3.5 ARTIGO 5:

Ultrastructural localization of basic proteins and carbohydrates in male accessory glands of two *Triatoma* species (Hemiptera, Reduviidae, Triatominae).

ULTRASTRUCTURAL LOCALIZATION OF BASIC PROTEINS AND CARBOHYDRATES IN MALE ACCESSORY GLANDS OF TWO *TRIATOMA* SPECIES (HEMIPTERA, REDUVIIDAE, TRIATOMINAE)

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ABSTRACT

The male accessory glands in *Triatoma* are tubular and produce substances with some functions related to production of the spermatophore. In the present study, the cytochemistry of male accessory glands was evaluated in starved *Triatoma brasiliensis* and *Triatoma melanica* adults. The storage of carbohydrates and proteins in ones *T. melanica* male accessory glands occurs before than in *T. brasiliensis*. In addition, the occurrence of eletron-lucent granules without carbohydrates and proteins, suggests that other substances are released by these glands, which may be used for lubrication of the male genitalia. *Triatoma brasiliensis* males have more intense secretory activity from the fifth day of adult life, what can indicate a higher reproductive capacity. The analysis of lipid production in male accessory glands can contribute to the knowledge of spermatophore formation in these species.

1. Introduction

The reproductive tract male of insects is constituted by a pair of testes, connected to the aedeagus by genital ducts and some accessory glands (Snodgrass, 1993; Chapman, 1998). Male insect accessory gland secretions play multifunctional roles in the transference, activation and protection of germ cells, as well as in changing female behaviour and stimulating ovarian development and oviposition (Davey, 1958; Fuchs et al., 1969; Pickford et al., 1969; Friedel and Gillot, 1977; Chen, 1984; Hartmann and Loher, 1999; Gillot, 2002; 2003).

The secretory products of these glands can be proteins, carbohydrates and lipids (Gillot, 2003). They may contain a variety of biologically active components, such as prostaglandins (Gillot, 1988), juvenile hormone (Shirk et al., 1980; Borovsky et al., 1994) and toxic compounds that have been suggested to serve as egg protectants (Blum and Hilker, 2002; Eisner et al., 2002).

In Triatominae (Reduviidae), there are four pairs of male accessory glands classified in anterior (AG I), external (AG II), internal (AG III) and dorsal (AG IV) portioned according to the insect body plan. The four glands opens together in the hilus from which arise a common gland duct that opens into the ejaculatory duct (Barth, 1958).

The anatomical aspect of male accessory glands in Triatominae changes according to aging. In the first day of adult life the glands are wither, without secretion storage. Afther three days, the glands have a shinny aspect, with enlarged volume. The glandular volume increases in five day old males due intracellular secretion storage (Khalifa, 1950; Freitas et al., 2007). The four accessory glands have the lumen lined by a single layer of secretory cells and well developed basement membrane. All glands are surrounded by thin muscle layer and tracheae, with tracheoles in close association with the glandular epithelium (Freitas et al., 2007).

The glandular activity dedicated to the production of spermatophore in triatomine bugs depends on the age and feeding status of the insects (Regis et al., 1985). In *Triatoma infestans* Klug, 1834, the secretion storage begin at sixth day after imaginal moult, and two blood meals are required to induce glandular activity. *Panstrongylus megistus* (Burmeister, 1835) behaviour is similar to *Rhodnius prolixus* Stal, 1859 males which, fed or not, produce spermatophores about eighth day after adult emergence (Khalifa, 1950; Regis et al., 1987).

Triatoma brasiliensis Neiva, 1911 is now the most important Chagas disease vector in Northeastern Brazil (Costa et al. 2003a). Recently, multidisciplinary studies of four distinct forms of this species proposed subdividing of *T. brasiliensis* into three species (*T. brasiliensis, T. melanica* and *T. juazeirensis*) and one subspecies (*T. brasiliensis macromelasoma*) (Costa et al., 2003b, Costa et al., 2006, Costa and Félix, 2007).

Preliminary studies on the ultrastructure of male accessory glands in *T. brasiliensis* and *T. melanica* were recently made (Freitas et al. unpublished). However, the nature of their secretion is unknown. The purpose of this investigation was to study the composition of the secretion found in male accessory glands of *T. brasiliensis* and *T. melanica* using cytochemical methods and with transmission electron microscopy.

2. Material and methods

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

Starved adults of two species with one, three and five days after ecdysis were dissected in saline solution for insects (0.1M NaCl; 0.1M KCl). Male accessory glands were isolated and transferred to 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.

For carbohydrate detection, the periodic acid-thiosemicarbazide-silver proteinate method (Thiery, 1967) was used. Ultrathin sections of glands were collected on gold grids and treated with periodic acid 1%, 20 min at room temperature. After rinsing in distilled water, the sections were condensed with thiosemicarbazide (1% thiosemicarbazide in 10% acetic acid for 48h at room temperature). After rinsing sequentially with 10, 5 and 1% acetic acid and with distilled water, the sections were exposed to silver proteinate 1% for 30 min at room temperature in the dark.

