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ULTRASTRUCTURAL STUDY ON *BIOMPHALARIA ALEXANDRINA* HAEMOCYTES INFECTED WITH *SCHISTOSOMA MANSONI* IN EGYPT AND ITS CORRELATION WITH NITRIC OXIDE LEVEL

By

EMAN G. HELAL[1], SHADIA M. EL- DAFRAWY[2], AMIRA H. MOHAMED[3], BASMA M. ABOU-EL-NOUR[4] and SAMAH IBRAHIM[5]

Department of Zoology, Faculty of Science, Al-Azhar University (Girls)[1,4], Department of Medical Malacology[2,5] Electron Microscopy[3], Theodor Bilharz Research Institute P.O. Box 30, Imbaba, Giza, Egypt

Abstract

Some snails of *Biomphalaria alexandrina* can resist the infection of *Schistosoma mansoni* so this study aimed to clearly this mechanism by using light and electron microscopy (EM) and determine the role of Nitric oxide in this mechanism. *B. alexandrina* snails used in this study were exposed individually to *S. mansoni* infection according to their response they were classified into susceptible group (shed cercariae) and resistant group (failed to shed cercariae). Snails not exposed to infection were included in this study as control group. Nitric oxide (NO) level was assayed directly in the soluble fraction of *B. alexandrina* haemolymph supernatants collected from each group of *B. alexandrina* snails were subjected to NO assay by the Greiss reaction. The level of NO in haemolymph of infected snails was significantly increased ($p<0.001$) than both control and non infected snails groups, however, in non infected snails group had significantly ($p<0.05$) compared to control group. This study when correlated the changes recognized by EM with NO level the pro apoptotic effect of high level of NO on the haemocytes. Characterization and identification of cell shape of haemocytes in both haemolymph and tissue were examined by light and electron microscopy. Examination of *B. alexandrina* snail's haemocytes revealed three types of different cells classified according to their shape and granular contents. These cells are granulocytes, amoebocytes and hyalineocytes. Electron microscope study also revealed the important role of granulocytes and amoebocytes as defense mechanism against snail infection. NO is considered an important anti parasite molecule; intra-molluscan stages of parasites switch off host NO defense response

Key Words: Apoptosis; *Biomphalaria alexandrina*; Electron microscope; Immune response.

Introduction

Schistosomiasis is an important health problem that affects over 200 million people worldwide. Among the schistosomes species that infect human beings; *S. mansoni* is transmitted by *B. alexandrina* and causes intestinal and hepatic schistosomiasis in Africa, Arabian Peninsula, and South America (Gryseels *et al,* 2006). The pathogenic trematodes that cause schistosomiasis, is endemic over 70 tropical and subtropical countries (Hotez *et al,* 2008; Montresor *et al*, 2012; Gryseels, 2012). NO is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes including vasodilatation, neuronal function, inflammation and immune function. NO is a short-lived molecule (with a half-life of a few seconds) that is produced from a group of enzymes known as nitric oxide synthesis (NOS). Since such a small molecule; NO is able to diffuse rapidly across cell membranes and, depending on the conditions, is able to diffuse for distances of more than several hundred microns. The biological effects of NO are mediated through the reaction with a number of targets such as haem groups, cysteine residues,

iron and zinc clusters. Due to the importance of NO, abnormal regulation or control of NO synthesis is capable of affecting a number of important biological processes and has been implicated in a variety of diseases (Bruckdorfer, 2005). However, in other parasitic diseases, the production of NO may allow progression of infection and is considered as a parasitic evasive mechanism (Vijay *et al*, 2011). There are three known isoforms of NOS enzymes, two are constitutive (cNOS) and "Ca^{2+} sensitive" and the third is inducible (iNOS) and "Ca^{2+} insensitive". Cloning of NOS enzymes indicates that, cNOS includes both neuronal constitutive (nNOS or NOS1) and endothelial constitutive (eNOS or NOS3) and the third is the inducible (iNOS or NOS2) enzyme (Trajanovska and Donald, 2011). NO had been demonstrated to play a role in a variety of biological processes including the regulation of cell death (apoptosis) and cell motility (Kim *et al*, 1999), neurotransmission (Herrero and Gagnon, 2001) and immune defense (Espinoza *et al*, 2002).

The present study was designed to study the immunological role of haemocyte and host parasite relationships during *S. mansoni* infection in Egypt.

Material and Methods

B. alexandrina snails used in this study were obtained from the laboratory-bred stock in Medical Malacology Laboratory, Theodor Bilharz Research Institute (TBRI) Egypt. Laboratory bred *B. alexandrina* snails were exposed individually to ten *S. mansoni* miracidia in the presence of 2 ml of dechlorinated aerated tap water under florescent light at temperature (25±1ºC). They remained in contact with miracidia over night and then transferred to their original aquaria, at laboratory temperature (25±1°C) throughout the infection process (Henning and Youssef, 1976). They were kept in the darkness at 26°C for about a month. The fresh lettuce leaves were supplied as food every couple of days and the dead snails were regularly removed. Thirty days post infection; the snails were checked individually for the cercarial shedding (McClelland, 1965; Kamel *et al*, 2006). Three groups (300 snails each) were used in this experiment for NO determination, light and electron microscopic examination.

