3.4 ARTIGO 4:

Ultrastructure of the male accessory glands of two species of *Triatoma* (Hemiptera, Reduviidae, Triatominae).

ULTRASTRUCTURE OF THE MALE ACCESSORY GLANDS OF TWO SPECIES OF *Triatoma* (HEMIPTERA, REDUVIIDAE, TRIATOMINAE)

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ABSTRACT

The four mesodermal male accessory glands of *T. melanica* and *T. brasiliensis*, have their secretion storage until the first days of adult life in starved males. In *T. melanica* small vacuoles are released into the gland lumen in one day old males, however afther three and five days few granules are found in the cell cytoplasm. The secretory granules in *T. brasiliensis* increase in size and amount, in the five days old adult, which may result in higher reproductive capacity. The secretory pathway in the male accessory glands of the two species seems to be merocrine and apocrine suggesting that different substances are being produced at different times. The cell microvilli in *T. brasiliensis* are longer and narrower presenting many secretory granules in the cytoplasm of five days old adults, these can be absent in some regions of the cell surface of *T. melanica*. During the maturation process of male accessory glands of *T brasiliensis* and *T. melanica*, granules with different sizes, shapes and electron density were seen suggesting synthesis of substances for spermatophore formation. The study of the functions of the male accessory glands, and the analysis of its secretions with association to mating strategies, can contributing to the knowledge of reproductive aspects in these species of Triatominae.

INTRODUCTION

The accessory reproductive glands of male insects produce secretions that are essential for the transfer of sperm to the female. The accessory glands components exert their effects at all phases of the reproductive biology of the mated female, from the moment that sperm is deposited in the reproductive tract to egg laying (Gillot, 2003). The glands are related which substances sperm protection, storage and activation, sperm competition, female behavior (reduction in attractiveness), fecundity, ovulation, oviposition and protection of laid eggs (Davey, 1958; Fuchs et al., 1969; Pickford et al., 1969; Friedel and Gillot, 1977; Chen, 1984; Hartmann and Loher, 1999; Gillot, 2002; 2003).

The morphological study of male accessory reproductive glands, besides the nature of their secretion, has been made by histological, histochemical and ultrastructural techniques (Barth, 1958; Bairati, 1968; Odhiambo, 1969; Louis and Kumar, 1971; De Loof and Lagasse, 1972; Dapples et al., 1974; Gadzama et al., 1977; Couche and Gillot, 1990; Dallai et al., 1999; Marchini et al., 2003; Freitas et al., 2007).

In Triatominae there are four pairs of male accessory glands classified in anterior, external, internal and dorsal portions which open together forming a hilus from which arises a common gland duct which opens into the ejaculatory duct (Barth, 1958).

In *Rhodnius prolixus* Stal, 1859, the three pairs of glands with transparent and viscous content are responsible for the production of spermatophores, whereas the fourth gland has an opaque and granular aspect, being its secretion responsible for the movements of the spermatozoa inside the female (Davey, 1958). Adult males of *Panstrongylus megistus* (Burmeister, 1835) begin to accumulate the accessory gland secretion from two days old, without difference of the secretion types between the dorsal and internal glands, whereas those from anterior and external glands present different protein patterns (Regis et al., 1985, 1987). The accessory reproductive glands of *Triatoma rubrofasciata* (De Geer, 1773), showed some ultrastructural features change according to the age due to the presence of secretory granules with different sizes, shape and electron density in the gland cells (Freitas et al., 2007).

Triatoma brasiliensis is now considered the most important Chagas disease vector in Northeasten Brazil (Costa et al. 2003a). Recently, multidisciplinary studies on four distinct forms of this species clarified the subdivision of *T. brasiliensis* into three species and one subspecies, respectively: *T. brasiliensis, T. melanica, T. juazeirensis,* and *T. b. macromelasoma* (Costa et al., 2003b, Costa et al., 2006, Costa and Félix, 2007).

The purpose of this investigation was to study the morphology of the accessory glands found in the male reproductive tract of *T. brasiliensis* and *T. melanica*, providing a detailed description of their ultrastructure during the process of sexual maturation, contributing to the knowledge of reproductive aspects in these species of Triatominae.

MATERIALS AND METODS

The insects were obtained from F1 colonies initiated with specimens collected in the types localities of the two species: *T. brasiliensis* (Caicó City, Rio Grande Norte) and *T. melanica* (Espinosa City, Minas Gerais) (Fig. 1). The colonies were maintained in the Sector of Morphology, Ultrastructure and Biochemistry of Arthropods and Parasites, Oswaldo Cruz Institute. Fifth instar male nymphs were maintained in a dark glass flask (30x15 cm), covered with nylon screen at 29 ± 1ºC, 80 ± 5% rh and 12 hours photoperiod. Folded filter paper was placed inside the flask to increase the contact surface and refuge, as well as removing the humidity excess. The insects were fed weekly with blood of Swiss mouse (Protocol CEUA - FIOCRUZ P0100-01).

Starved males of both species that were one, three and five days old were dissected in saline solution for insect (0.1M NaCl, 0.1M KCl). The male accessory glands were isolated and fixed in 2.5% glutaraldehyde in sodium cacodilate buffer 0.1M, pH 7.2 for 1h and post-fixed in 1% osmium tetroxide in the same buffer for 1h. The samples were dehydrated in a graded acetone series and embedded in Epon resin.

Thin sections were stained with toluidine blue 1% and visualized in a light microscope. Ultra-thin sections were stained with 5% uranyl acetate and 1% lead citrate and analyzed in a transmission electron microscope Zeiss EM 109.