For basic protein detection, the phosphotungstic acid method (PTA) was used. After glutaraldehyde fixation, glands were dehydrated (without post fixation with O_sO_4) in ethanol and incubated for 2h at room temperature in 2% PTA in ethanol, washed in ethanol and embedded in Epon resin. For both methods, unstained sections were observed under a transmission electron microscope Zeiss EM 109.

3. Results:

In both species, the epithelium is rich in polymorphic mitochondria which are scattered in the entire cytoplasm. Rough endoplasmatic reticulum (RER) and secretory granules are abundant, in one, three and five days-old adults. The granules vary in size and amount, according to the stage of maturation of the glands, as well as the RER cisterns that seem more extensive in one day-old male.

The cytochemical tests used showed positive electron-dense granules. Some electron-lucent structures, not delimited by membrane, were here considered glycogen storage granules. The electron-lucent membrane bounded granules found here, were negative to the cytochemical tests used.

The four glands of one, three and five day-old male of *Triatoma brasiliensis,* glycogen storage granules in the entire cytoplasm (Fig. 1, 2 and 3). Positive granules for carbohydrate (Fig. 4) were seen, only in the AG I and AG II for all studied insects.

Electron-lucent granules were found in the entire cytoplasm, of the four glands. The small granules in one day old males increasing their volume until the fifth day (Figs 1, 2, 3 and 5). Some of these granules were released to the gland lumen (Fig. 3 and 5), in AG I in adults one and five days-old (Fig 3 and 5), AGII in adult one day old day, and AG IV in the third day-old, while only in AG II, the released material was found to be positive for carbohydrates, in the three days-old males (Fig. 6).

In the lumen of the four glands was seen accumulation of contents positive for carbohydrates, in the five day-old (Fig. 2 and 3).

The four glands were positive for proteins in the basal portion of the glandular epithelium, during the entire adult lifespan (Fig. 7). However, in AG I, the presence of positive granules occurred in the first day of adult life, while in AG III only in the fifth day (Fig. 8).

Electron-lucent granules are was present in cytoplasm of gland cells for all days studied (Fig. 9). Some presenting proteins inside (Fig. 10). In AG I release of the granule electron-lucent was frequently observed into the gland lumen, in all insects, whereas in AG IV only five days-old insects showed granules in the lumen (Fig. 11).

In AG I and AG IV, in five days-old insects, lipids could be seen in the gland cells (Fig. 12), but this content was not found in other glands.

In *Triatoma melanica* the four male accessory glands had positive granules for carbohydrates scattered in the cytoplasm (Fig. 13). Particles and granules of glycogen storage are seen in AG I, AG III and AG VI (Fig. 14 and 15), except in AG II.

Electron-lucent granules are present in the entire cytoplasm of the four glands, starting from the third day of adult life (Fig. 14). However only in AG I and AG II, at three days-old insects, some granules were released into the glandular lumen (Fig. 16).

The accessory gland IV had secretion electron-lucent content in the first day-old adult (Fig. 17). The release of carbohydrates was found in AG I, AG II, starting from the third day-old insect, and in AG IV in the first day-old *T. melanica* male (Fig. 16 and 18).

In the AG I of five days-old adults, electron-lucent granules containing carbohydrates and being released into the glandular lumen were found (Fig. 14).

The test for protein detection showed that the four glands, in all studied insects had positive granules scattered in the entire cytoplasm, as well as the presence of electron-lucent granules (Fig. 19-22). In the AG III and AG IV, these electron-lucent granules are released into the gland lumen of the insects in the all ages (Fig. 21, 22 and 24), and in AG I, only in the five-day old insects. The gland lumen had positive reaction for protein, with a granular content in AG I, AG II and AG III in all insects, while in AG IV this was found only in for five day-old insects (Fig. 19 and 21).

The presence of protein release in small vesicles was found only in AG II for the five-day old *T. melanica* (Fig. 23).

With the granular diversity, and its occurrence in species of *Triatoma* with different ages, a comparison of the results obtained is presented in Table.

4. Discussion:

In Triatominae, the storage of male accessory glands contents occurs early in adult life, so that the four glands double in size in four days-old insects (Regis et al., 1985). The secretions produced by the male accessory glands of insects are constituted by heterogeneous substances (Odhiambo, 1969; Gerber et al. 1971; Dailey et al., 1980); this is corroborated by the results obtained here that showed granules with positive reactions for carbohydrates and proteins simultaneously.

Data obtained from measuring the male accessory glands of two days-old *T. infestans*, either fed or starved, showed that the first mating is directly related to the need of more than one blood meal to complete the gland activity (Regis et al., 1985). In the present study, the male accessory glands of *T. brasiliensis* and *T. melanica* showed secretory granules containing carbohydrates and proteins in the glandular epithelium and being released into the gland lumen, in one day-old starved insects, suggesting a possible role of these glands in spermatophore production, because this is constituted by carbohydrates and proteins (Gillot, 2003). This fact may indicate that, for *T. brasiliensis* and *T. melanica* blood meal is not necessary for glandular activation. However, the viability of the spermatophore produced is unknown.