Two groups from these exposed snails (300 snails each) has been taken, the snails that shed cercariae were considered susceptible to infection (G1), while those failed to shed cercariae were found alive under microscopic examination; these snails were considered resistant to infection (G2). 300 snails were not exposed to miracidia used as control (G3).

Haemolymph and tissue of snails were collected after 40 days (during cercarial shedding) post exposure to *S. mansoni* miracidia and then analyzed and examined.

Collection of Haemolymph: Haemolymph samples were collected from all experimental group outlined (Michelson, 1966), via removing a small portion of the shell and inserting a capillary tube into the heart. Haemolymph was pooled from 300 snails collected in ten vial tubes (1.5 ml) each containing 30 snails and kept in ice-bath for NO determination and kept with 4% glutaraldehyde and Sodium cacodylate for examination by light & electron microscope.

Measurement of nitric oxide: NO level was assayed directly in the soluble fraction of *B. alexandrina* haemolymph supernatants collected from each group of *B. alexandrina* snails were subjected to NO assay by the Greiss reaction of Promega, Madison, WI, USA (Green, 1982; Tafalla *et al,* 2002; 2003). Briefly, 100µl of freshly prepared Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamide dihydrochloride in distilled water, and 2.5% orthophosphoric acid) was added to 100µl of supernatants sample obtained from each experimental group. After 10 minutes, each reaction tube was centrifuged at 3000×g for 5 min and 200 µl of the supernatant was transferred to 96-well plates. NO was measured at 540 nm by using automated spectrophotometer

(ELISA reader model EL312e, Bio-Tek, Winooski, VT, USA). The nitrite concentration in samples was calculated with reference to a sodium nitrite NaNO2 linear standard curve µM/ml.

Examination by light and Electron Microscope: The collected haemolymph were centrifuged and both the sedimented cells and collected tissue were fixed in 4% glutaraldhyde with sodium cacodylate. Two hours later, the cells were post fixed in 2 % osmium tetraoxide, dehydrated with ascending concentration of alcohol and embedded in epoxy resin. Specimens were examined in a transmission Zeiss EM-9 electron microscope, which was operated at an acceleration voltage of 50 kV. Semi-thin sections one micron thickness were performed by ultramicrotome from the made capsules and stained with methylene blue azur II and examined by light microscopy. Apoptotic cells were identified morphologically by (Savill, 1997). Also, the sections were examined by light microscopy to choose the areas of ultra thin, section which were mounted on copper grids and double stained with uranyl acetate and lead citrate and examined by Phillips electron microscope.

Statistical analysis: data were analyzed using the computer system (SPSS, 18). One way analysis of variance (ANOVA) was used to test the significance of difference between multiple means for each group. Scheffe's test was done to determine which group was responsible for the significant difference in the multiple analyses SPSS/ PC+ Professional statistics 18.0.

Results

The NO levels that were indirectly estimated in the soluble fraction of *B. alexandrina* haemolymph during the *S. mansoni* infection through the measurement of nitrite are shown in the study. The measured level of NO in haemolymph of infected snails was significantly increased (p<0.001) in comparison with control and non infected snails however, non infected snails had significantly increased (p<0.05) compared to control group.

Table 1: Nitric oxide level in haemolymph in groups

Groups	Mean ± SE
Exposed [infected snails (G1)]	33.03 ±0.72**
Exposed [non-infected snails (G2)]	21.73 ±1.22*
Unexposed [control (G3)]	15.76±0.48

*p<0.05 comparison with control group, **p<0.001 comparison with control & non-infected group.

Light Microscope: Examination of *B. alexandrina* tissue by light microscopy revealed that granulocytes: In control group, we found collection of normal granulocytes, amebocytes and hyalinocyte. Granulocytes cells were characterized by granular cytoplasm with different size granules. These cells may be hypo or hypergranular. Amoebocytes: These cells are the largest and characterized by extending of many pseudopodia, central clear nucleus. Hyalinocyte: These are translucent non granular cells (Fig.1). In infected snail tissue group**,** few granulocytes cells with dense nuclear chromatin condensation as an early sign of apoptosis and many hyalinocytes were seen, while amebocytes couldn't be seen. Marked hypocellularity was observed as compared to control group with severe degeneration and vacuolation. Hyalinocytes could be seen between granulocytes and sever vacuolated amebocytes. Many apoptotic bodies could also be detected (Fig. 2). In non infected snail tissue group, the granulocytes were the predominant cells in the center and surrounded by a zone of amebocytes at the periphery, hyalinocytes couldn't be seen. The infiltrated amebocytes were detected to the center to interact with the granulocytes, also many reactive amebocytes with large vacuolated cytoplasm and many pseudopodia were seen (Fig.3).

Electron microscope of control *B. alexandrina* snail's haemocytes in haemolymph not exposed to infection could detect granulocytes with high granules in the cytoplasm and irregular nucleus (Fig.4a) and regular nucleus (Fig.4b), amoebocytes with irregular nucleus and large cytoplasm containing normal mitochondria and many rough endoplasmic reticulums (Fig.4c) and in amoebocytes with irregular nucleus (Fig. 4d). Hyalinocytes detected undifferentiated cells were circular, transparent and agranular with different sizes (Fig. 4e).

