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Ultrastructure study of vitellogenesis of experimentally recovered *Fasciola gigantica*

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ABSTRACT

The ultrastructure of experimentally recovered *Fasciola gigantica* shows that vitelline cells are grouped in vitelline follicles. Vitelline cells developed through a series of developmental stages. At stage I of vitelline cell development, the nucleo-cytoplasmic ratio is high and the cytoplasm has many elongated mitochondria. In the stage II, the cisternae of endoplasmic reticulum is appeared, the formation of shell protein globules is started and the cell increases in size, while the nucleo-cytoplasmic ratio decreases. At the final stage of development, stage III, the prominent structure is the domination of shell protein globules as the vitelline cell is fully matured and ready to deliver its shell protein globules to the fertilized eggs. This study is the first to show the fine structure of vitelline cells of *F. gigantica* recovered from experimentally infected mice. The present work opens the way for more studies on experimentally recovered digenean worms and also exploring strategies for fighting the diseases caused by these worms.

INTRODUCTION

Many ultrastructural studies were done on the different structures of *Fasciola* spp. like the tegument (Threadgold, 1963 & 1967; Bennett and Threadgold, 1975; Davies, 1978; Skuce and Fairweather 1989; Hiekal, 1992; Stoitsova and Gorchilova, 1997; Sobhon *et al*., 1998; Mckinstry *et al.* 2007), the caecal epithelium (Thorsell and Björkman, 1965; Gallagher and Threadgold, 1967; Halton, 1967; Bennett and Threadgold, 1973; Robinson and Threadgold, 1975; Bennett, 1975; Davies, 1978; Threadgold, 1978; Ashour *et al*., 2001; Meemon *et al.* 2010 and Savage *et al*. 2014).

Sprmatogenesis of adult *F. gigantic* was studied by Ashour *et al*., (2002) and Ndiaye *et al*., (2004). However, Ashour *et al*. (2003) demonstrated that the migrating *F. gigantica* worms recovered from body cavity of experimentally infected mice reach their maturity; as all spermatogenesis and spermiogenesis stages were observed in these migrating worms.

Some studies were done on the female reproductive system and different structures of adult female *Fasciola* spp. that recovered from naturally infected hosts (Holy and Wittrock, 1986). Many studies on the structure and development of vitelline cells and egg shell formation were done by many authors like Stephenson (1947), Yosufzai (1953), Burton 1963, Bjorkman and Thorsell (1963), Irwin and Threadgold (1970 and 1972), Hendow and James (1989), Colhoun *et al*. (1998), Meepool *et al.* (2006) and Savage *et al*. (2014).

Some studies were done on the structure and development of vitelline cells of some digenean trematodes (Gupta *et al*., 1987; Skuce and Fairweather, 1988; Wells and Cordingley, 1991; Colhoun *et al*., 1998; Robinson *et al*., 2001; Meepool *et al*., 2006; Sampour, 2008; Taeleb, 2013a & b and Greani *et al*., 2014). The mature vitelline cells are derived from stem cells in the vitelline follicles (Stephenson, 1947; Irwin and Threadgold, 1972; Threadgold, 1982 and Colhoun *et al*., 1998). It was shown that the synthesis of shell protein globules begins in the maturing vitelline cells of the digenetic trematode *Haploporus lateralis* (Sampour, 2008), *Opisthorchis viverrini* (Khampoosa *et al*, 2012). The egg shell precursor proteins are synthesized by the vitelline cells (Threadgold, 1982). The vitelline cells also provide the developing embryo with nutrients in the form of glycogen and yolk material (Threadgold, 1982; Martinez-Alos *et al*., 1993 and Swiderski and Xylander 2000).

In the present study, the ultrastructure of the vitelline cells of experimentally recovered *F. gigantica* worms is demonstrated for the first time.

MATERIALS AND METHODS

Worm collection

Adult worms of *Fasciola gigantica* Cobbold, 1855 were collected from the bile ducts of buffaloes (*Bubalus bubalis),* at Basateen abattoir, Cairo. These worms were transferred into a warm saline solution (0.85% NaCl) for oviposition. Deposited eggs were washed with distilled water and were incubated at 26 - 28°C.

Snail infection

After 14 - 21 days post incubation, eggs hatch into miracidia. Laboratory bred snails *Lymnaea natalensis* Krauss, 1848 were individually put into vials with 3 - 5 miracidia and left for 24 hours to guarantee the snail's infection. After 35 days post infection (pi), the cercariae were shed out from the infected snails. Cercariae were adhered on the vials' walls and bottoms forming metacercariae. The metacercariae were gently collected, counted and maintained in clean vials with distilled water and stored at 4° C.