Although the four accessory glands of *T. brasiliensis* and *T. melanica* present a similar epithelial morphology (Freitas et al. unpublished data), the use of ultrastructural cythochemistry showed different secretory characteristics, among the glands, during the maturation period. Positive granules for carbohydrates were found in all ages studied in the four glands of *T. melanica*. However, carbohydrates were present only in AG I and AG II *T. brasiliensis*. Therefore, in *T. melanica*, from the first day-old insect the four male accessory glands play a role in carbohydrate metabolism, possibly for spermatophore production.

The protein granules were found, only in the AG I and AG III of *T. brasiliensis*, while these granules were present in the four glands in *T. melanica*, in all studied ages. According to Leopold (1976) carbohydrates and proteins are characteristic of the secretions of the male accessory glands, and they have been recorded in the secretions of these glands in Orthoptera (Odhiambo, 1969; Braswell et al., 2006), Hemiptera (Davey, 1958), Coleoptera (Anderson, 1950; De Loof and Lagasse, 1972; Frenk and Happ, 1976), Lepidoptera (Thibout, 1971), Hymenoptera (Jeantet, 1972; Cruz-Landim and Dallacqua, 2005), Diptera (Leopold, 1970; Federer and Chen, 1982; Marchini et al., 2003) and Ixodidae mites (Ricardo et al., 2007). Supported by data here obtained, we suggest that the male accessory glands of *T. melanica* begin spermatophore production precursors before than *T. brasiliensis* ones, in which that process is asynchronous when compared to *T. melanica*.

Glycogen stored in clots or dispersed in the cytoplasm, was observed in the four male accessory glands of *T. brasiliensis*, in all studied ages. However, in *T. melanica* glycogen was absent in the AG II. Because glycogen is a reserve to be transformed in glucose, according to the needs of the cell, we can suppose that AG II of *T. melanica* storage glycogen from the five days-old insects.

In *R. prolixus,* the spermatophore is produced by the three pairs of male accessory glands that contain a transparent material, while the secretion of the fourth pair, of opaque aspect, is responsible for the movement of the spermatozoa inside of the reproductive organ of the female (Davey, 1958). In the present study we observed that, in *T. brasiliensis*, AG III and AG IV do not participate in the production of carbohydrates, until the fifth day of adult life, suggesting that one of these glands, or both, may play the same role of the opaque glands of *R. prolixus*. These finding corroborates the data of Regis et al. (1985) where the authors suggest that AG II in *T. infestans* play the same role of the opaque glands of *R. prolixus*.

Rhodnius and *Triatoma* males have a large edeagus that is evaginated and occupies the female's vagina during mating; therefore, there is not a direct contact between the edeagus and the female spermatheca and the secretions of the male accessory glands are used in spermatophore production which occupies the vagina of the female, where the sperm mass is released (Khalifa, 1950). The presence of many electron-lucent granules, negative for carbohydrates and proteins, released into the lumen of male accessory glands of *T. brasiliensis* and *T. melanica*, suggests that other products are being produced and secreted. These can contain substances to stimulate egg-laying, and/or for lubrication of male genitalia (Gregory, 1965; Pickford et al., 1969).

Males of *T. melanica* have earlier secretory activity when compared to *T. brasiliensis*, since the four male accessory glands produce and release substances from the first day of adult life. However, males of *T. brasiliensis* have a more intense secretory activity, supported by the presence of many large secretory granules from the fifth day of adult life, suggesting a high reproductive capacity.

The main products of the male accessory glands are carbohydrates, proteins and lipids (Frenk and Happ, 1976; Gillot, 2003), however these products have been poorly studied. In addition to carbohydrates and proteins present in different periods, in the different glands, lipid granules were observed in AG I and AG IV of *T. brasiliensis*, starting in five days-old adults (data not shown). Therefore, a cytochemical and biochemical study for detection of lipids should be accomplished for the confirmation of the origin of this products in both species.

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Table 1: Ultrastructural cytochemical detection of carbohydrates and proteins in the four male accessory glands (I, II, III and IV) of the *Triatoma brasiliensis* and *Triatoma melanica,* with different ages. 1d – one day old males. 3d – three days old male. 5d – five days old males.

	Triatoma brasiliensis												*Triatoma melânica*											
	Carbohydrates												Carbohydrates											
	Anterior (I)			External (II)			Internal (III)			Dorsal (IV)			Anterior (I)			External (II)			Internal (III)			Dorsal (IV)		
	1d	3d	5d	1d	3d	5d	1d	3d	5d	1d	3d	5d	1d	3d	5d	1d	3d	5d	1d	3d	5d	1d	3d	5d
Electron-dense granules inside citoplasm	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Electron-dense material in constitutive secretion	-	-	-		+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-
Electron-luscent granules with electron-dense material in glandular lumen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Granular content positive in the glandular lumen	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	Proteins												Proteins											
	Anterior (I)			External (II)			Internal (III)			Dorsal (IV)			Anterior (I)			External (II)			Internal (III)			Dorsal (IV)		
	1d	3d	5d	1d	3d	5d	1d	3d	5d	1d	3d	5d	1d	3d	5d	1d	3d	5d	1d	3d	5d	1d	3d	5d
Electron-dense granules inside citoplasm	+	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Granular aspect positive in glandular lumen	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	+
Electron-dense material in constitutive secretion	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Granulation dispense in portion basal of the epithelium	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Electron-dense material in constitutive secretion	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-