Electron microscope demonstrated apoptotic changes in snails' haemocytes in susceptible (infected snails) and non susceptible (non infected snails) in haemolymph and tissue.

A- Haemocytes of *B. alexandrina* (susceptible for infection) in haemolymph and tissue after exposure to the infection: EM of haemolymph in infected *B. alexandrina* showed the granulocyte in early apoptotic changes with peripheral chromatin condensation (Fig. 5a). Granulocyte in early apoptotic changes with peripheral chromatin condensation, and many cells in late apoptotic change represented by intracellular apoptotic bodies (Fig. 5b). Cells in late apoptotic were filled with the apoptotic bodies (Fig. 5c & d) and increasing number of hyalino cells (Fig. 5e). Amoebocyte had many degenerated mitochondria (Fig. 6a). Many hyalino cells, most of them in mitosis, while other haemolymph cells showed apoptotic changes (Fig. 6b). High magnification showed the mitotic division of the hyalino cells in infected snail tissue (Fig. 6c). EM of tissue in infected *B. alexandrina* snails showed two granulocytes in early apoptotic changes with peripheral chromatin condensation (Fig. 7a) and granulocyte with many degenerated mitochondria (Fig. 7b). Late apoptotic changes were represented by the apoptotic bodies in its way to be released from the cell (Fig. 7c). Late apoptotic changes were represented by the extra cellular release of apoptotic bodies (Fig. 7d).

B- Haemocytes of *B. alexandrina* (non-susceptible for infection) in haemolymph and tissue after exposure to the infection: Electron micrograph of the haemolymph showed activated granulocyte represented by: numerous extending pseudopodia and many phagolysosomes (Fig. 8a), numerous mitochondria in the cytoplasm (Fig. 8b) and extending pseudopodia and two large phagolysosomes (Fig. 9a), showed extending pseudopodia and two large phagolysosomes, interaction between activated amoebocyte and granulocyte (Fig. 9b). Many extended pseudopodia in its way to form the phagolysosomes (Fig. 9c).

Discussion

The numerous species and strains of *Biomphalaria* vary in their compatibility as a schistosome host such that some display resistance to infection while others are susceptible. The present study showed the impact of resistance of *B. alexandrina* snails on the mechanisms of interaction between haemolymph and the tissue reactions against *S. mansoni* infection. When resistant snails are exposed to *S. mansoni* miracidia, haemocytes (snail blood cells) migrate towards the recently transformed sporocysts and enclose them in a multilayered cellular encapsulation of the parasites or take part in the repair of damaged tissue caused by the parasite. Soon after, the sporocysts are killed by a cytotoxic reaction which most likely involves free radicals such as hydrogen peroxide and/or nitric oxide (Hahn *et al*, 2001). In contrast, susceptible snails were not able to defend against *S. mansoni* larvae and an infection might develop following miracidial exposure.

The internal defense system (IDS) is one of the factors that influence the susceptibility pattern of the snails. This system is stimulated by the excretory secretory products of the penetrating miracidia (Zahoor *et al*, 2010). The comparison of uninfected and infected snails has allowed any changes occurring within infected snails to be more evident by using the EM, which showed that

haemocyte of *B. alexandrina* haemocytes contained three morphologically distinct types of haemocytes, that agreed with Matricon-Gondran and Letocart (1999), who identified three subpopulations of haemocytes in haemolymph of *B. glabrata* snails based on their size and ultrastructural aspects, Kamel *et al*. (2006) who identified subpopulations of haemocytes in haemolymph of *B. alexandrina*, these three haemocyt are designated as round small hyalinocyte cells with circular shape and clear cell membrane, while granulocytes which are characterized by their moderate size with different size granules and amoebocytes are characterized by their large size, extending many pseudopodia and central clear nucleus. By optical microscopy, Martins-Souza *et al*. (2006) identified three circulating heamocyt subsets in *Biomphalaria* species. Contrary to the present study Mohamed (2011) who indicated that the *B. alexandrina* heamocyt are classified according to cell size and shape into two cell types, designated as small round hyalinocytes and granular spreading haemocytes.

In the present study, ultrastructural findings revealed definite morphological differences between the haemocytes in both haemolymph and tissue of infected and non-infected snails. The formal signs are haemocytes activation in non-infected group which are represented by extended pseudopodia and increase phagolysosomes and mitochondria in the cytoplasm, while the apoptotic features were the main signs seen in infected group which are represented by surface membrane ruffles and blebs, intense perinuclear chromatin condensation, fragmented nucleus, vaculated cytoplasm, apoptotic bodies with intact cell membrane and finally release of apoptotic body. Our results are in consistence with (Russo and Madec, 2007, Kiss, 2010; Lapied *et al,* 2011).

Also, there was difference in the type of the cells in these two groups. Hyalinocytes were the main cells observed in infected group while granulocytes and amoebocytes were the main cells in non-infected group. The results agreed with Sparks (1972) who found that the successful elimination of potential infective agents requires granulocytes to engulf particles and further eliminate living pathogens through enzymatic or oxidative degradation. However, he thought that hyalinocytes were responsible primarily for wound repair, requiring aggregation at an injury site.

