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Cycle of the seminiferous epithelium of the bat *Molossus molossus*, characterized by tubular morphology and acrosomal development

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ABSTRACT

Objective: To describe the seminiferous epithelium cycle (SEC) by tubular morphology method, and the acrosomal development of individualizing spermatids, and to explore the distinction of the stages between two generations of spermatids. **Methods:** Testicular fragments were fixed in Karnovsky, embedded in glycol methacrylate and examined under light microscopy and transmission electron microscopy. Sections in 3 μm thickness were stained with toluidine blue for the characterization of the stages of the SEC by the tubular morphology method, or submitted to the PAS reaction for the visualization of the acrosomal formation. Additional details on the acrosomal formation were observed under transmission electron microscopy. **Results:** Through the eight stages described by tubular morphology method, 10 steps of acrosomal formation were observed in the spermatid development, called acrosomal steps. As the spermatids were produced in stage V of the tubular morphology method, it was at this stage from which began the steps of acrosomal development. **Conclusions:** We propose association of the acrosomal steps for the first time, with the different stages by tubular morphology method. This method presents an alternative to the existent methods, allowing interspecific comparisons of the SEC, not only among different species of bats, but also among the other mammals.

1. Introduction

Bats are the second largest group of mammals in terms of species richness[1]. They act on the environment in several ways, either as seed dispersers, pollinators, vectors of zoonozies or controllers of insect populations[2], including here the insectivorous species *Molossus molossus* (*M. molossus*), which is widely distributed throughout Brazil.

The reproductive biology of bats can be as variable as the habits where they are found, and they also have different reproductive strategies[2-4]. Knowledge of testicular morphophysiology is important for the biological understanding of reproduction. Several studies have been conducted on testicular function in different species of bats, especially those of temperate regions[5-9], and a few discuss spermatogenesis. Spermatogenesis is a complex and well-organized process of cell division by a spermatogonia to form the spermatozoon. This process occurs in the seminiferous tubules of sexually mature animals, where the spermatogenic cells are arranged in a series of well-defined cellular associations, or stages[10].

The stages follow one another in a particular region of the seminiferous epithelium over the course of time. This sequence is called the cycle of the seminiferous epithelium[11]. One system used for studying these stages of the seminiferous epithelium cycle is that based on the shape and location of the nuclei of spermatids and spermatocytes, and on the presence of figures of meiotic divisions. This system, designated as the tubular morphology method by Berndtson[12], divides the cycle into eight stages for

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mammals. Another method of identification is based on morphology of the spermatids, in particular the formation of the acrosomal system. In this method, described for the first time by Leblond and Clermont[11], stages are arbitrarily referred, and the number of stages for each species is variable, averaging 14.

The tubular morphology method can facilitate comparison between species, while this comparison is more difficult in the acrosomal system method, which is species-specific. However, using the former method it is difficult to identify the stages with two generations of spermatids, while the latter method of observing the formation of the acrosomal system makes it possible to monitor all differentiation stages of spermatids, thus enabling different generations to be distinguished. So, this study aims to describe the cycle by the tubular morphology method, and propose individualizing spermatids in an association with their steps of acrosomal formation, as an alternative to the existents methods, using as a model of study the bat *M. molossus*.

2. Material and methods

2.1. Animals

Five adult male *M. molossus*were captured in the summer in the south-eastern state of Minas Gerais, City of Viçosa, Brazil (20° 45′14″S e 42° 52′53″W). Samples were collected at dusk, using mist nets set up near the animal roosting sites. The captured bats were placed in cages, which were kept protected from light. A diet composed of *Tenebrio* sp. coleopteran larvae and water *ad libitum* was offered, from capture in the field until euthanasia in the laboratory on the day following the night of capture. Euthanasia was performed by injecting sodium pentobarbital (Nembutal) at a concentration of 40 mg/kg intraperitoneally, followed by injection of a saturated solution of potassium chloride. The captures were authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA-MG-139/06-NUFAS-MG) and by the State Institute of Forests from Minas Gerais (IEF-MG-121/06). The present experimentation methodology has been evaluated and approved by the Ethics Commission of the Veterinary Department-UFV, document number 93/011.