Mice infection

Mice were bought from Schistosoma biological supply program (SBSP) unit at Theodor Bilharz institute, Giza, Egypt. Mice were starved 24 hours before infection then fed individually on a small piece of bread containing metacercariae. After 50 days pi, mice were dissected and liver was carefully examined for the presence of *F. gigantica* worms.

Electron microscopy

Worms were sliced transversely into halves and fixed in 3% glutaraldehyde for two hours at room temperature, specimens were washed 2 - 3 times in phosphate buffer (pH 7.2), and post fixed in 1% osmium tetraoxide for two hours at 4° C then washed in the phosphate buffer. Specimens were dehydrated in an ascending series of ethanol then acetone and then embedded in resin (Epon 812). Ultrathin sections were stained with uranyl acetate and lead citrate and examined under JEOL (JSM-6300) transmission electron microscope at the regional centre of fungi, El Azhar University, Cairo, Egypt.

RESULTS

Vitelline cells aggregated in vitelline follicles (Figs. 1 - 3), and contained the shell protein clusters which are peripherally arranged underneath the cell membrane of the mature vitelline cells (Figs. 3 & 12). Some of the mature vitelline cells contained vacuoles filled with yolk granules (Fig. 3). The shell protein clusters were consisted from many accumulated small shell protein globules (Figs. 5, 12, 4 - 11). The number and size of shell protein globules in the vitelline clusters varied among different clusters as they ranged from 2 to 15 globules (8.62 ± 3.67) per cluster; and from 0.074 µm to1.48 µm. Vitelline cells are active cells due to abundance of rough endoplasmic reticulum (RER) within their cytoplasm;

RER is concentrated around the nucleus (Figs. 9 & 10).

Vitellogenesis in the experimentally recovered *Fasciola gigantica* cells start from a stem cell. The stem cell measured 8.14 x 3.14 µm, with a very large nucleus (6 x 2.71) that occupies most of the cytoplasm (high nucleo-cytoplasmic ratio) and contained aggregated chromatin (Fig. 6 & 13). Vitellogenesis started in vitelline follicles through three developmental stages: I, II and III.

In stage I of vitellogenesis (Figs. 5 & 6), the vitelline cells took a variable elongated appearance (Fig. 5) or semi polygonal appearance (Fig. 6). Elongated vitelline cell reached 13 x 3.4 µm; the nucleus was also elongated and reached 6.6 x 2.6 µm. The stage I of vitelline cells had a high nucleo-cytoplasmic ratio and contained a high content of chromatin (Figs. 5, 6, 12 & 14). Many elongated mitochondria were also observed in the cytoplasm of these vitelline cells (Figs. 5, 6 & 14); these mitochondria have obvious cristae (Figs. 7 & 8).

In stage II of vitellogenesis (Figs. 4, 9 - 12 & 15), the cell reached to 13.73 x 8.4 µm. The nucleus (6 x 4.26 µm) was gradually displaced from the cell's centre and the chromatin aggregations started to decrease (Fig 11). On the other hand, the formation of shell protein globules is started. Globules accumulated either at one pole of stage II vitelline cells or surrounded their nuclei (Figs. 4, 12). In this stage of development, RER is found in the cytoplasm and surrounding the nucleus. Rough endoplasmic reticulum consists from many parallel cisternae which took a perinuclear position (Figs. 4 & 9).

In the stage III of vitellogenesis (Figs. 12 & 16), the cell reached 19 x 9 µm; at this stage, the vitelline cells became mature. The cells of stage III of development are filled with shell protein globules which accumulated in clusters. Each shell protein cluster was formed from small globules of various sizes and arranged beneath the cell membrane and surrounded an electron-lucent matrix (Figs. 1 - 3, 12 & 16). In stage III of vitelline cell development, the nucleus started to decrease in size and the electron-lucent matrix started to be formed (Fig. 11); the fully mature vitelline cell had no nucleus and the electron lucent matrix dominated the vitelline cell. The individual shell protein globules attached to each other within the cluster and the number of shell protein clusters is varying among mature vitelline cells as they ranged from 7 to 18 (12.25 ± 3.89) clusters per cell. The cluster size varied in the mature vitelline cells as it ranged from 0.25 to 0.54 µm (0.34 ± 0.07). Mitochondria and RER are absent from the mature vitelline cells (stage III of development). Yolk granules were also observed in vacuoles within the electron-lucent matrix of the stage III of vitellogenesis (Fig. 3).