Again, NO is another signaling molecule involved in the regulation of immune system through its effect on cell apoptosis. The effects of NO on apoptosis vary greatly depending upon the level of NO, the cell type, and the physiological status of the cell, and can be either pro- or anti-apoptotic. The present statistical analysis showed that the measured level of NO in haemolymph after 40 days (during cercarial shedding) post exposure to *S. mansoni* miracidia in infected snails was significantly increased in infected snail group than in non-infected group. Migration and recognition/adhesion of haemocytes to transforming miracidia/ developing sporocysts are important determinants of resistance response (Davids *et al,* 1999). Integrin-like cell surface receptors are known to regulate haemocyte adhesion and motility (Walker *et al,* 2010), and a tandem-repeat galectin has been found to bind haemocytes and the tegument of *S. mansoni* sporocysts making it a candidate anti-schistosome pattern recognition receptor (Yoshino *et al,* 2008). NO and its reactive intermediates play an important role in host defense responses against pathogens (Zahoor *et al,* 2009). Investigation of NO generation by haemocytes from schistosome-susceptible and schistosome-resistant *B. glabrata* has revealed notable strain-dependent differences in the capacities of these cells to produce NO under basal conditions, and in the presence of *S. mansoni* excretory-secretory products (ESPs). NO output appears to be regulated by the extracellular signal regulated kinase (ERK) signaling pathway, which might be important for the outcome of infec-

tion, particularly as *S. mansoni* differentially influences ERK activation in these defense cells (Zahoor *et al,* 2008).

Barcia and Ramos-Martinez (2008) found that NO production in molluscan haemocytes is elevated following exposure to parasites or pathogens, or following exposure to the cytokine, IL-2 then recovered after the first few days of *S. mansoni* infection. In our study, the combination of the high level of NO with the apoptotic picture found in infected group and the significant decrease of its level in non-infected group in the presence of activated cells could confirm the pro apoptotic effect of high level NO. Wright *et al. (*2006) stated NO as an antiparasite molecule and considered to be an ancestral defense molecule; also Brunet (2001) found that NO limits parasite development not only in vertebrate definitive and intermediate hosts, but also in invertebrate intermediate hosts.

It is worth noting that, experimental evidences of reactive oxygen species (ROS) and/or NOS participation in killing of *S. mansoni* sporocysts by *B. glabrata* haemocyt were obtained using specific oxidant scavengers or enzyme inhibitors (Conte and Ottaviani, 1995). Oxygen-dependent killing mechanisms in molluscs such as *B. glabrata* are known to play a crucial role in killing schistosome sporocysts (Hahn *et al*, 2001). It has been hypothesized previously that differences exist between schistosome-resistant and schistosome-susceptible *B. glabrata* strains in their oxygen-dependent killing mechanisms. *S. mansoni* ESPs are known to affect the physiology of haemocytes; cell motility, phagocytosis and the production of reactive oxygen metabolites which are differentially modulated in susceptible and resistant *B. glabrata* strains by *S. mansoni* ESPs (Connors *et al,* 1995). Besides, the identification of ornithin decarboxalyase (ODC) in resistant snails may also imply activation of arginine metabolic pathways, which play an important role in inflammation and wound healing (Witte and Barbul, 2003). Increased levels of ODC in resistant snails indicate production of substrate L-ornithine, inferring the depletion of L-arginine by arginase activity and subsequent inhibition of NO preventing damage to host cells.

Conclusions

Apoptosis is important for the functioning of the molluscan immune system as indicated by the apoptosis picture observed in circulating and resident haemocytes in infected group. Thus, the anti-apoptotic factors play a role in host protection against parasites by limiting the spread of the pathogen while preventing inflammatory cells damage.

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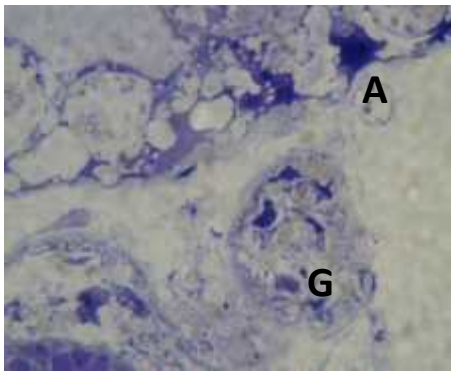
Fig 1: Semi thin section of normal control snail showing collection of granulocytes (G) and amebocytes (A) (X 40)