2.2. Tissue samples

After euthanasia, the reproductive organs were removed and fixed by immersion in Karnovsky solution[13] for 24 h, and transferred to ethanol 70%. The testes were weighed, dehydrated in crescent ethanol series, and embedded in glycol methacrylate (Historesin, ©Leica Microsystems, Nussloch, Germany). Sections of 3 μm thickness at intervals of 40 μm were obtained in order to avoid the same seminiferous tubule. For analysis using the tubular morphology method, the preparations were stained with toluidine blue and sodium borate 1% (©Merck, Darmstadt, Germany). For visualization of the acrosomal system, preparations were stained with periodic acid-Schiff (PAS) and counter-stained with hematoxylin. The slides were mounted with Entellan (©Merck, Darmstadt, Germany), and analyzed under light microscopy. For characterization of the ultrastructure of spermatids, testicular fragments were post-fixed with 1% osmium tetroxide (EMS, Hatfield, USA) for 3 h, dehydrated in acetone and embedded in resin Epon 812 (EMS, Hatfield, USA). Ultrathin sections were stained with 3% uranil acetate (EMS, Hatfield, USA) and 3% lead citrate (EMS, Hatfield, USA) and observed using a transmission electron microscope Jeol 1011 (Deutschland, Germany).

2.3. Analysis of results

The results obtained were submitted to descriptive analysis, comparing the acrosome formation along the stages of the seminiferous epithelium cycle characterized by the tubular morphology method.

3. Results

The seminiferous epithelium of the bat *M. molossus* showed different arrangements between the different generations of germ cells. The distribution of these arrangements, or stages, is presented segmentally along the length of the seminiferous tubule. There is usually therefore only one stage per tubular cross-section. Different generations of germ cells were observed in cross-sections of the seminiferous epithelium and were compartmentalized, so that the spermatogonia were observed near the tunica propria, as well as younger generations of primary spermatocytes. The primary spermatocytes of older generations composed an intermediate layer, and spermatids were observed to be distributed in the layer closest to the lumen of the seminiferous tubule. Sertoli cells were observed to have nuclei often with a rounded or oval shape near the basal compartment of the epithelium at all stages, with loose chromatin and a well developed nucleolus (Figure 1).

Using the tubular morphology method, the cycle of the seminiferous epithelium in *M. molossus* was divided into eight stages, and represented in Roman numerals. However, for an accurate characterization of these stages, this method was associated with the acrosomal development, which is used to describe the different steps in acrosomal morphology throughout its development, represented in Arabic numerals. Thus, through the eight stages described using the tubular morphology method, 10 steps were observed in the spermatid development of *M. molossus* (Figure 2), called acrosomal steps. As the spermatids are produced in stage V of the tubular morphology method, it is at this stage from which begin the steps characterized by the acrosomal development. Thus, in stage I the presence of step 5 is observed, step 6 in stage II, step 7 in stage III, and step 8 in stage IV. Starting from stage V of the tubular morphology method two generations of spermatids are observed, at this stage being found as a newly formed generation, representing acrosomal step 1, and another older generation, representing acrosomal step 9. Thus, in stage VI, spermatids were observed in step 2 and step 10, steps 3 and 10 in stage VII, and steps 4 and 10 at stage VIII (Figure 2). A description of these associations follows, and the ultrastructural characteristics of the 10 steps of acrosomal formation is shown in the Figure 3.

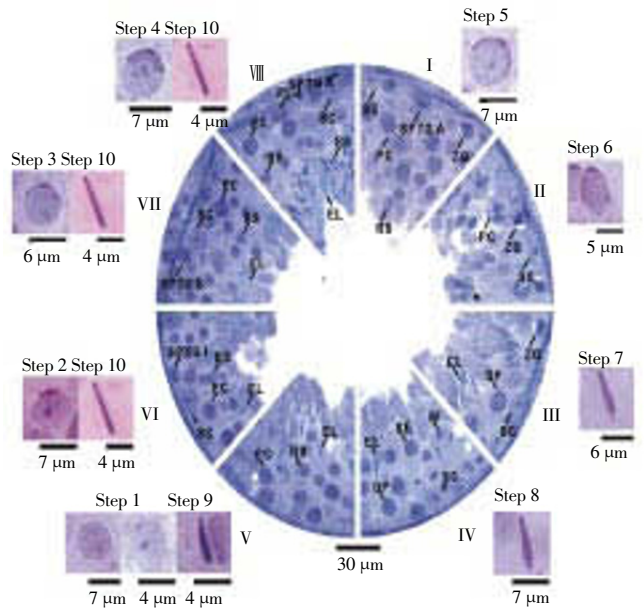

Figure 1. Photographic mounting showing the eight stages of the seminiferous epithelium cycle in the bat *M. molossus* under light microscopy (Roman numerals, Toluidine blue).
The different steps of acrossomal formation are showed in boxes (Arabic numerals, PAS). SC: Sertoli cell; SPTG A: type A spermatogonia; SPTG I: intermediate spermatogonia; SPTG B: type B spermatogonia; PL-L: primary spermatocyte in pre-leptotene to leptotene; ZG: primary spermatocyte in zygotene; PC: primary spermatocyte in pachytene; DP: primary spermatocyte in diplotene; M: metaphasic figure; SE: secondary spermatocyte; RS: round spermatids; *: elongating spermatids; ES: elongated spermatids; RB: residual bodie.