RESULTS

The four accessory glands of the male reproductive tract of these two species are elongated sac-like (Fig. 2a-b). This anatomical aspect changes according to the age of bugs. In the first day of adult life the glands are wither, without secretion storage. At three days old, the glands have a shine aspect, with enlarged volume which increases at the five days old males due to secretion storage.

In both species, the four accessory glands have the same structure. The epithelium consists of a single layer of secretory cells, seated on a thin basal membrane and surrounded by a muscular layer (Figs. 3a-d). The anterior (AGI) and external (AGII) glands have high epithelial cells (Fig. 3a-b e 4a-b) while the internal (AGIII) and dorsal (AGIV) glands have cubic ones (Fig. 3c-d e 4c-d).

The epithelium is composed by a single layer of secretory cells with basal plasma membrane infolds, showing granular homogeneous aspect (Fig. 5-7). Focal contacts can be seen in association with the basal membrane (Fig. 8). All glands are surrounded by a thin muscular layer and trachea, whose tracheole are in close association with the glandular epithelium (Fig. 5).

The cytoplasm of secretory cells has ribossomes that may be free or bound to the endoplasmic reticulum membranes (RER) (Fig. 9), Golgi complexes and mitochondria, many of them large and polymorphic (Fig. 10). The apical surface of the cell has microvilli irregularly arranged (Fig. 11, 12). The adjacent cells are maintained by *zonula adherens* and septate junctions with narrow intercellular space (Fig. 11, 13). The nucleus, localized in the middle region of the cell, has predominance of descondensed chromatin and one or two nucleoli (Fig. 14).

Some ultrastructural features of the male accessory glands change according to the age. During five days after adult emergence, the maturation of the four glands of both species presents the following aspects:

Triatoma brasiliensis

The epithelial cells of the four accessory glands have basal plasma membrane infoldings that are few and short never reaching the middle cell region (Fig. 6 and 15). Mitochondria rarely were found associated the basal plasma membrane infoldings (Fig. 15).

The maturation is synchronous in the four glands. In the first day old adult male, the four glands have the cell cytoplasm rich in mitochondria and rough endoplasmic reticulum (RER) (Fig. 15). Three day old adult males present cell cytoplasm with mitochondria, RER, free polyribosomes and small granules with different electron densities (Fig. 11 and 16). Vacuoles with myelinic figures are released into the glandular lumen.

In the five day old adult males the four accessory glands have the cytoplasm filled with secretory granules with different aspects (Fig. 3a-d, 17 and 18), that release their content in to the gland lumen (Fig. 19). At the apical cell region, in the four glands, occur narrow microvilli (Fig. 11 and 20).

Triatoma melanica

The four accessory glands in one day old males present cells with numerous mitochondria and RER (Fig. 7, 9 and 10). The RER cisterns are widened in the AGII (Fig. 7) than in other glands (Fig. 9). In the basal cell region there are some cytoplasm electron-lucent inclusions without surrounding membrane (Fig. 9 and 21) with some membrane-like structure inside them (Fig. 22). One day old adult males present vesicles closely associated with the apical cell surface (Fig. 12).

Three and five days old adults present the four accessory glands similar to those described above with numerous mitochondria, RER and small granules with different degrees of electron density (Fig. 5, Fig. 23 and 25). Only in AGII in the three day old adults was found lysosomes (Fig. 23). Vacuoles and vesicles with medium electron-density released into the gland lumen were found in four glands (Fig. 24-26).

The apical cell surface of the four glands has microvilli (Fig. 12, 24-26), although, in some regions the cellular surface has few or no microvilli.

DISCUSSION

The four male accessory glands of *T. melanica* and *T. brasiliensis* have mesodermal origin because they have no cuticular intima lining the gland lumen (Leopold, 1976; Chapman, 1998; Dallai al., 1999; Marchini et al. 2003).

In Triatominae, the secretion stored in the male accessory glands occurs quickly, probably until the fourth day adult life because in five day old males these glands almost double in volume when compared with one and three day old males of both species. The first mating of *T. infestans* males need more than one blood meal to complete the glandular activity, already in *P. megistus*, the secretion stored in the male accessory glands occurs in the second day of adult life in starved insects (Regis et al., 1985). In starved males of *T. rubrofasciata* secretory granules are released into the glandular lumen from the third day of adult life (Freitas et al., 2007), indicating that, in *P. megistus* and *T. rubrofasciata* the gland activity is independent of the nutritional state of the insect. Here, the maturation of male accessory glands in *T. brasiliensis* and *T. melanica* is also independent of feeding, since from the third day of adult life secretory granules are found in the cell cytoplasm and are released into glandular lumen. In *T. melanica* small vacuoles are released into the gland lumen in one day old males, however in the three and five day old males few granules are found in the cell cytoplasm, suggesting regular secretory activity when compared with *T. brasiliensis*, granules released to the gland lumen are lacking. However, the secretory granules in *T. brasiliensis* increase in size and amount in the five days old adult, which may result in higher reproductive capacity.

The secretory pathway in the male accessory glands of the two species seems to be merocrine, but in some cases we found membrane-like structures inside vacuoles in glandular lumen suggesting apocrine secretion. The occurrence of both secretion pathways were found in the male accessory glands of *Leptinotarsa decemlineata* (De Loof and Lagasse, 1972), *Periplaneta americana* (Adiyodi and Adiyodi, 1974), *Tenebrio molitor* (Gadzama et al., 1977) and *T. rubrofasciata* (Freitas et al., 2007), suggesting that different substances are being produced in different times.

In both species, in the four male accessory glands, vacuoles release their content by exocytosis. However, in some cases, these vacuoles are released entirely in the glandular lumen.

The cell microvilli have different morphologic aspects in both species. In *T. brasiliensis,* they are longer and narrower than those found in *T. melanica,* where they can be absent in some regions of the cell surface. This feature indicates that microvilli in *T. brasiliensis* increase the secretion surface, corroborating the fact that in the five days old adults cell the cytoplasm has many secretory granules.