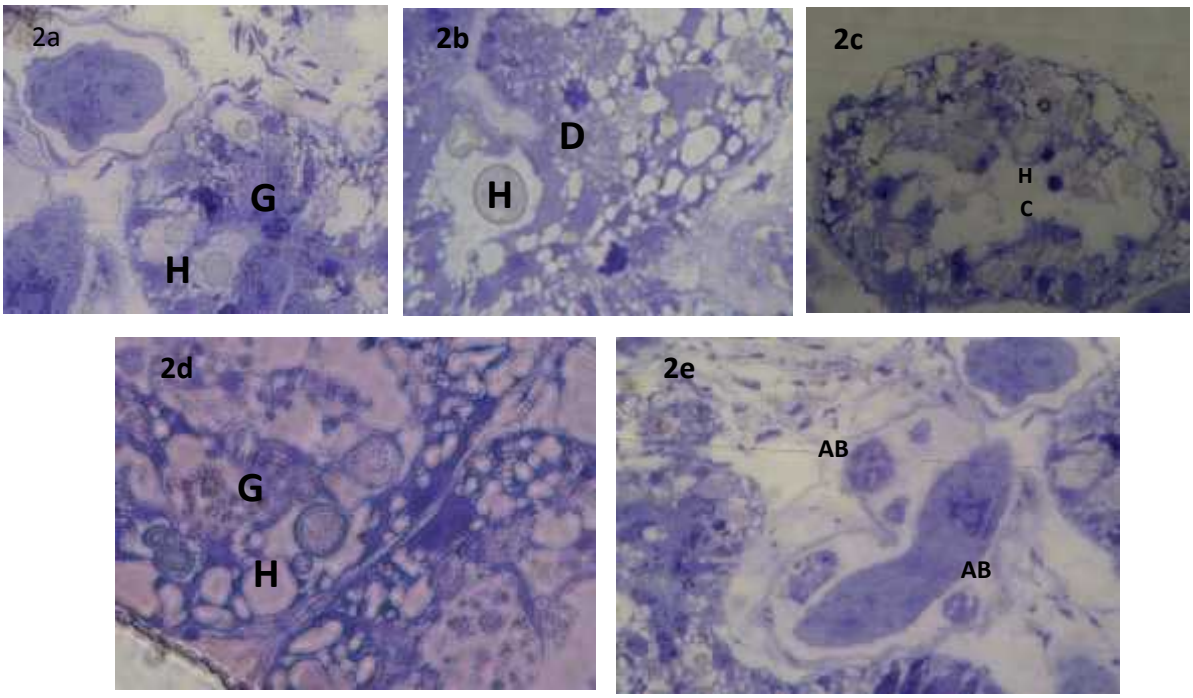


Fig 2: Semi section of infected snail tissue showing that: (2a) few granulocytes with dense nuclear chromatin condensation as an early sign of apoptosis (G) and many hyalinocytes in centre (H), amebocytes can't be seen (X 40). (2b) marked degeneration (D) and vacuolation, many hyalinocytes (H) seen (X 40). (2c) severe hypocellularity(HC) (X40). (2d) hyalinocytes (H) between granulocytes (G) and vacuolated amebocytes (X 40). (2e) many apoptotic bodies (AB) (X 40).

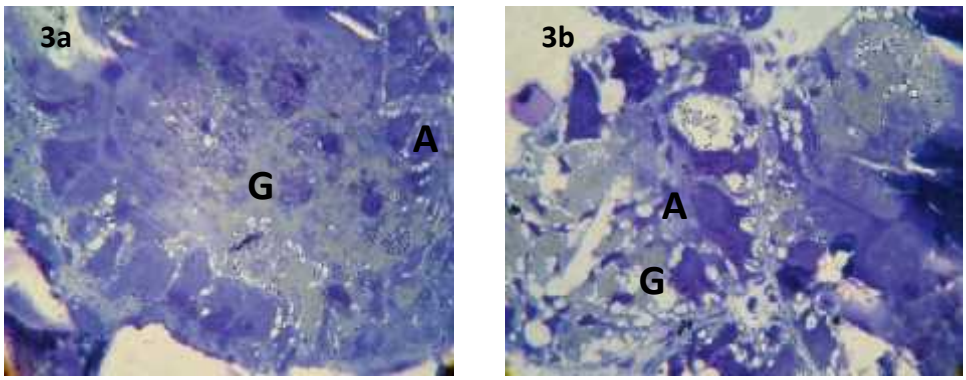


Fig. 3: Semi thin section of non-infected snail tissue showing that: (3a) Granulocytes (G) predominant cells in center and surrounded by a zone of amebocytes (A) at periphery, hyalinocytes not seen(X 40). (3b) Infiltrated amebocytes (A) to center to interact with granulocytes (G) (X40).