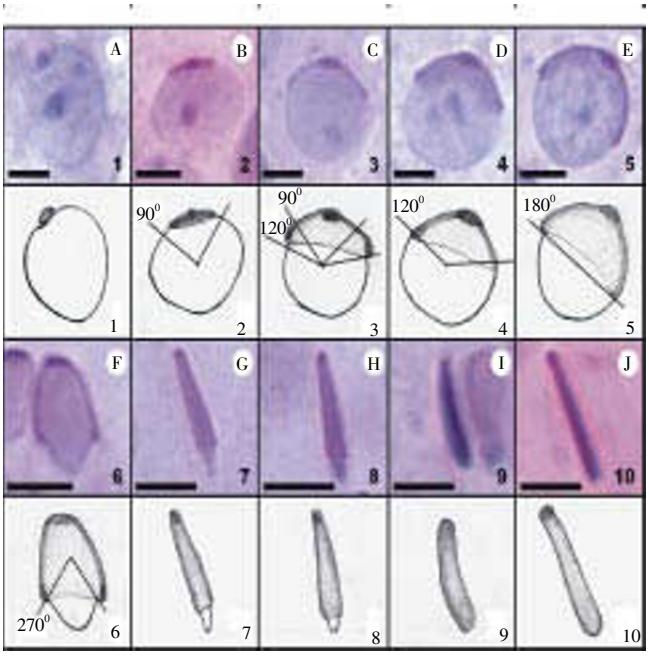

Figure 2. Different steps of acrosome formation in the nuclear surface of spermatids of the bat *Sturnira lilium* by light microscopy (upper figures) and their schematic drawings (lower figures).
A) Step 1, Bar: 3 μm. B) Step 2, Bar: 3 μm. C) Step 3, Bar: 3 μm. D) Step 4, Bar: 3 μm. E) Step 5, Bar: 3μ m. F) Step 6, Bar: 5 μm. G) Step 7, Bar: 5 μm. H) Step 8, Bar: 6 μm. I) Step 9, Bar: 6 μm. J) Step 10, Bar: 7 μm.

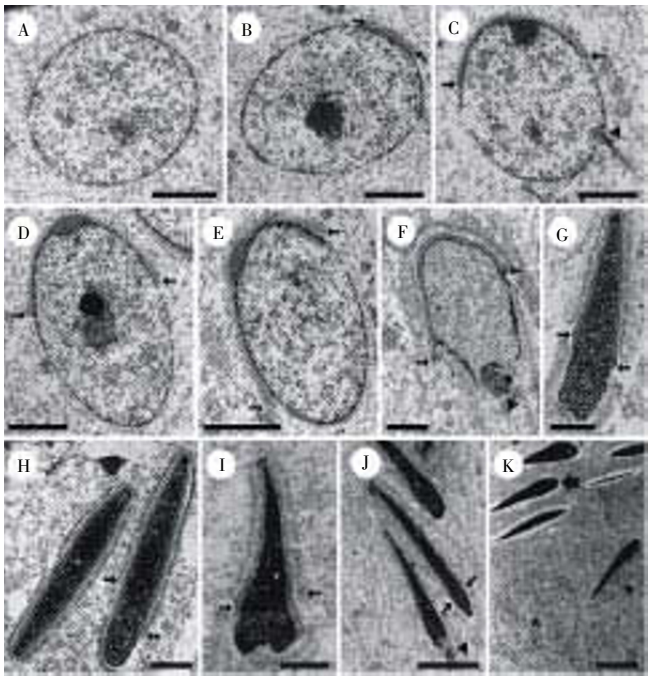

Figure 3. Ultrastructure of the 10 steps of acrosomal formation.
→: acrosomal boundary; ▸: centriole. A) Step 1, Bar: 2 μm. B) Step 2, Bar: 2 μm. C) Step 3, Bar: 2 μm. D) Step 4, Bar: 5 μm. E) Step 5, Bar: 2 μm. F) Step 6, Bar: 1 μm. G) Step 7, Bar: 1 μm. H) Step 8, Bar: 1 μm. I) Step 9, Bar: 2 μm. J) Step 10, Bar: 2.5 μm. K) Stage 5 of the seminiferous epithelium cycle with spermatids showing the acrosomal steps 1 (*) and 9 (➥). Bar 3 μm.

