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Ultrastructure changes in the haemocytes of *Galleria mellonella* larvae treated with gamma irradiated *Steinernema carpocapsae* BA2

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ABSTRACT

The ultrastructure studies on the haemolymph of 5th larval instar of *Galleria mellonella* showed five types of haemocytes; Prohaemocytes, Plasmatocytes, Granulocytes, Oenocytoids and Spherulocytes. After treatment with *Steinernema carpocapsae* BA2, the haemocytes underwent considerable structural changes. More destructive effects were observed in the haemocytes of *G. mellonella* treated with gamma irradiated *S. carpocapsae*.

1. Introduction

Entomopathogenic nematodes (*Heterorhabditidae* and *Steinernematidae*) are considered environmentally safe and IPM compatible alternative to chemical insecticides for the control of pests. Entomopathogenic nematode from the genus *Steinernematidae* and *Heterorhabditidae* was characterized by a symbiotic association with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively. The bacteria are contained in the intestine of the free-living infective juveniles (IJS) of these nematodes (Ansari, Tirry, & Moens, 2003).

Abbreviations: De, desmosomes; Dg, dense granules; DN, distorted nucleus; G, Golgi complex; Lm, lysed membrane; M, mitochondria; N, nucleus; Nu, nuclei; Ps, pseudopodia; Rer, Rough endoplasmic reticulum; Sg, structured granules; V, vacuoles.

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The IJS enter the haemocoel of the insect and release the symbiotic bacteria (Kaya & Gaugler, 1993) that multiply in the haemolymph causing insect death within 48–72 h which and establishing conditions for nematode development in the cadaver by providing nutrients (Nickle & Welch, 1984).

The immediate response against nematodes is encapsulation and against bacteria is phagosytosis, or nodulation in the case of a large load Duphny and Bourchier (1992).

Since the hemolymph is the main site of action, hematological studies are important in the field of insect physiology because certain vital activities are performed by haemocytes.

Generally, the present work is primarily concerned to show the biological effect of gamma irradiated *Steinernema carpocapsae* BA2 on *Galleria mellonella*, In addition the changes in the ultrastructure of the haemocytes.

2. Materials and methods

2.1. Insect

The greater wax moth, *Galleria mellonella* larvae were obtained from the infested hives and reared in the laboratory at 28 ± 2 °C and a relative humidity of 65 ± 5% as described by Hussein (2004, 203 pp.).

2.2. The entomopathogenic nematode

Steinernema carpocapsae BA2 was originally obtained from the National Research Center (NRC), Pests & plant Protection Department and reared *in vivo* according to Glazer and Lewis (2000).

2.3. Irradiation technique

Irradiation *S. carpocapsae* BA2 was carried out using Gamma Cell Irradiation Unit (caesium, Cs^{137} source) located in the National Center for Radiation Research and Technology (NCRRT). The dose rate was 0.83084 Rad/s. In the present study, all results were calculated as a Gray unit (Gy); where Gy = 100 rad.

2.4. Bioassay experiments

2.4.1. Ultrastructure studies of the haemocytes

For EM studies, haemolymph was diluted (1:1) with a cold physiological saline buffer containing 6% (v/v) glutaraldehyde and chilled for 30 min (Horohov & Dunn, 1982). The fixed cells were centrifuged (6500 rpm, 2 min), the pellet suspended in 0.1 M cacodylate buffer, pH 6.5, containing 2% osmium tetroxide and the mixture incubated for 2 h at 4–8 °C. The post-fixed cells were washed with distilled water and stained overnight with 0.5% (w/v) uranyl acetate, dehydrated, and embedded in Epon 812 (Luft, 1961). Ultrathin sections were cut using, stained with lead citrate (Reynolds, 1963) for 15 min and examined using Transmission Electron Microscope.

The haemocytes were identified according to Ribeiro and Brehélin (2006) and Neuwirth (2005).

3. Results

3.1. Normal haemocytes

In the present study, five types of haemocytes were identified in 5th larval instar of *G. mellonella*; Prohaemocytes, Plasmatocytes, Granulocytes, Oenocytoids and Spherulocytes (Fig. 1).

Prohaemocytes were small rounded cell with variable sizes. Plasma membrane was generally smooth, and the nucleus (N) was large, centrally located, almost filling up the whole cell. The cytoplasm was basophilic with scattered chromatin and evident nucleoli (Nu) showing a large amount of free ribosome, but only small rough endoplasmic reticulum (Rer) cisternae. Few mitochondria (M) and rare Golgi complex (G) were observed.

Plasmatocytes were oval and variable in size. The elongate or lobate nucleus exhibit variable sizes and centrally localized, showing scattered chromatin masses and up to two nucleoli. The cytoplasm showed well-developed Rer, Golgi system, mitochondria and vacuoles (V).

Granulocytes were the most frequently observed cell type in larvae and were spherical cells. The nucleus was round, centrally located, with scattered chromatin masses and nucleolus. Two types of membrane bound granules were observed: dense granules (Dg), containing electron-dense and homogenous content, and structured granules (Sg), with crystalloid content. Vacuoles of variable sizes and shapes were also present. The developed Rer, the Golgi apparatus, mitochondria and glycogen particles were dispersed in the cytoplasm.