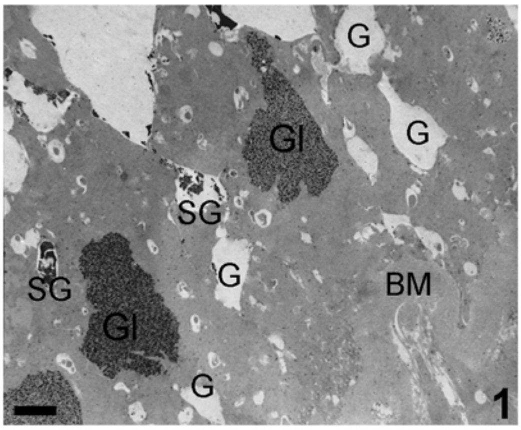
Figure legends

Figures 1-6: Male accessory glands of *Triatoma brasiliensis* submitted to the periodic acid-thiosemicarbazide-silver proteinate technique for detection of carbohydrates. Unstained sections. (1) General view of glandular epithelium of the AGI (5th day). (bar 2µm). (2) General view of glandular epithelium of the AGIII (5th day). (bar 2µm). (3) Apical portion of AG I (5th day). (bar 1µm). (4) Basal portion of AG II (3th day). (bar 0.5µm). (5) Apical portion of AG I (1th day). (bar 2µm). (6) Apical portion of AG II (3th day). (bar 0.5µm). Basal membrane (BM), electron-lucent granules (G), glycogen particles (Gl), storage granules glycogen (SG), nucleus (N), microvilli (Mv), secretion (S), lumen (L), positive reaction in granules (arrowhead), mitochondria (M), constitutive secretion (arrow).

Figures 7-12: Male accessory glands of *Triatoma brasiliensis* submitted to the ethanolic phosphotungstic acid technique for detection of proteins. Unstained sections. (7) Basal portion of AG III (1th day). (bar 1µm). (8) Positive reaction in granules (G) (bar 0.5µm). (9) Basal portion of AG IV (1th day). (bar 5µm). (10) Electron-lucent granules (G) with positive reaction (bar 1µm). (11) Apical portion of AG IV (5th day). (bar 2µm). (12) Medium portion of AG I (5th day). (bar 1µm). Trachea (T), basal membrane (BM), mitochondria (M), positive reaction (arrowhead), electron-lucent granules (G) nucleus (N), lumen (L), Lipids (Li).

Figures 13-18: Male accessory glands of *Triatoma melanica* submitted to the periodic acid-thiosemicarbazide-silver proteinate technique for detection of carbohydrates. Unstained section. (13) Glandular epithelium of the AGII (5th day). (bar 1µm). (14) General view of glandular epithelium of the AGI (5th day). (bar 1µm). (15) Storage granule glycogen (G). (bar 0.5µm). (16) Apical portion of AG I (3th day). (bar 0.5µm). (17) Apical portion of AG IV (1th day). (bar 1µm). (18) Apical portion of AG IV (1th day). (bar 0.5µm). Nucleus (N), mitochondria (M), positive reaction in granules (arrowhead), electron-lucent granules (G), glycogen particles (Gl), storage granule glycogen (SG), mitochondrias (M), microvilli (Mv), secretion of granules with positive reaction (S), lumen (L), constitutive secretion (arrow), rough endoplasmatic reticulum (RER), *zonula adherens* (ZA).