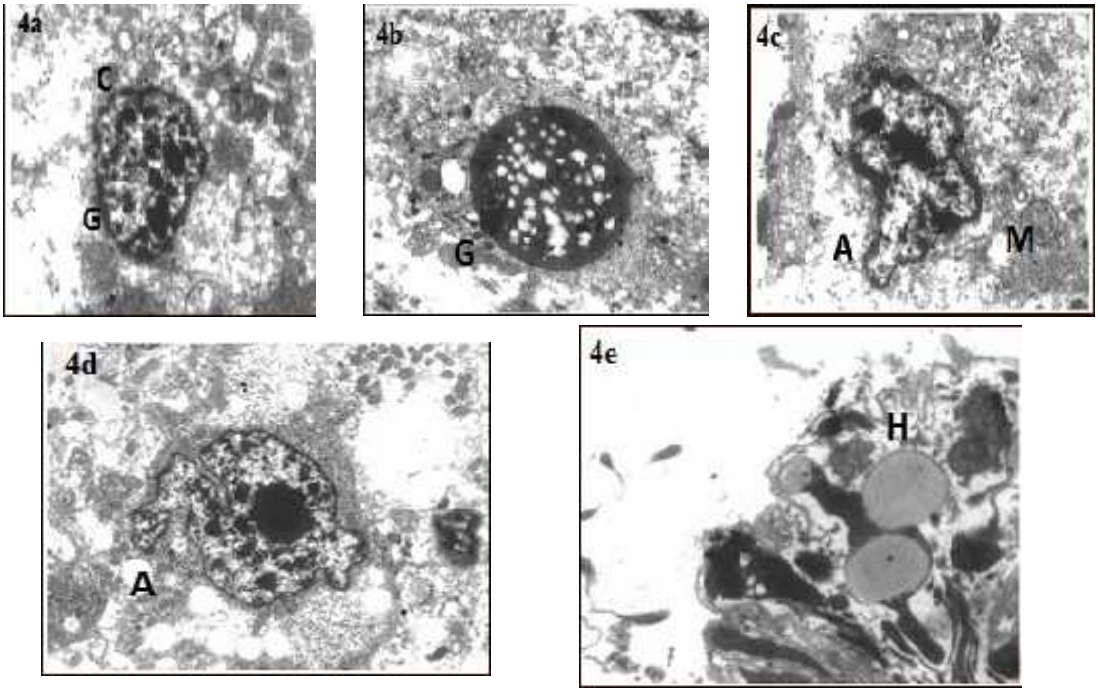


Fig. 4: Electron micrograph of control *B. alexandrina* haemocyte in haemolymph not exposed to infection showing that: (4a) Granulocyte (G) with high granular cytoplasm (C) and irregular nucleus (X15, 000). (4b) Granulocyte (G) with high granular cytoplasm and regular nucleus (X15, 000). (4c) Amoebocyte (A) with irregular nucleus and large cytoplasm containing normal mitochondria (M) and many rough endoplasmic reticulum (X15, 000). (4d) Amoebocyte (A) with irregular nucleus (X15, 000). (4e) Hyalinocyte (H) transparent and agranular with different size (X10, 000).

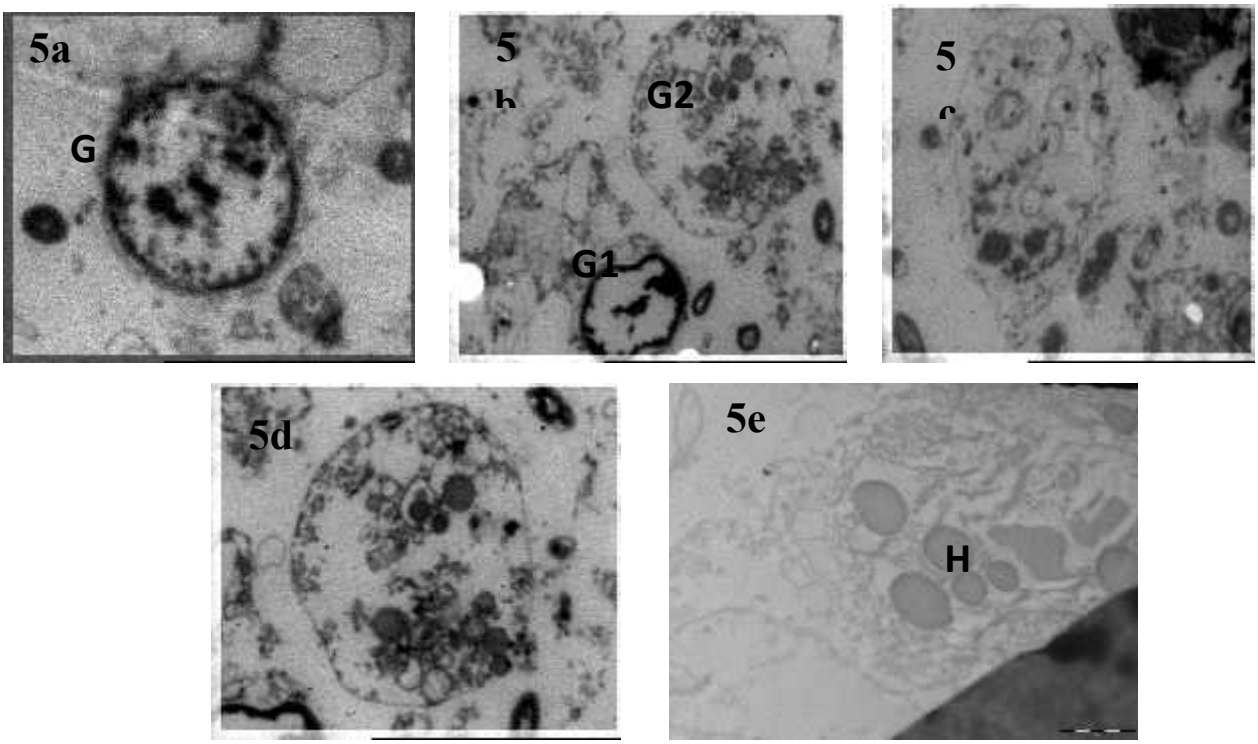


Fig. 5: Electron micrograph of infected *B. alexandrina* in haemolymph after exposure to infection showing that (5a) Granulocyte (G) in early apoptotic changes with peripheral chromatin condensation (X15, 000). (5b) Granulocyte in early apoptotic changes with peripheral chromatin condensation (G1) and other cell in late apoptotic changes represented by intracellular apoptotic bodies (G2) (X15, 000). (5c) & (5d) cell in late apoptotic change filled with apoptotic bodies (X15, 000). (5e) Increasing number of hyalino cells (H) (X10, 000).