Cells with high secretory activity have secretory granules, rough endoplasmatic reticulum, Golgi complex and mitochondria (Alberts et al., 2004). During the maturation process of the male accessory glands of *T brasiliensis* and *T. melanica*, granules with different sizes, shapes and electron density indicate the necessity of cytochemical analyses to determine the secretory nature of these glands, because the four accessory glands play an important role in spermatophore production during mating. Moreover, proteins and carbohydrates are indispensable components in the male accessory glands, since they had been described in the secretion of the accessory glands of Orthoptera (Odhiambo, 1969), Dictyoptera (Vijayalekshmi and Adiyodi, 1973), Hemiptera (Davey, 1958), Coleoptera (Anderson, 1950; DeLoof and Lagasse, 1972) and Diptera (Leopold, 1970).

The spermatophore production in Triatominae is essential for the transport and maintenance of the spermatozoa inside the female reproductive tract. In this way, the functions of the male accessory glands, and the analysis of their secretions, associated with mating strategies, can contributing to the knowledge of reproductive aspects in these species of Triatominae.

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FIGURE LEGENDS:

Figure 1. Collection sites number 1 (Caicó City, RN) and 2 (Espinosa City, MG) are the type-localities of *Triatoma brasiliensis* and *Triatoma melanica,* respectively.

Figure 2. Male reproductive system of *Triatoma brasiliensis*. (a) Schematic drawing according to insect body plan, with accessory glands detailed in black. (b) Scanning electron micrograph of the accessory gland (bar 200µm). Testes (T), vas deferent (vd), vesicle seminal (vs), glandular duct (gd), anterior gland (G1), external gland (G2), internal gland (G3), dorsal gland (G4), trachea (Tr).

Figures 3a-d: Section of a portion of the epithelial cell of the male accessory glands of *Triatoma brasiliensis.* Anterior gland (a); external gland (b); internal gland (c); dorsal gland (d). Basal lamina (BL); secretory granules (S); nucleus (N); lumen (L); secretion (arrowhead); muscle (Mu); microvilli (Mv) (bar (10µm).

Figures 4a-d: Section of a portion of the epithelial cell of the male accessory glands of *Triatoma melanica.* Anterior gland (a); external gland (b); internal gland (c); dorsal gland (d). Basal lamina (BL); nucleus (N); lumen (L); secretion (arrowhead); trachea (T); secretory granule (S); muscle (Mu) (bar (20µm).

Figures 5-9: Male accessory glands of *T. brasiliensis* and *T. melanica.* (5) Basal region of the epithelium (bar 2µm). (6-7) Basal plasma membrane folds (arrowhead) (bar 0.5µm). (8) Electron dense material in focal contacts (arrowhead) (bar 0.1µm). (9) Median portion of the epithelium (bar 1µm). Muscle (Mu); trachea (T); basal membrane (BM); secretory granule (S); mitochondria (M); rough endoplasmatic reticulum (RER); stored granule (SG).

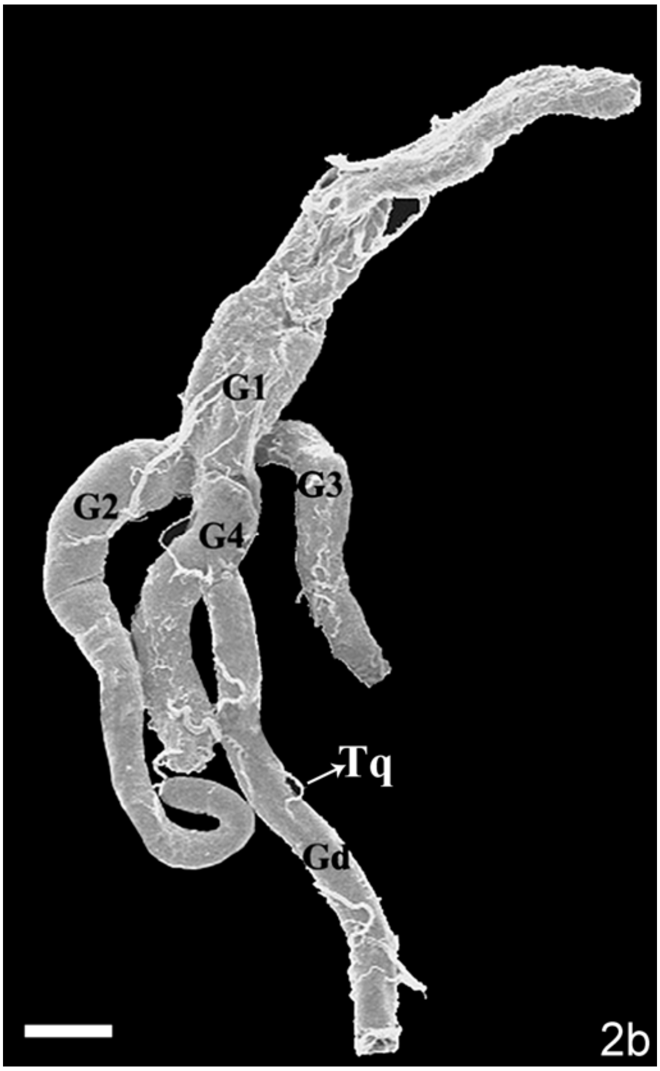
Figures 10-14: Male accessory glands of *T. brasiliensis* and *T. melanica.* (10) mitochondria (M) large and polymorphic (bar 0.5µm). (11) Apical portion of the cell (bar