Figures 19-24: Male accessory glands of *Triatoma melanica* submitted to the ethanolic phosphotungstic acid technique for detection of proteins. Unstained sections. (19) Apical portion of AG II (1th day). (bar 0.5µm). (20) Basal portion of AG IV (1th day). (bar 2µm). (21) General view of the epithelium glandular of AG III (1th day). (bar 5µm). (22) General view of the epithelium glandular of AG IV (5th day). (bar 5µm). (23) Apical portion of AG II (5th day). (bar 0.5µm). (24) Apical portion of AG IV (1th day). (bar 1µm). Electron-lucent granules (G), positive reaction in granule (arrowhead), microvilli (Mv), lumen (L), basal membrane (BM), mitochondria (M), nucleus (N), Muscle (Mu), electron-lucent secretion (S), constitutive secretion of material positive for protein (arrow), *zonula aderens* (ZA).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SG
SG
G
BM
Gl
G
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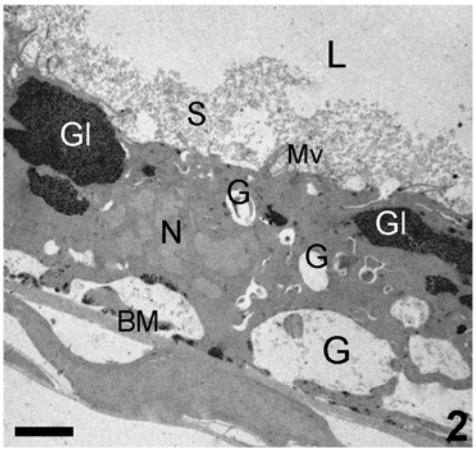
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BM
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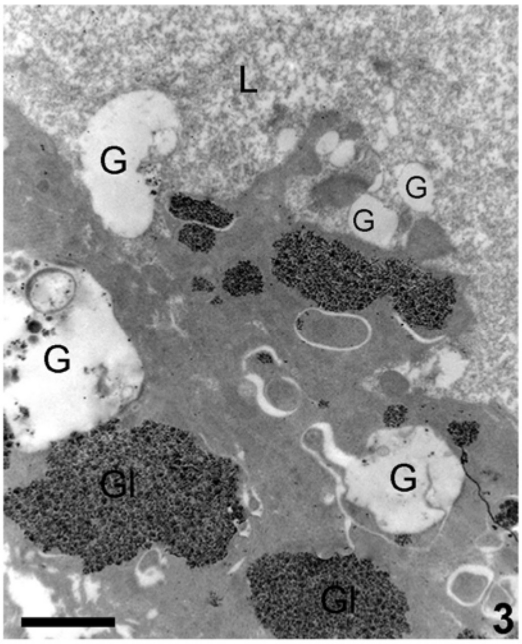
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G
G
Gl
Gl
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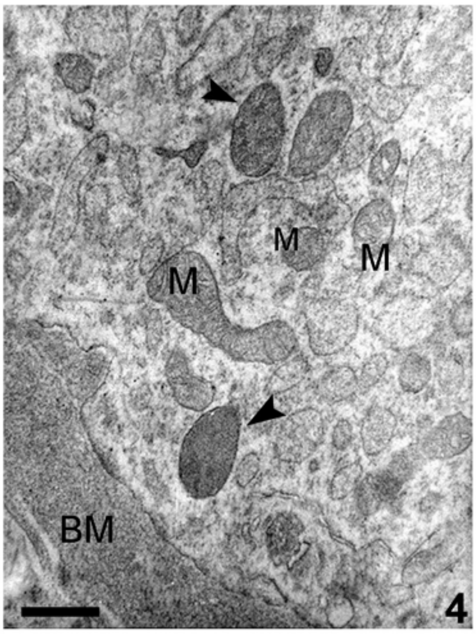
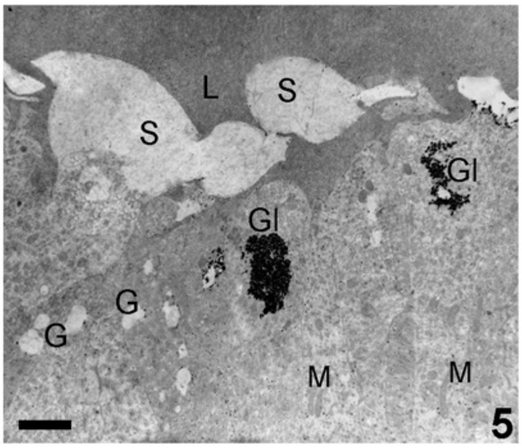

M
M
M
BM
4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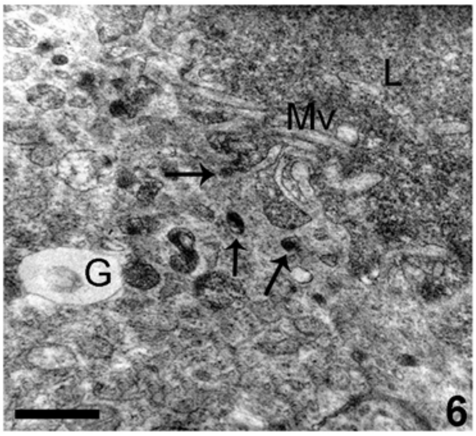
S
S
Gl
Gl
G
G
M
M
5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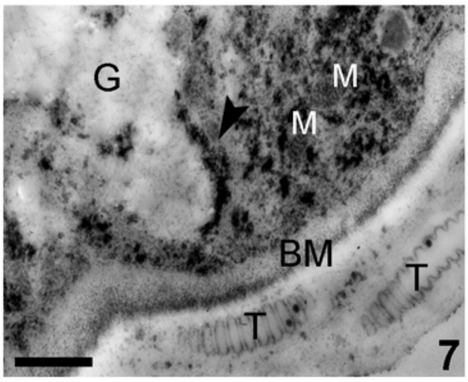
Mv
G
6