3.1. Stage I

Type A spermatogonia and primary spermatocytes of tree generations were found to be present at this stage, in the phase of pre-leptotene to leptotene or already in zygotene, close to the tunica propria, and another at pachytene, in an intermediate location in the epithelium. The spermatids observed in this stage were rounded and located in at least two layers of cells near the lumen (Figure 1-I). These cells revealed the formation of the acrosome in an advanced stage of development (acrosomal step 5), with the formation of an acrosomal cap involving approximately 180° of the nuclear surface (Figure 2-E; Figure 3-E), directing it toward the base of the seminiferous tubule.

3.2. Stage II

Beyond the presence of type A spermatogonia, at this stage primary spermatocytes at zygotene near the basal lamina and primary spermatocytes at pachytene in the intermediate region of the epithelium were observed. The most remarkable aspect was the beginning of elongation of the nuclei of round spermatids (Figure 1-II), which showed acrosome occupying an area of up to 270° of the nuclear surface, characterizing acrosomal step 6 (Figure 2-F). At the start of elongation, spermatid nuclei initiate contact with the plasma membrane, and the cytoplasm also appears elongated, extending toward the lumen and surrounding part of the flagellum in formation (Figure 3-F).

3.3. Stage III

Two generations of primary spermatocytes were present at this stage, these being the spermatocytes in zygotene and in diplotene. The nuclei of spermatids continued to elongate and formed bunches, deeply inserted in the seminiferous epithelium (Figure 1-III). Spermatids observed in this stage continued the process of elongation and chromosome condensation, becoming more elongated than in the previous step, and with an occupancy close to 270° of the nuclear surface, a feature of acrosomal step 7 (Figure 2-G; Figure 3-G).

3.4. Stage IV

Figures of meiotic division are typically observed in stage IV, characterizing the transition from primary spermatocytes in diplotene to secondary spermatocytes, and from these to round spermatids (Figure 1-IV). As in the preceding stage, bunches of spermatids were observed, now even more elongated with an almost complete coverage of the nuclear surface acrosome, characterizing acrosomal step 8 (Figure 2-H; Figure 3-H).

3.5. Stage V

At this stage, there is only one generation of primary spermatocytes in the transition from zygotene to pachytene, since the spermatocyte generation later originated a generation of spermatids. These newly formed round spermatids have the characteristics of acrosomal step 1, showing no acrosome on their surfaces, since the pro-acrosomal vesicles are being formed in the cytoplasm, and have not yet made contact with the spermatid nucleus (Figure 2-A; Figure 3-A; Figure 3-K). The generation of late spermatids present at this stage is found in compact bunches more deeply embedded in the seminiferous epithelium. In these cells, the acrosome covers almost the entire nuclear surface: this is acrosomal step 9 (Figure 2-I; Figure 3-I).

3.6. Stage VI

At this stage are observed the intermediate spermatogonia, originate from spermatogonia A. These have smaller and darker nuclei compared to spermatogonia A. The present generation of primary spermatocytes at this stage is in pachytene, and the bunches of spermatids have become more superficial in the epithelium compared to the previous stage (Figure 1-VI). It becomes difficult to distinguish the generations of germ cells once they reach the point of being very similar to the previous stage. However, the round spermatids have characteristic features of acrosomal step 2, with the acrosome vesicles and granules in contact with the nucleus, and a slight flattening in the nuclear surface, extending to approximately 90° of the nuclear surface (Figure 2-B; Figure 3-B). The elongated spermatids of the later generation are within acrosomal step 10 when their nuclei, while still in the process of stretching and condensation, were considered to be in complete or nearly complete formation. In this phase the acrosome shows a round shape well distinguishable in the apical surface of the nucleous of the elongated spermatid (Figure 2-J; Figure 3-J).