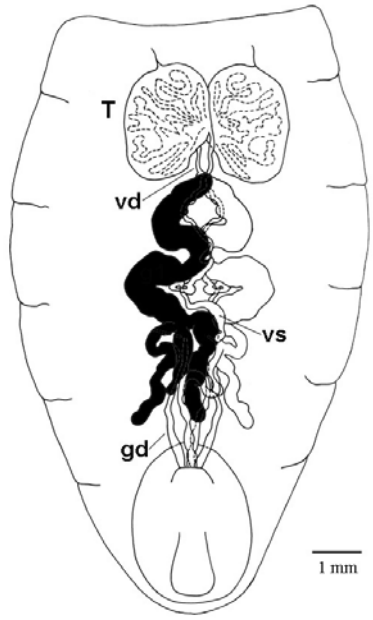
2µm). (12) Exocitosis (arrowhead) in apical portion of the cell (bar 0.5µm). (13) septate junction (SJ) with narrow intercellular space (bar 0.1µm). (14) Nucleus (N) in middle region with descondensed chromatin and one or two nucleoli (Nu) (bar 2µm). Rough endoplasmatic reticulum (RER); microvilli (Mv); lumen (L); z*onula adherens* (arrowhead); secretory glanule (S).

Figures 15-20: Male accessory glands of *Triatoma brasiliensis*: (15) Basal portion of epithelium (one day old), arrowhead showing basal plasma membrane folds (1µm). (16) General view of glandular epithelium (three day old), arrowhead showing free polyribosome (5µm). (17-19) General view of the epithelium cell (five day old) (bar 1µm). (20) Apical portion of the cell (bar 0.5µm). Trachea (T); basal membrane (BM); mitochondria (M); rough endoplasmatic reticulum (RER); secretory granule (S); vacuole (V); microvilli (Mv); lumen (L).

Figures 21-26: Male accessory glands of *Triatoma melanica*: (21) Basal portion of epithelium (one day old), arrowhead showing free polyribossome (1µm). (22) Storage granule (SG) with membranous vacuole (bar 0.5µm). (23) General view of the glandular epithelium (three day old) (bar 0.5µm). (24) Apical portion of the cell (three day old), arrowhead showing exocitosis (bar 1µm). (25) Apical portion of the cell (five day old) (bar 1µm). (26) Apical portion of the cell (five day old) (bar 2µm). Trachea (T); basal membrane (BM); mitochondria (M); storage granule (SG); rough endoplasmatic reticulum (RER); secretory granule (S); lysosome (Ly); microvilli (Mv); vesicles (V); lumen (L).