DISCUSSION

The high egg production of the liver fluke, *F. hepatica*, with its high number of vitelline cells revealed that, *F. hepatica* has to consume and produce materials for its reproductive needs (Meepool and Sobhon, 2009). In the present work, the finding of a large number of vitelline cells and a great number of clusters of shell protein globules may also indicate high rates of egg production in *F. gigantica*. The presence of prominent RER in the cytoplasm of vitelline cells indicates higher activity rates in protein production and this is in agreement with the findings of Irwin and Threadgold (1972) and Colhoun *et al*. (1998). In the present study the large number of individual globules within the shell clusters also indicates a high rate of egg production that needs a continuous supply for the shell formation (protein material) which is translated in the abundance of RER in the vitelline cells.

In *F. hepatica*, it has been estimated that the adult worm produces ~ 25,000 eggs / day (egg / 3.46 sec.) Happich and Boray (1969); According to Meepool and Sobhon (2009), each egg consists of one ovum and about 30 vitelline cells and as it was recorded by Happich and Boray (1969) that an egg was produced every 3.46 sec., so one vitelline cell will be produced every ~ 0.12

sec. (Meepool and Sobhon, 2009). When the shell protein globules of *F. hepatica* are formed they migrate to the periphery of vitelline cells where large clusters accumulate there and a certain degree of globules' fusion within these clusters is reported (Irwin and Threadgold, 1972). In the present study the coalescing of shell protein globules in clusters may happen in a similar process to that reported by Irwin and Threadgold (1972). The presence of shell protein globules at the cell periphery indicates that the vitelline cells are mature and they are ready to discharge the protein globules onto the newly formed eggs to form their shells.

Egg shell production in *Schistosoma mansoni* has been proposed by Wells & Cordingley (1991), as eggshell protein globules were released from the vitelline cells within the ootype then followed by their subsequent union to form the eggshell; union and tanning of these components produces eggshell. Egg formation in *F. hepatica* takes place in the ootype, which is surrounded by the Mehlis' gland cells. Each egg involves the combination of an ovum, approximately 30 vitelline cells and secretions from the Mehlis' gland. The vitelline cells release shell protein globules which coalesce around the cell cluster to form the eggshell Colhoun *et al*. (1998). The results of Colhoun *et al*. (1998) suggested that the mechanism for eggshell formation in *F. hepatica* is similar to that proposed for *S. mansoni* by Wells & Cordingley (1991) and may be common to other trematodes as well.

Greani *et al*. (2012) studied the ultrastructural organization of the female reproductive system of *Metadena depressa*, a digenean intestinal parasite of *Dentex dentex*, and they found that, vitellogenesis is divided into four stages: stage I, vitellocytes have a cytoplasm mainly packed with ribosomes and few mitochondria; stage II, the cells started launching the synthetic activity; stage III, synthesis of active clusters of shell globules began; stage IV, maturation of vitellocytes which are filled with shell globule clusters and generally have many large lipid droplets; and glycogen granules are grouped at the border of the cell. In the present study, the development of vitelline cells occurs through three stages; stage I with large nucleus and some mitochondria, with no ribosome were observed. In stage II, the synthetic activity of shell protein globules are started as the endoplasmic reticulum appeared and loaded with ribosomes; in this stage the shell protein globules are accumulating. In stage III, the shell protein clusters are the dominated structures with an electron-lucent matrix; so the developmental stages of experimentally recovered *F. gigantica* and *Metadena depressa* are nearly similar as both worms are digeneans and this may confirm the hypothesis of Colhoun *et al*. (1998) for the similarity of vitelline cell development in trematodes.

In the present study, the average number of the shell protein globules within the cluster was about 8 globules which much lower than those counted by *Greani et al*. (2012) in their study of vitelline cells of *Metadena depressa*, as they recorded approximately 45 globules per cluster. On the other hand the average cluster diameter of vitelline cells of the present study is smaller (0.34 μm) than that of vitelline cells of *Metadena depressa* (3 μm) (Greani *et al*. 2012). The vast majority of shell protein globules in the present study are attached together in the form of spherical cluster; while shell protein globules are individually grouped in clusters in vitelline cells of *Metadena depressa* (Greani *et al*. 2012).

In the present study, the nucleo-cytoplasmic ratio is decreased with vitelline cell development; this observation was also noticed in the development of vitelline cells of *F. hepatica* (Colhoun *et al*., 1998) and *Metadena depressa* (Greani *et al*., 2012). The mature vitelline cells of experimentally recovered *Fasciola gigantic* in the present study have an electron-lucent matrix which is greater than its counterpart in *M. depressa* (Greani *et al*., 2012).