3.7. Stage VII

Type A and type B spermatogonia were found at stage VII, the nuclei of which had an ovoid or rounded shape, primary spermatocytes in pachytene, and round and elongated spermatids (Figure 1-VII). The generation of round spermatids has an acrosome occupation of 95° to 120° of the nuclear surface, and is considered to be acrosomal step 3 (Figure 2-C; Figure 3-C); and the generation of elongated spermatids were considered to be acrosomal step 10 (Figure 2-J; Figure 3-J), due to their degree of development, with bunches more separated and closer to the tubular lumen.

3.8. Stage VIII

In the basal region of the seminiferous epithelium type A spermatogonia were observed, and a new generation of primary spermatocytes were observed in pre-leptotene. Primary spermatocytes in pachytene and round spermatids were present in the middle region of the epithelium, and the most characteristic feature of this stage was the presence of elongated spermatids near the tubular lumen that were ready for spermiation, with their tails very evident, and also the presence of residual bodies stained at the luminal border of the seminiferous epithelium (Figure 1-VIII). The round spermatids of stage VIII presented an angle of acrosome occupation of the nuclear surface of between 120o and 175o, representing acrosomal step 4 (Figure 2-D; Figure 3-D), while elongated spermatids were also considered to represent acrosomal step 10 (Figure 2 -J; Figure 3-J).

4. Discussion

We proposed here, for the first time, the description of the stages of the seminiferous epithelium cycle characterized by the tubular morphology method, detailing the acrosomal formation mainly in the stages in which two generations of spermatids are present, what we have named acrosomal steps.

The seminiferous epithelium of the bat *M. molossus* was observed to have the pattern described for other mammalian species, with only one stage per tubular cross-section and eight stages described by the tubular morphology method[9,10,14-17]. Additionally, the ultrastructural features of spermatids in *M. molossus* showed similarities with those observed for spermiogenesis of the bat *Platyrrhinus lineatus*[18] and also with the previous descriptions for *M. molossus*[19].

The acrosomal developmental steps in spermatids during spermiogenesis can be grouped into four phases, namely: the Golgi, cap, acrosomal and maturation phases[5,8]. The onset of acrosomal development starts with the Golgi phase, in which the pro-acrosomal granules from the Golgi complex are observed. These granules fuse together to form the acrosome vesicle, characterizing the cap phase, which extends to occupying 50% of the nuclear surface; this initiates the acrosome phase, in which there is a change in the shape of the spermatid nucleus, which becomes elongated. Once the acrosome covers the entire surface, the nuclear maturation phase begins, culminating in spermiation[8,20].

The tubular morphology method is standardized regardless of the species studied, since it is based on the morphology

and position of the different generations of germ cells in the epithelium. It is therefore the method of choice for interspecific comparative studies. It however presents difficulties for describing the various stages when two generations of spermatids, ie, at stages V to VIII, are present, as the general morphology of round and elongated spermatid generations is similar. The acrosomal system method, although more accurate in the differentiation of stages, is based on the development of the acrosome, and is therefore highly specific to each individual species, generating a different number of acrosomal steps and consequently stages, making it difficult to use in interspecific comparative studies.

Beguelini[9], in an attempt to standardize the description of the seminiferous epithelium cycle in six species of bats of the suborder Microchiroptera, developed their own methodology for the definition of spermatogenic steps, which although not based on acrosomal development, allows the interspecific comparison of these microchiropterans. Singwi and Lall[5] describe the cycle of seminiferous epithelium in the micro-chiropteran Rhinopoma kinneari based on the acrosomal system method, finding 11 stages, formed from 16 steps of spermiogenesis. In turn, Morigaki[8] also use the acrosomal system method in the mega-chiropteran *Pteropus vampyrus* and the micro-chiropteran *Rhinolophus cornutus* to describe 11 stages of the cycle with 13 steps of spermiogenesis for *Pteropus vampyrus*, and 10 stages with 13 steps for *Rhinolophus cornutus*. In comparison, in this study we defined 10 acrosomal steps, with correspondence between step and phase as follows: step 1 to the Golgi phase; steps 2 to 5 to the cap phase: steps 6 to 9 to the acrosome phase; and all the maturation phase was grouped in step 10.

Thus, although interspecific comparations are difficult when only the acrosomal system is used, in all cases it is possible to identify eight stages in the seminiferous epithelium based on the tubular morphology method. Associating the acrosomal steps with the different stages of the tubular morphology method presents an alternative to the existing methods, allowing interspecific comparisons of the spermatogenic process. Thus, the usual methods of the tubular morphology gains now important tool for describing the cycle of the seminiferous epithelium, not only among different species of bats, but also among other mammals already studied.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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