Figure 1





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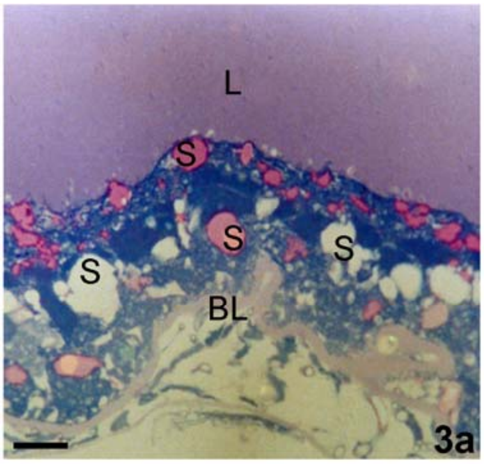
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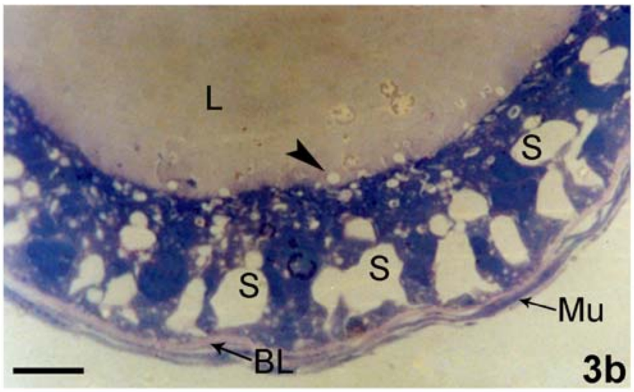
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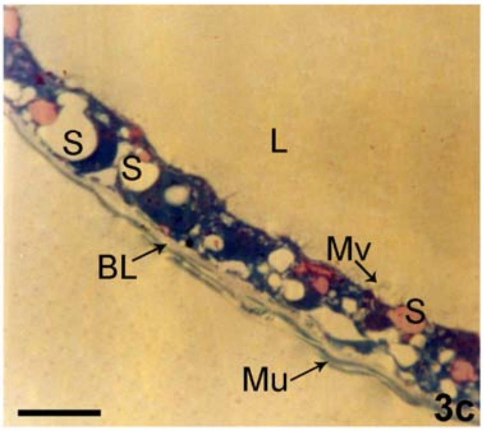
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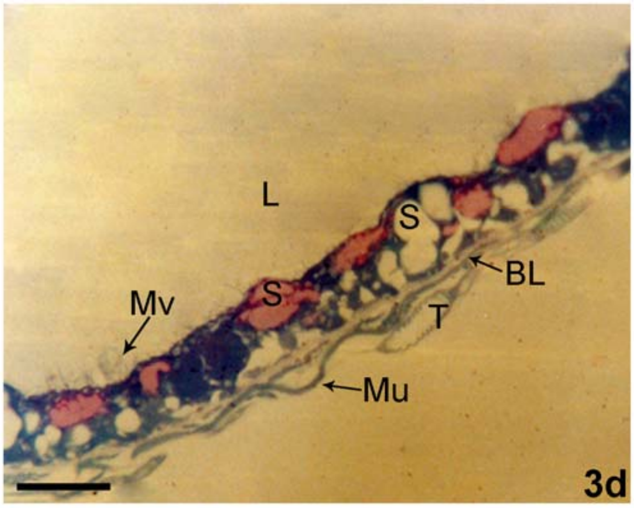
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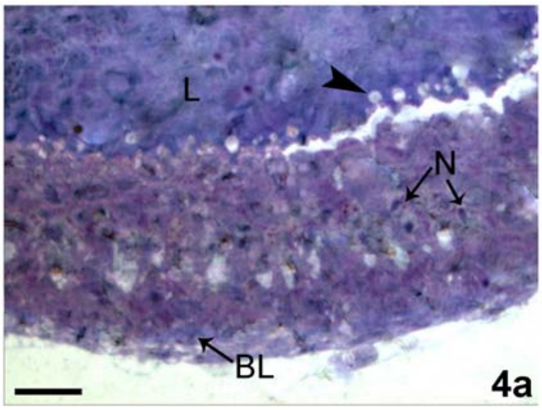
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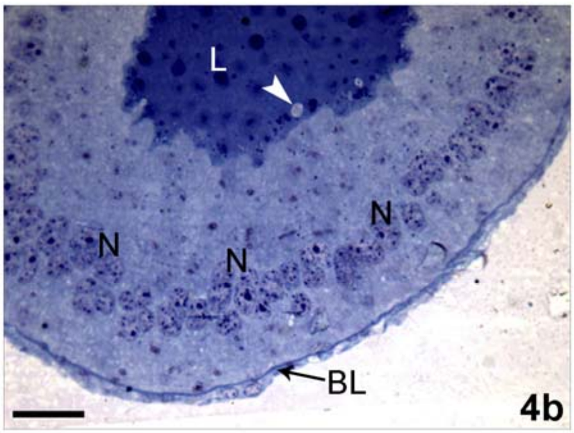
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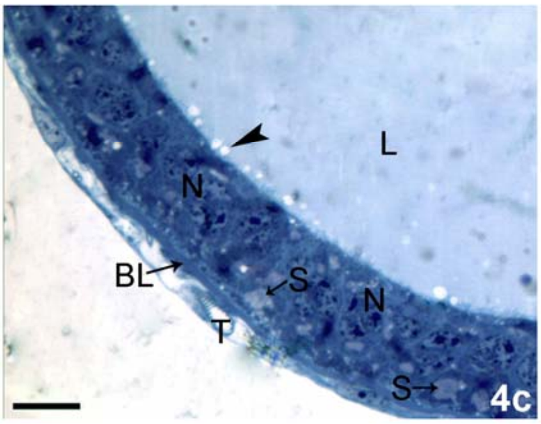
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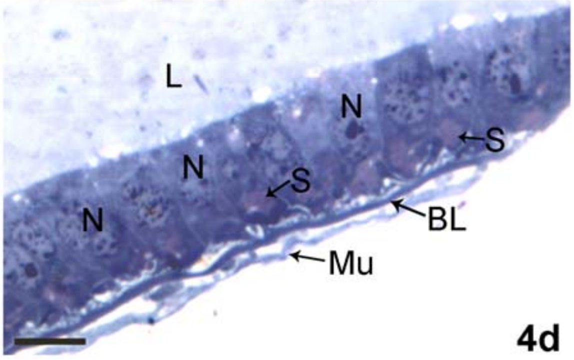
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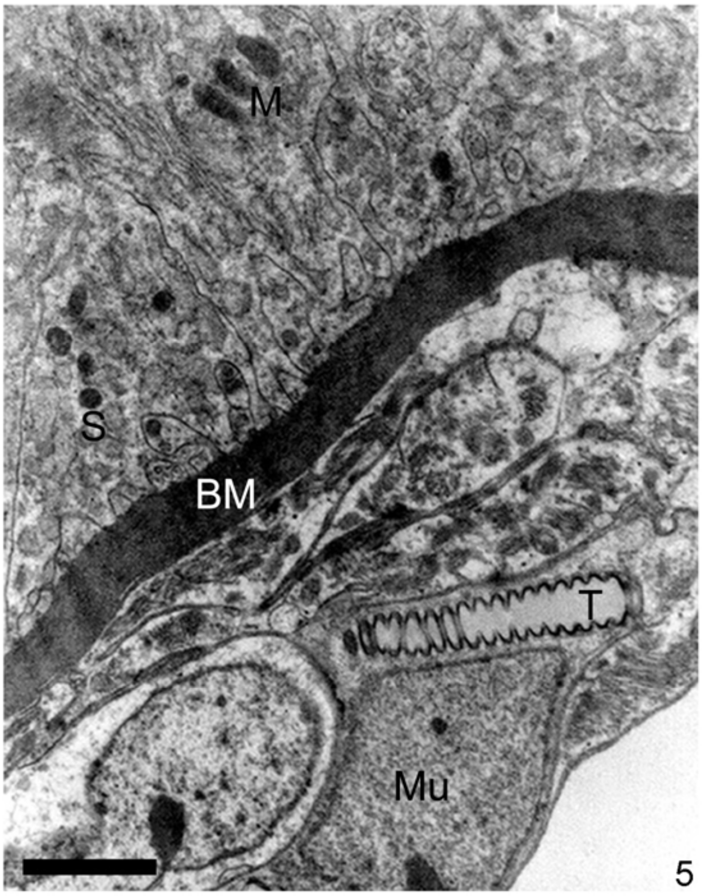