CONCLUSION

The present study proved that the experimentally recovered *F. gigantica* worms that recovered from experimentally infected mice can reach maturity, produce eggs and have normal development of vitelline cells. This work also represents a new trend for more studies on the reproductive organs of digenetic trematodes from experimentally infected animal models.

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LAST OF FIGURES

Figures 1 - 3: Vitelline cells grouped in vitelline follicles which are surrounded by muscle fibers and parenchyma tissue; yolk granules (white arrow heads in Fig. 3) are observed inside vacuoles within vitelline cells. (Scale bar: Fig. 1= 5µm; Figs. 2 & 3 = 10 µm). vf: vitelline follicles; vc: vitelline cells; mf: muscle fibres and pt: parenchyma tissue.

Figure 4: Vitelline cell with shell protein globules surround the nucleus in a circular manner. Note the stem cell (white arrow) and the vitelline cell is in the stage II of development. (Scale bar = 2 µm). N: nucleus.

Figures 5 & 6: Stage I of vitellogenesis; vitelline cell has a large nucleus with dense patches of chromatin. Cytoplasm contains many elongated mitochondria at one pole of the cell. (Scale bar: Fig.5 = 2 µm; Fig. 6 = 500 nm). Chr: chromatin; M: mitochondria and N: nucleus.

Figures 7 & 8: High magnification of a vitelline cell at stage I of development showing aggregations of mitochondria with clear cristae (Scale bar = 500 nm). M: mitochondria and white arrow heads: cristae.

Figures 9 & 10: Higher magnifications of figure (4) showing stage II of vitellogenesis. A perinuclear rough endoplasmic reticulum and clusters of shell protein globules surround the nucleus and line the cell membrane of vitelline cell; Nucleus has patches of chromatin. (Scale bar = 500 nm). N: nucleus; RER: rough endoplasmic reticulum; Chr: chromatin and SPG: shell protein globules.

Figure 11: Stage III of vitellogenesis; vitelline cell with a small nucleus; clusters of shell protein globules line the cell membrane. An electron lucent matrix appears within the cytoplasm. Note a vitelline cell at stage II of development and the presence of many mitochondria after rupturing of some vitelline cells (Scale bar = 2 µm). M: mitochondria and ELM: electron lucent matrix.

Figure 12: Stage III of vitellogenesis; vitelline cell without a nucleus; clusters of shell protein globules are found underneath the cell membrane. An electron lucent matrix dominates the vitelline cell. Note vitelline cells at stages I and II of development. (Scale bar = 2 µm); ELM: electron lucent matrix.

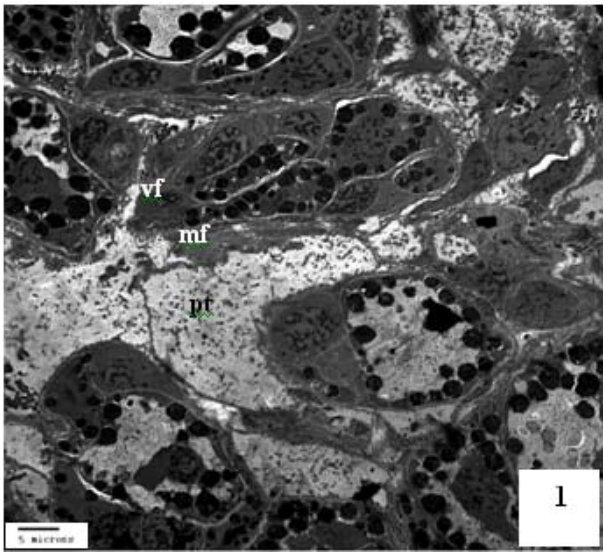
Figures 13-16: Diagrammatic drawings showing vitellogenesis

Figure 13: Stem cell. N: nucleus

Figure 14: Stage I of development. Chr: chromatin, M: mitochondria

Figure 15: Stage II of development. N: Nucleus, RER: rough endoplasmic reticulum, M: mitochondria, SPG: shell protein globules