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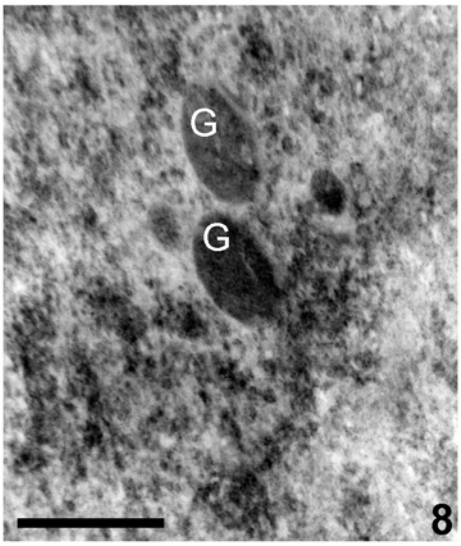

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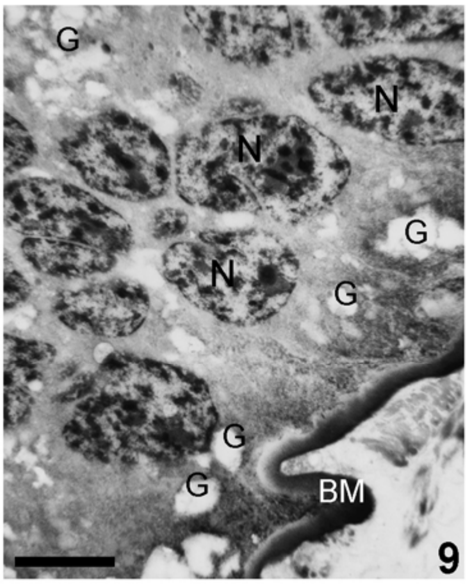

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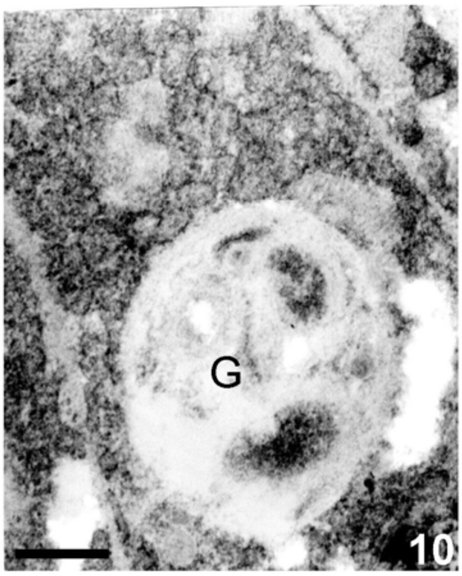

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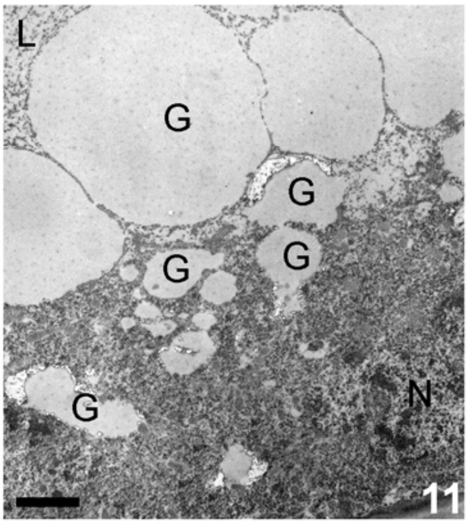

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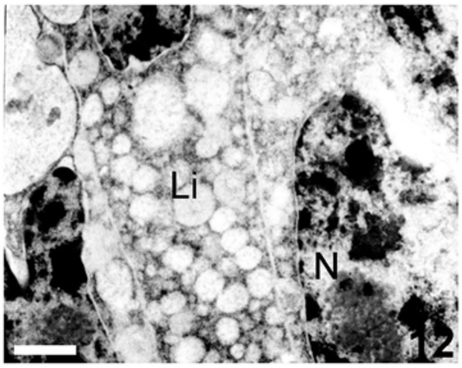