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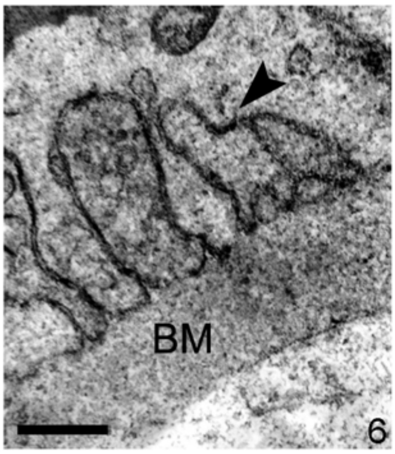

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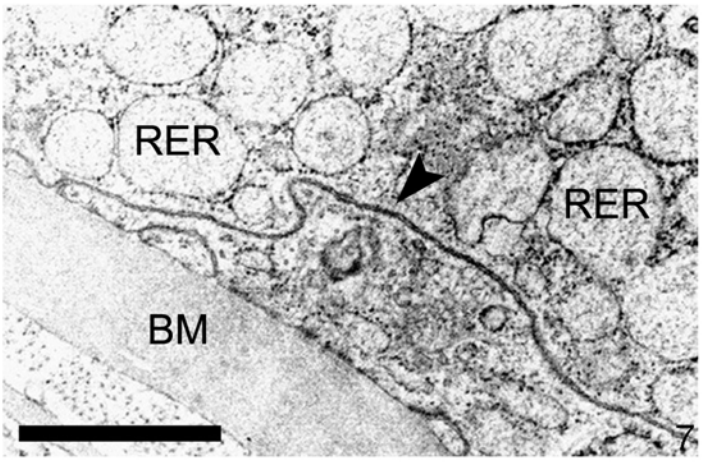

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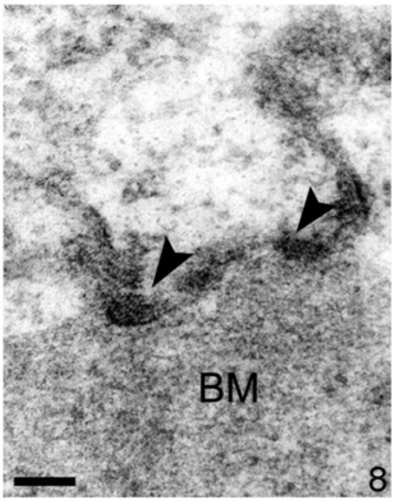

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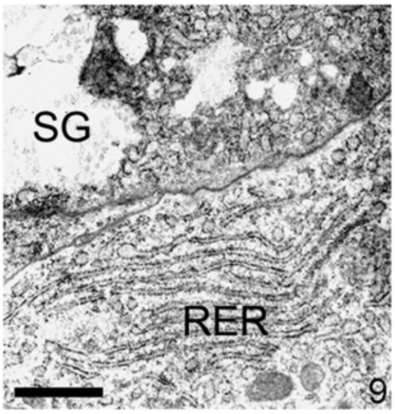

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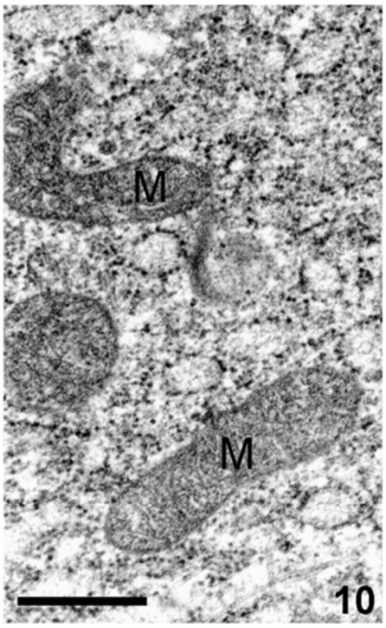
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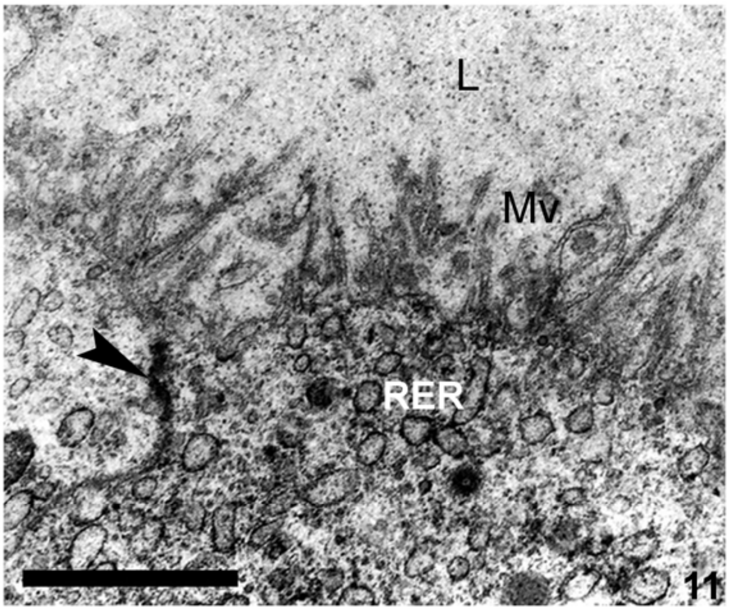
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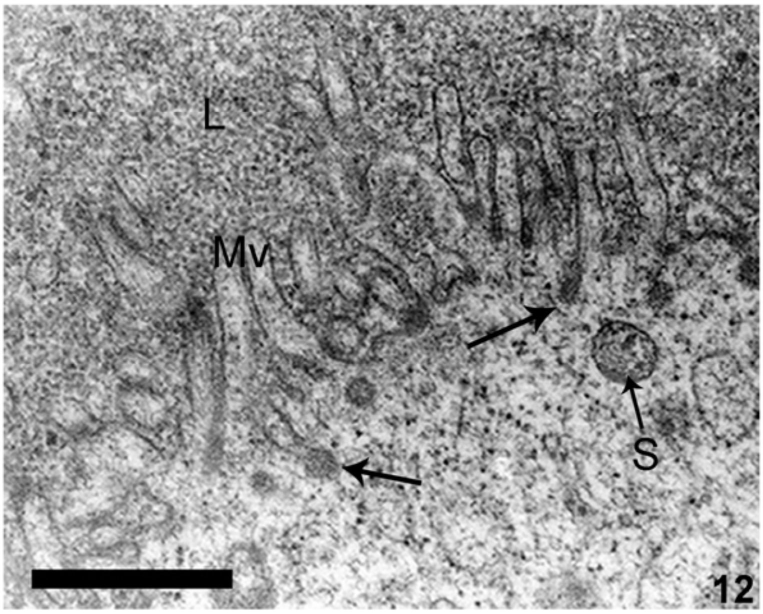
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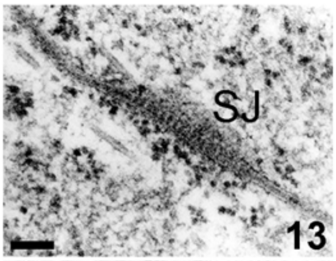
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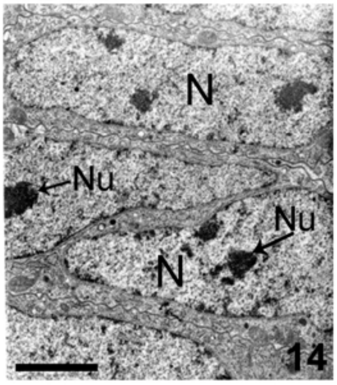
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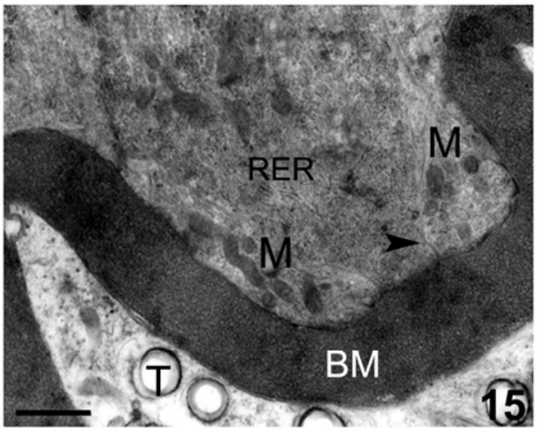