Figure 16: Stage III of development. SPG: shell protein globules, ELM: electron lucent matrix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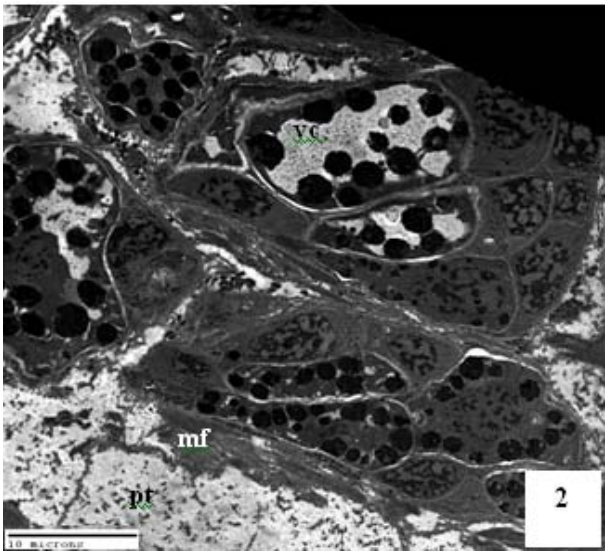
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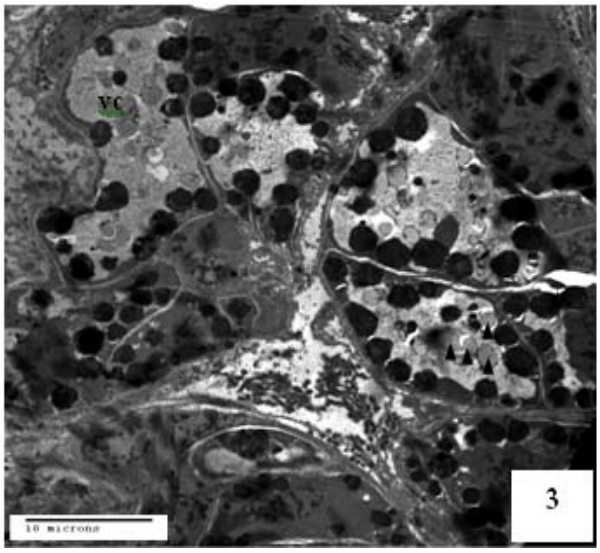
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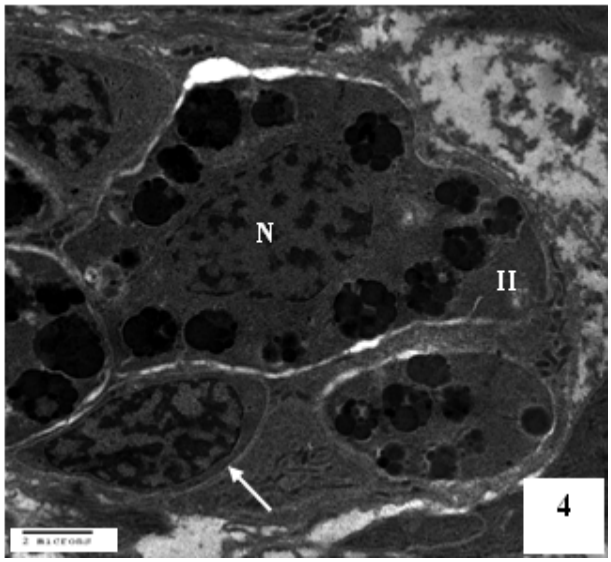
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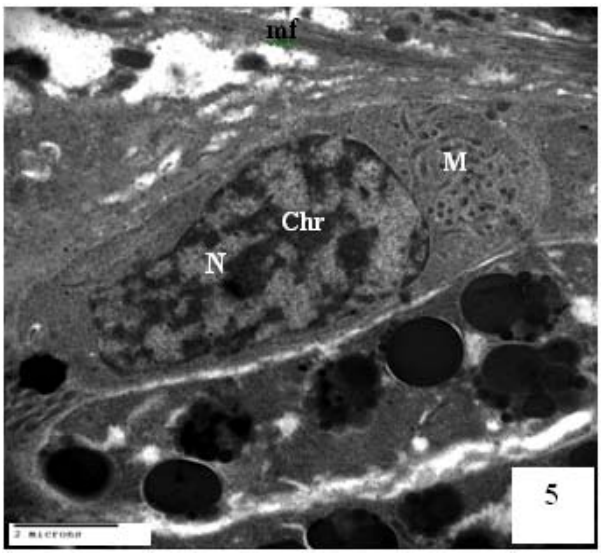
mf
M
Chr
N