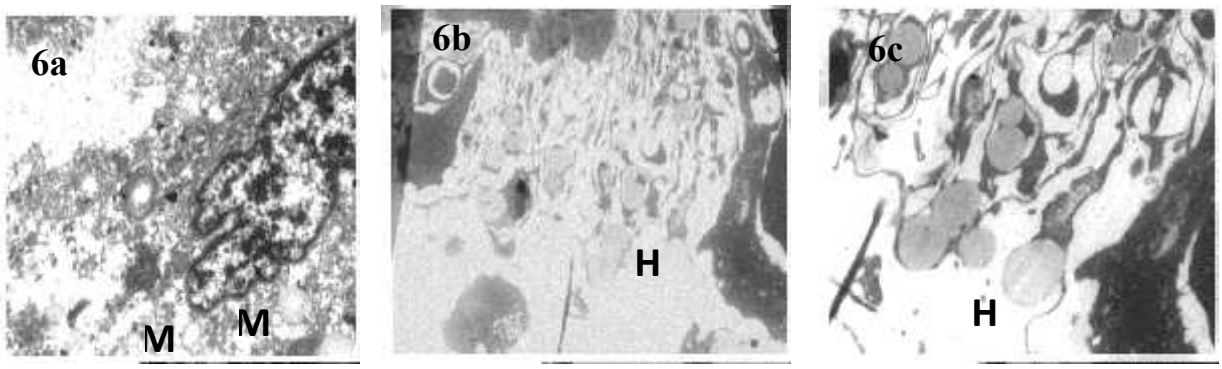


Fig. 6: Electron micrograph of infected *B. alexandrina* in haemolymph after exposure to infection showing that (6a) Amoebocyte with many degenerated mitochondria (M) (X15, 000). (6b) Many hyalino cells (H), most of them in mitosis, while, many other haemolymph cells showing apoptotic changes (X7, 000). (6c) High magnification showing the mitotic division of the hyaline cells (H) (X10, 000).

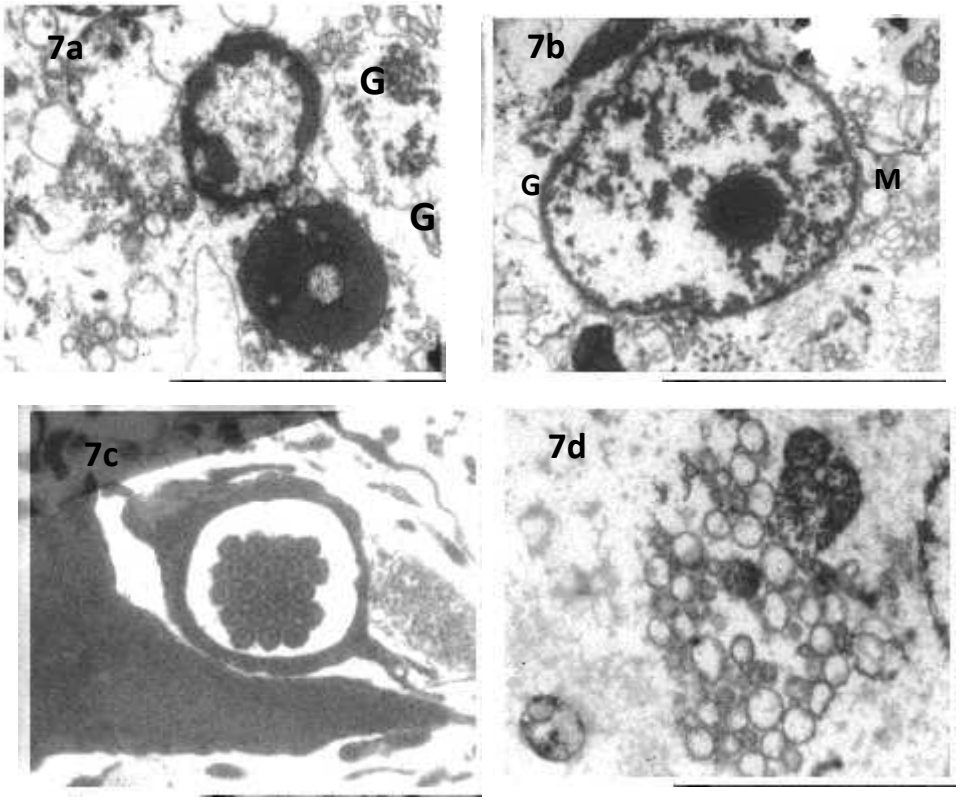


Fig. 7: Electron micrograph of infected *B. alexandrina* in tissue after exposure to infection showing that (7a) Two granulocytes (G) in early apoptotic changes with peripheral chromatin condensation (X10, 000). (7b) Granulocyte (G) with many degenerated mitochondria (M) (X15, 000). (7c) Late apoptotic changes represented by apoptotic bodies in its way to be released from the cell (X15, 000). (7d) Late apoptotic changes represented by the extra cellular release of apoptotic bodies (X15, 000).