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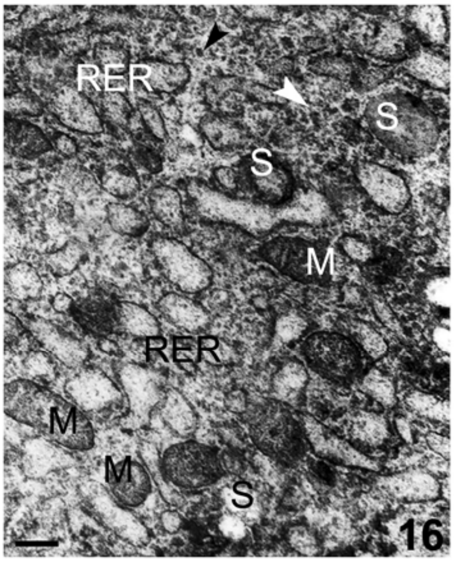

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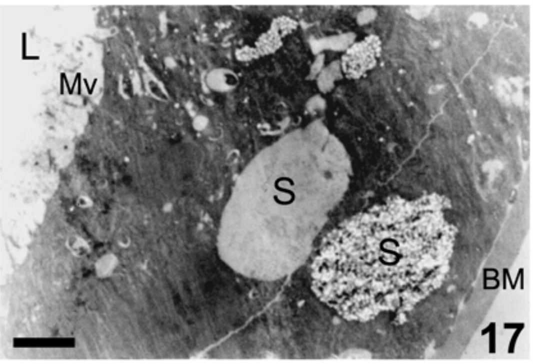

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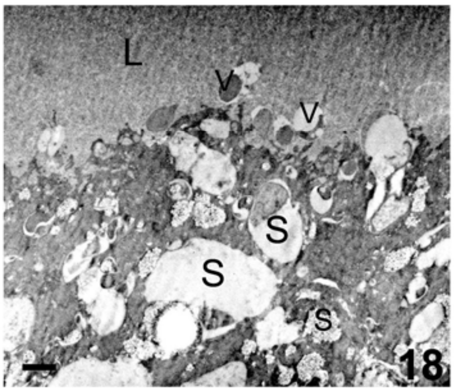

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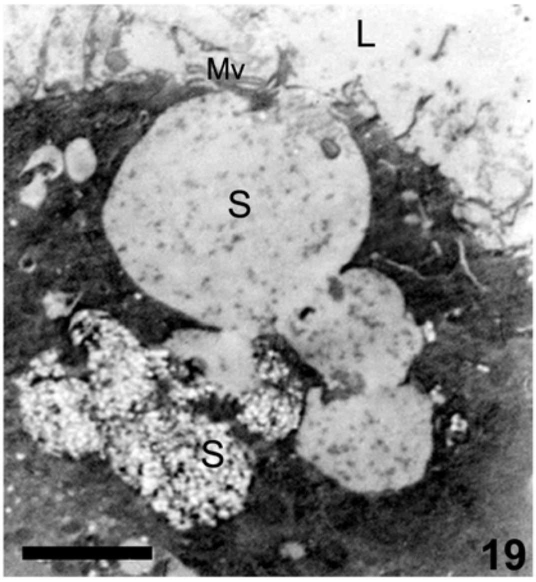