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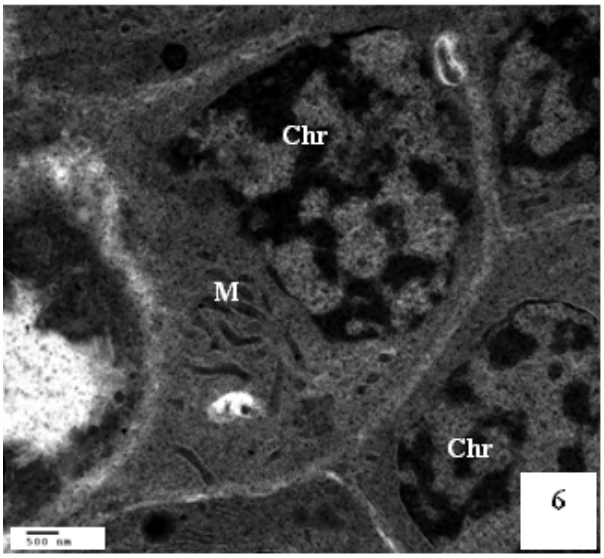
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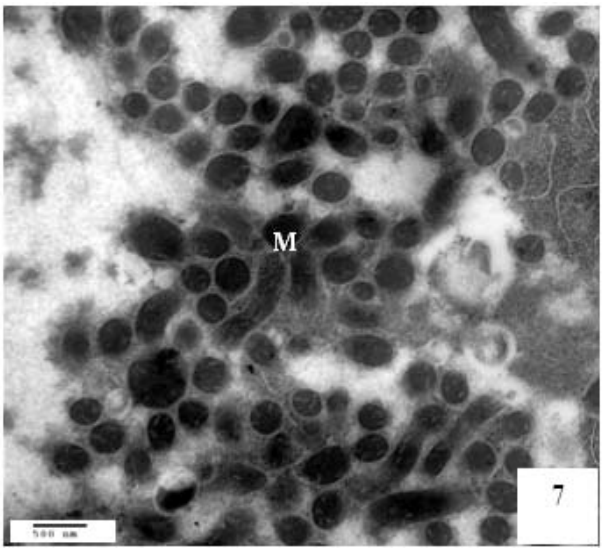
M
Chr
6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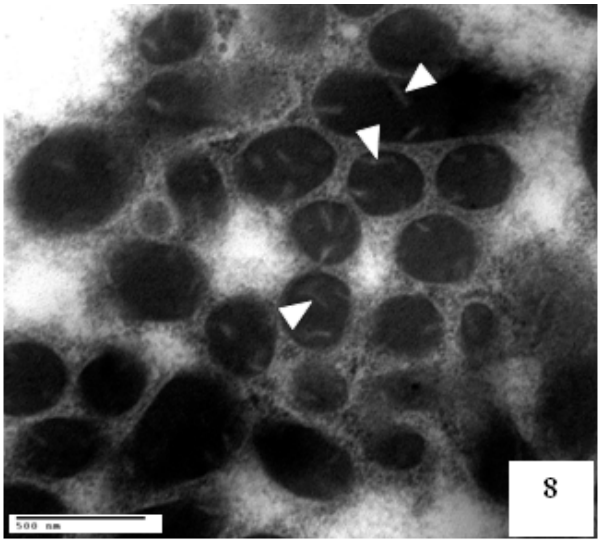
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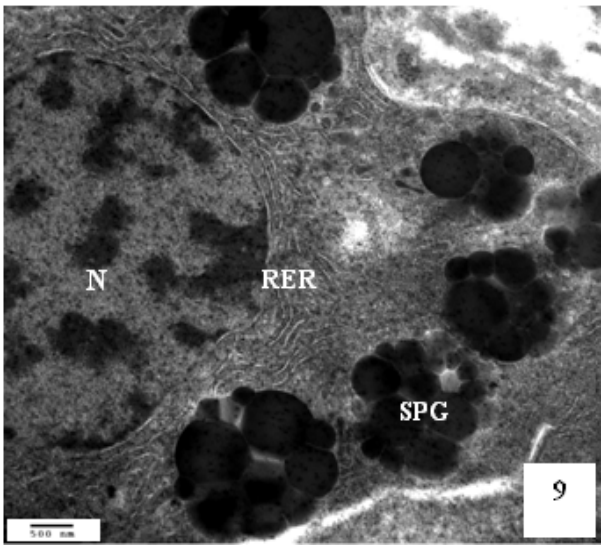
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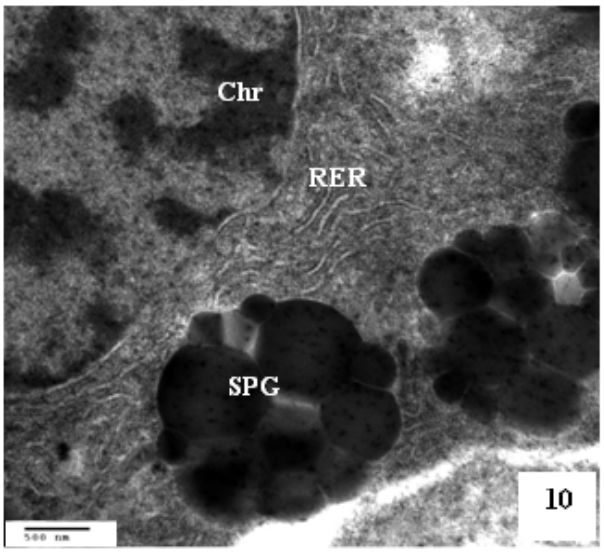
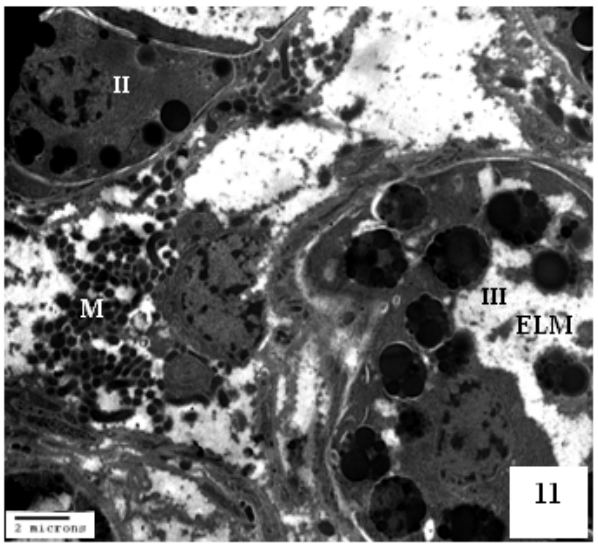
RER
SPG
9