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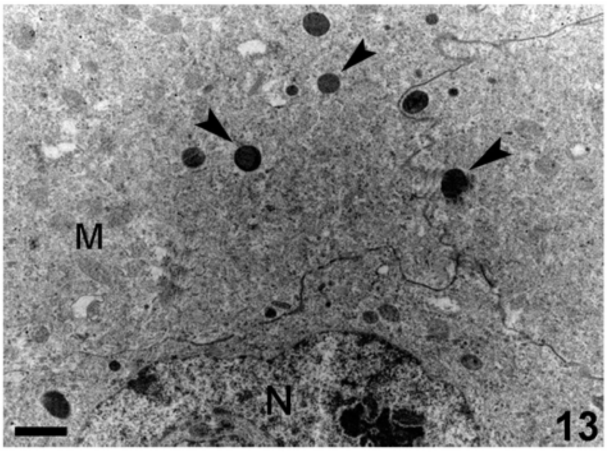
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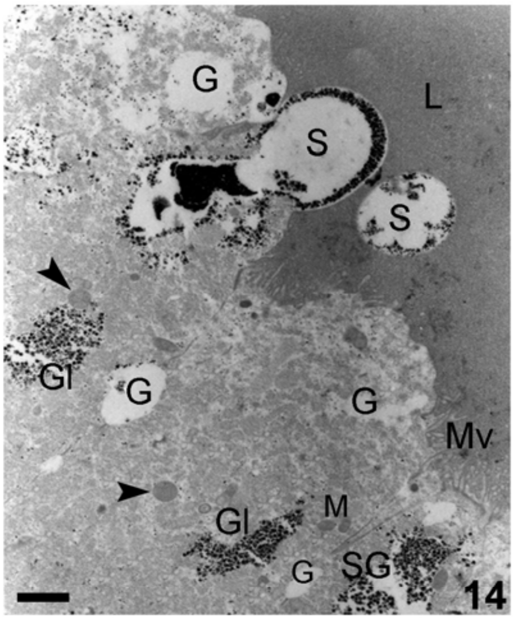
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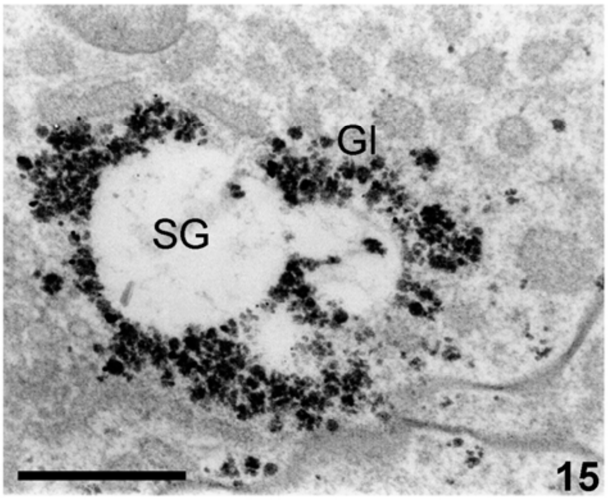
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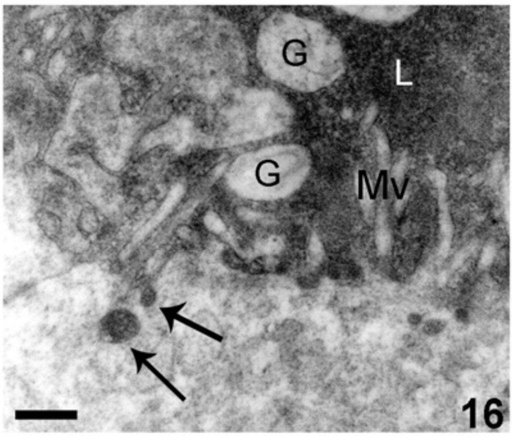
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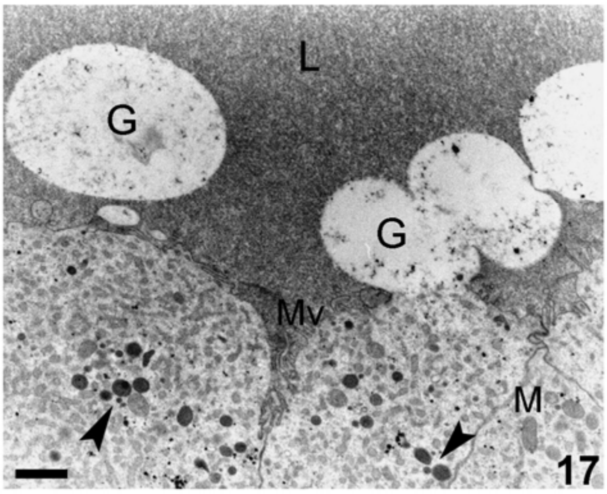
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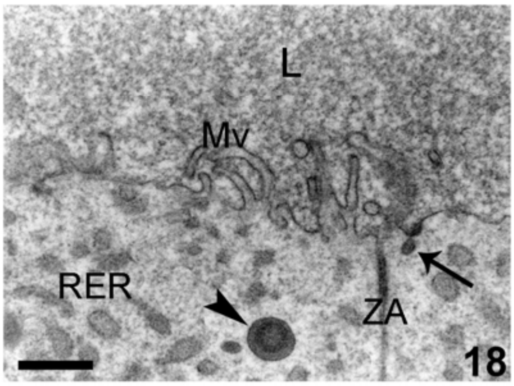
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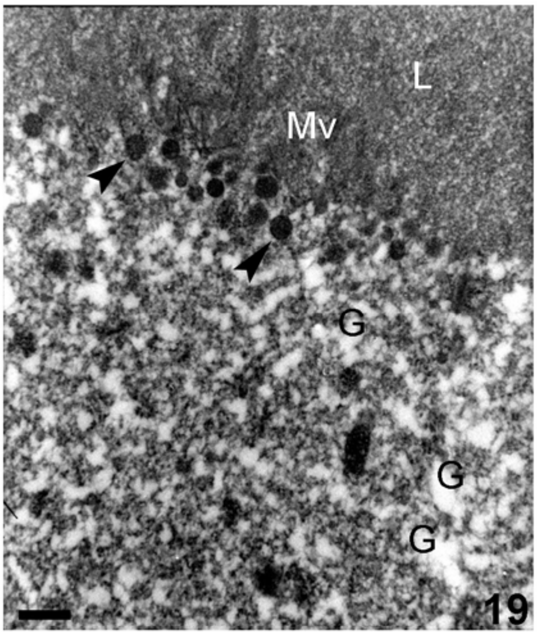
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G
G
