged and delineate the spermatic structure of the invertebrates give the way to the body of the spermatozoid (Baccetti & Afzelius 1976). The ridges have circumferential shape in Chinese hamster (Fawcett 1970).

The spermatozoa of opossum (Fawcett 1970) and fish (*Gadus morhua*) (Reboursa & Ottesenb 2013) have spiral membranes inside the mitochondrial nucleus. Membranous structures are observed in the mitochondria of rhea (Phillips & Asa 1989).

However, no structure comparable to similar

lipid bilayers in spermatozoa of turtles were identified in another species yet. Some mitochondrial changes were reported in other species, such as the bat, in which the spermatozoa has quiescence behavior in the female genital tract (Wimsatt et al. 1966, Mori & Uchida 1972).

Under appropriate conditions, mammalian spermatozoa may use phospholipids as source of energy (Fawcett 1975). Thus, it is speculated that there is an unusual laminated mitochondria in spermatozoa of turtle for maintain the sperm during prolonged storage in the female oviduct.

The reptiles are useful models for these types of experimental studies because they share features consisting of different phylogenetic lineages, and thus, this feature provides a reliable performance in relation to other classes of animals, and may help to distinguish abnormalities between different germinative cells within testes and, absence or interruption of certain phases of development of spermatozoa.

In conclusion, this study demonstrated that the process of elongation of cytoplasm in the spermatid is able to remove content excess required, allowing the efficient movement of spermatozoids to reach the ova in the female reproductive tract. In addition, the mitochondria have structure and shape poorly defined, with ridges and dense nucleus surrounded by concentric lipid bilayers.

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