Chr
RER
SPG
10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III
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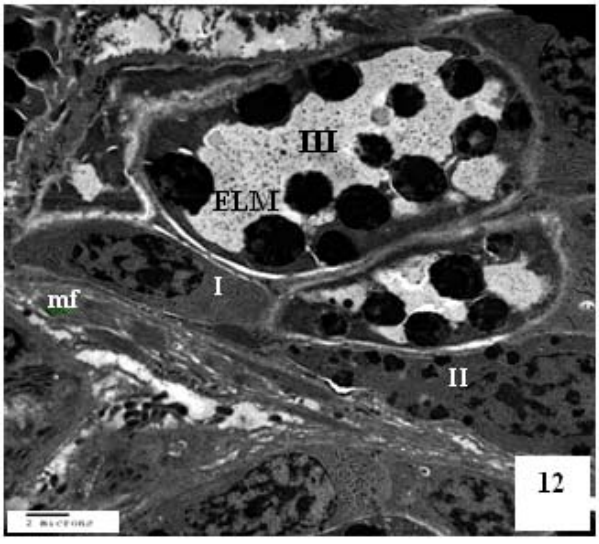
11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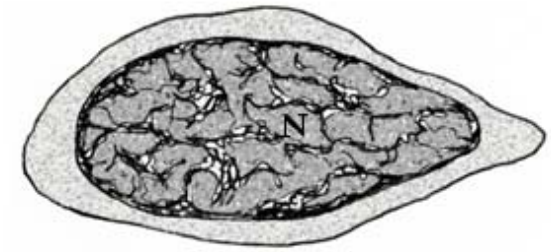
I
mf
II
12