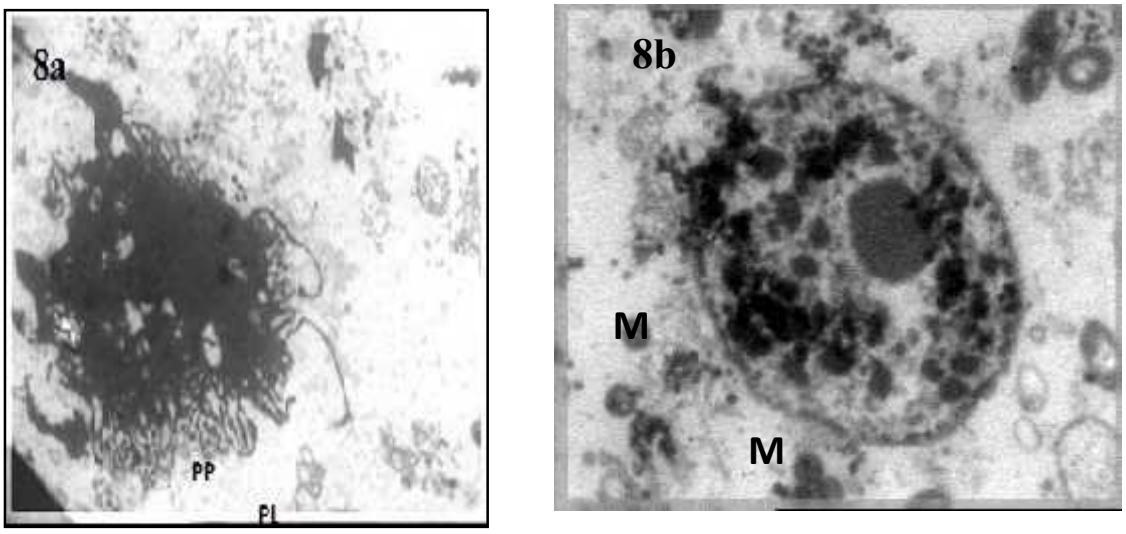


Fig. 8: Electron micrograph of non-infected *B. alexandrina* in haemolymph after exposure to infection showing that activated granulocytes with: (8a) numerous extending pseudopodia (PP) and many phagolysosomes (PL) (X15, 000). (8b) numerous mitochondria (M) in cytoplasm (X15, 000)

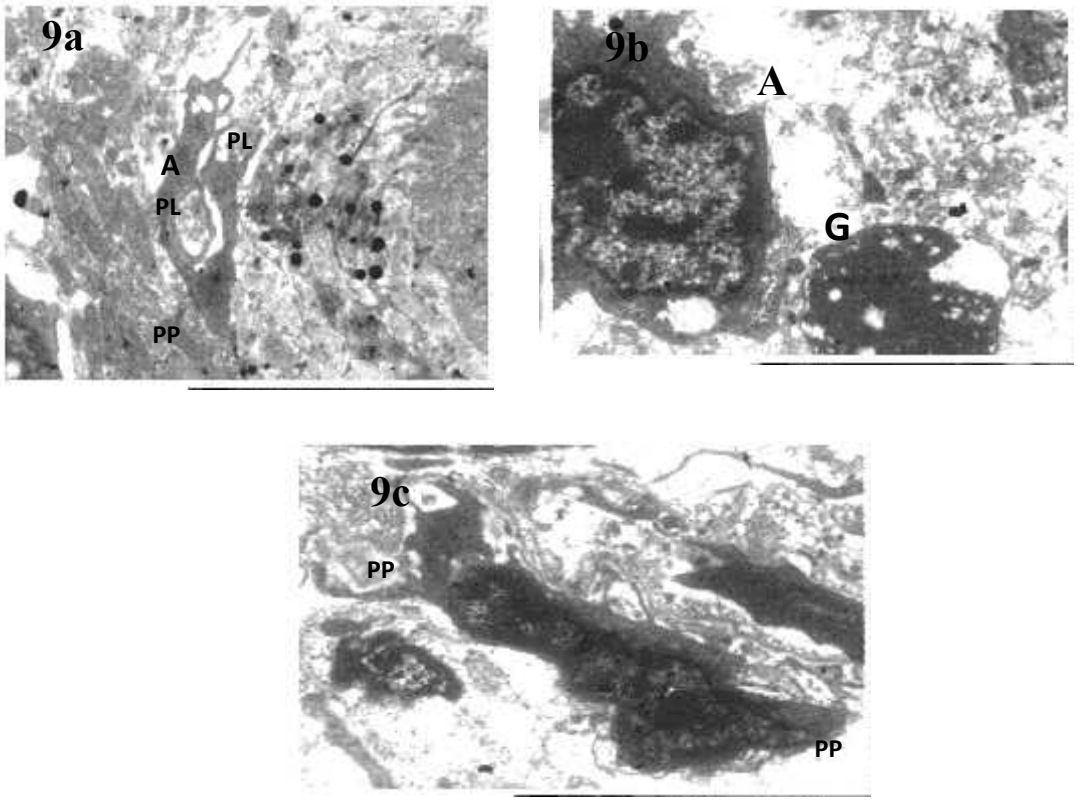


Fig. 9: Electron micrograph of non-infected snail *B. alexandrina* in tissue after exposure to infection showing that (9a) Activated amoebocyte (A) with extending pseudopodia (PP) and two large phagolysosomes (PL) (X10, 000). (9b) Interaction between activated amoebocyte (A) and granulocyte (G) (X15, 000). (9c) Many extending pseudopodia (PP) in its way forming phagolysosomes (X15, 000)