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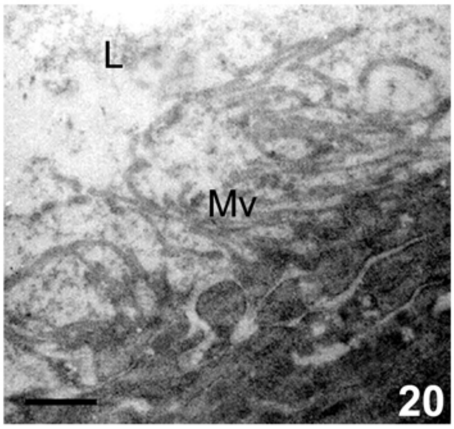

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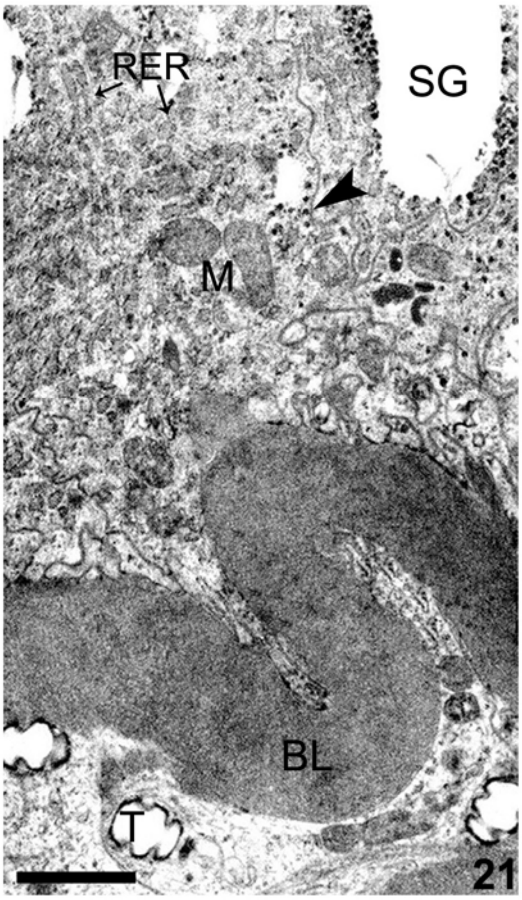
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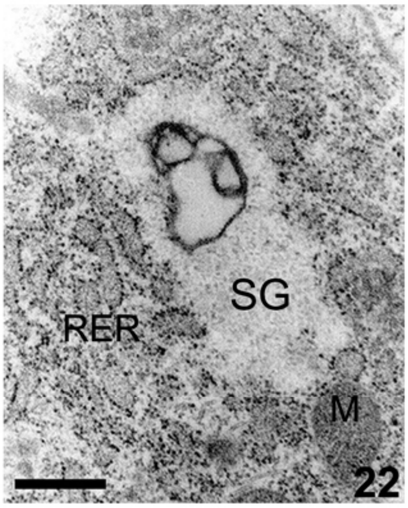
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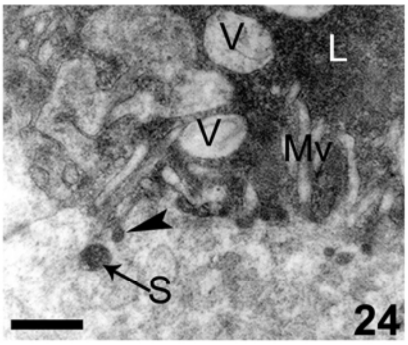
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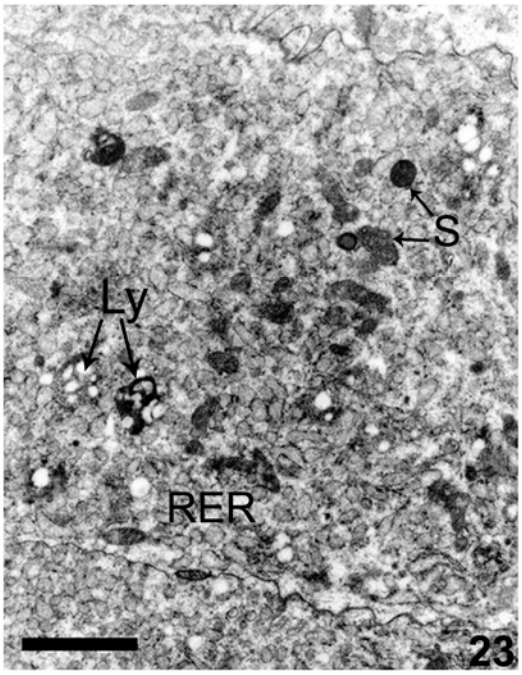
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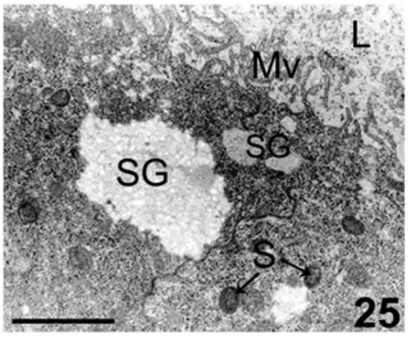
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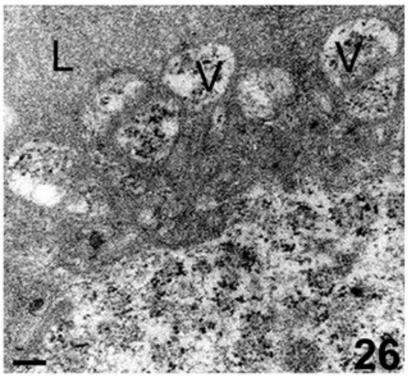
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