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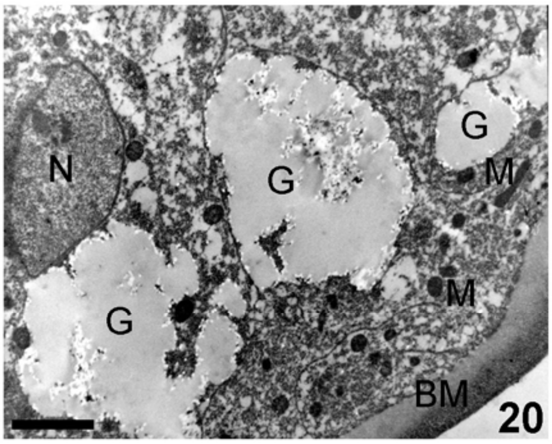
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BM
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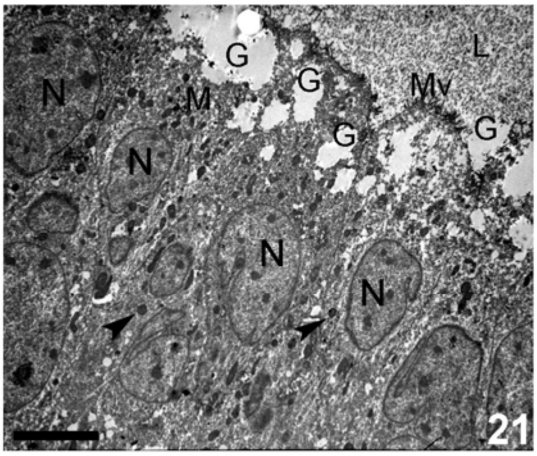
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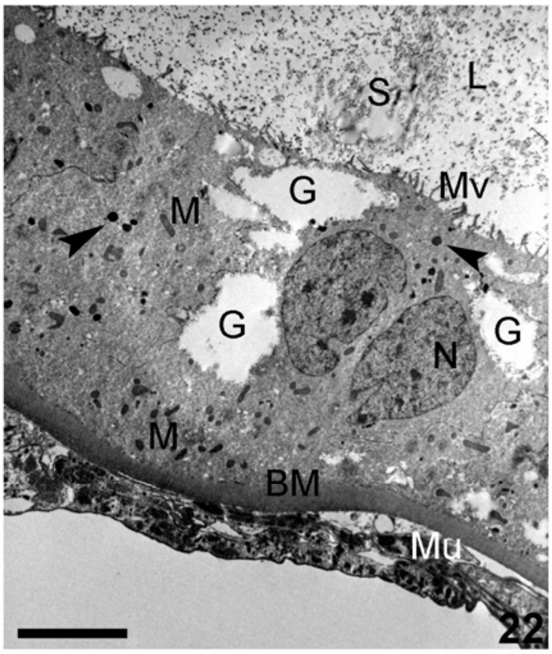
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Mu
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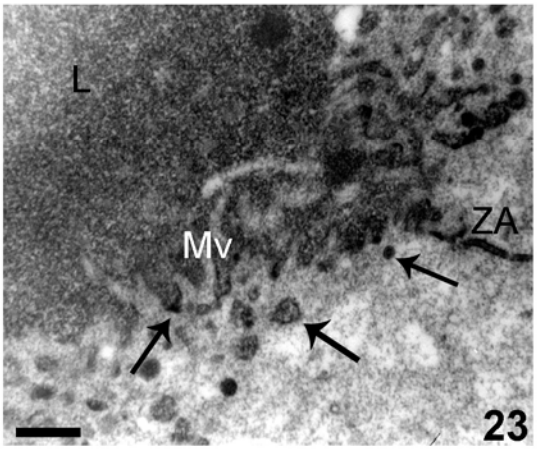
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Mv
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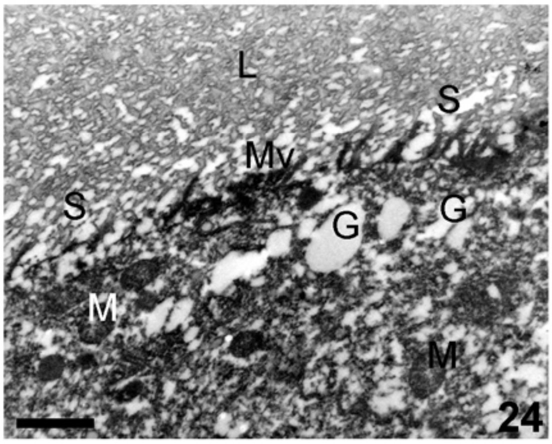
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Mv
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