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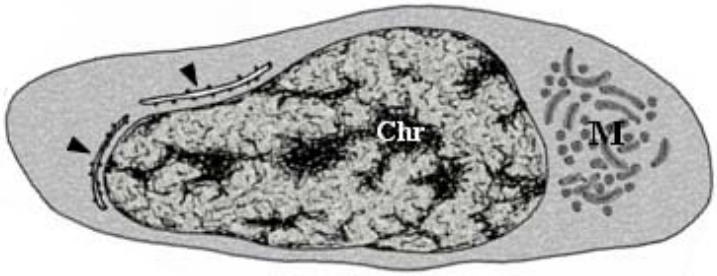

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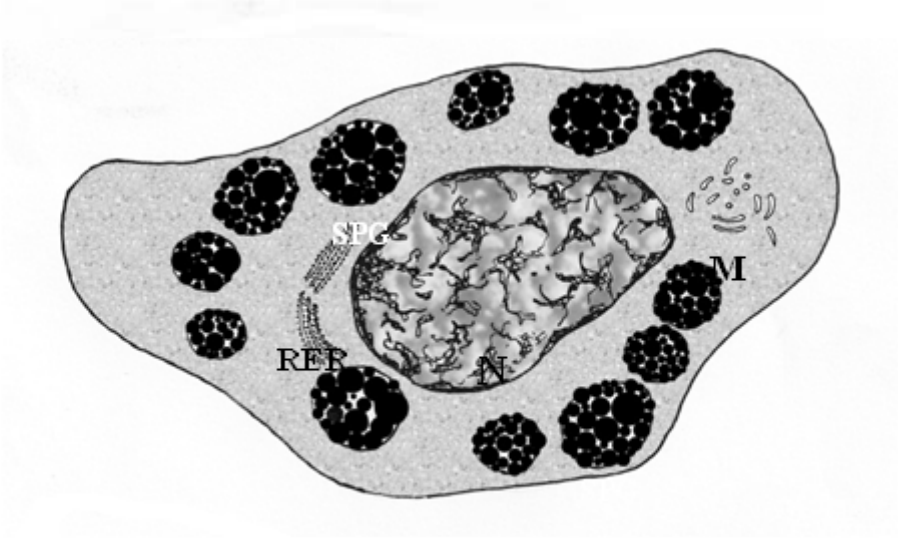

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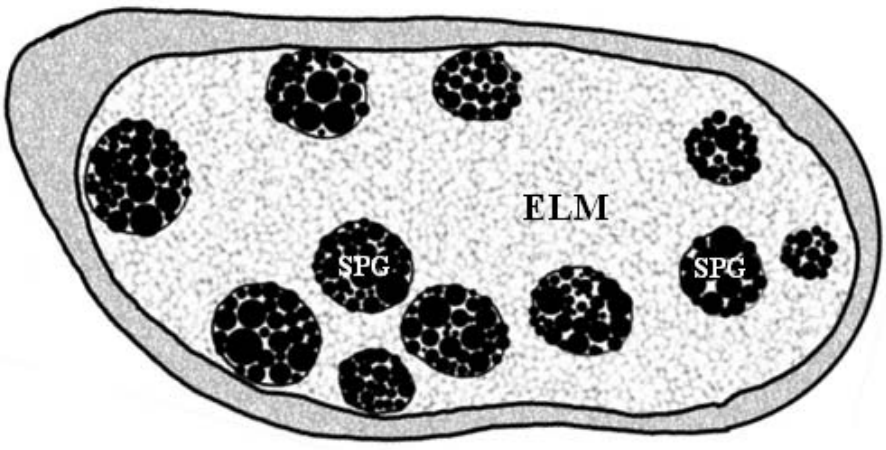

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ARABIC SUMMERY

دراسة التركيب الدقيق لتكوين الخلايا المحية في الفاشيولا جيجانتيكا المستخرجة تجريبياً

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التركيب الدقيق لديدان الفاشيولا جيجانتيكا المستخرجة تجريبياً يوضح أن الخلايا المحية تتجمع في بصيلات محية. وتتطور هذه الخلايا المحية عبر سلسلة من مراحل النمو. ففي المرحلة الأولى من تطور الخلايا المحية ، لُوحظ كبر نسبة النواة إلى السيتوبلازم وأن السيتوبلازم يحوي العديد من الميتوكوندريا المستطولة. بينما في المرحلة الثانية ، ظهرت صهاريج من الشبكة الإندوبلازمية، وبدأ تشكيل كريات بروتين القشرة وزيادة في حجم الخلايا ، في حين أنخفضت نسبة النواة إلى السيتوبلازم. وفي المرحلة النهائية من التطور- المرحلة الثالثة - سادت كريات بروتين القشرة حيثُ أصبحت الخلايا المحية ناضجة تماما وأصبحت على استعداد لتقديم كريات بروتين القشرة إلى البويضات المخصبة. هذه الدراسة هي الأولى التي تظهر التركيب الدقيق للخلايا المحية للفاشيولا جيجانتيكا المستخرجة من الفئران المصابة تجريبياً. والدراسة الحالية تفتح الطريق أمام المزيد من الدراسات على الديدان ثنائية العائل المستخرجة تجريبياً وأيضا إستكشاف إستراتيجيات لمكافحة الأمراض التي تسببها هذه الديدان.