Oenocytoids were rounded cells. The cellular membrane was smooth. The nucleus was small, eccentric, and showed a distribution pattern with alternate condensed and discondensed chromatin. The cytoplasm was homogenous with rounded structured granules (low electron-dense). Dense mitochondria, generally ring-shaped was observed.

Spherulocytes were characterized by their inclusions and membrane-bound spherules took up almost all the cytoplasm. The cellular surface was homogenous but exhibits cytoplasmic protrusion corresponding to the spherules. The nucleus was small, eccentric, mostly deformed by the spherules. The spherules contained moderate electron-dense and flocculent material, with a quite electron-dense core region. Besides the spherules, the cytoplasm contained few organelles around the nucleus, such as Rer and few mitochondria.

3.2. Haemocytes ultrastructure after infection with S. carpocapsae

The infection of *G. mellonella* with *S. carpocapsae* induced several pathological detritions.

During infection, Oenocytoids and Spherulocytes vanished from the haemolymph, and the other haemocytes underwent considerable structural changes.

After 12 h. of the infection with *S. carpocapsae*:

- The prohaemocytes were enlarged or took elongate shape (Fig. 2A).

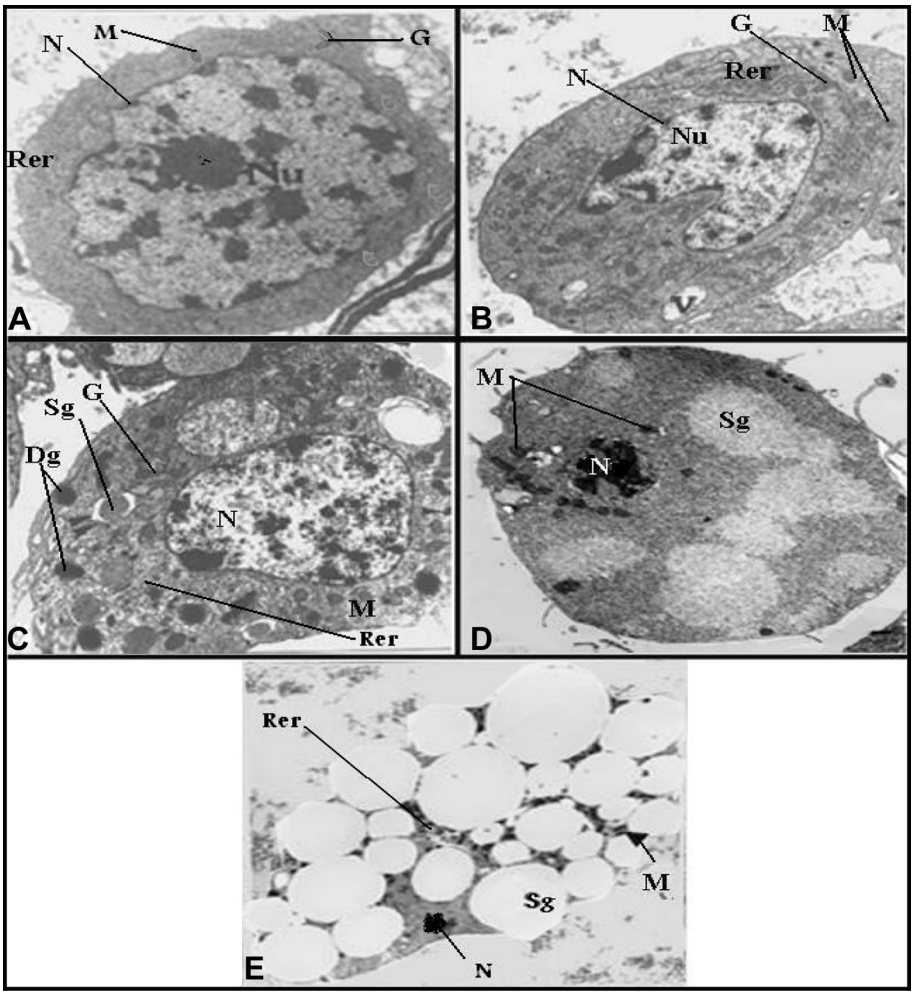

Fig. 1 – Ultrastructure of normal haemocytes of 5th larval instar of *G. mellonella* (TEM mag. = 12 Kx, bar: 2 nm). (A): Prohaemocytes; (B): Plasmatocytes; (C): Granulocytes; (D): Oenocytoids; (E): Spherulocytes.

- The cell membrane forming thin pseudopodia (Ps) (Fig. 2A and B).
- The contents of the cytoplasm seemed to swell giving the cells an extremely vacuolated (V) appearance (Fig. 2A and B).

Similar observations were reported under the infection with irradiated *S. carpocapsae* in addition to:

- Haemocytes that have phagocytized the bacteria (*Xenorhabdus nematophila*) tend to adhere to one another to contact, and to form aggregations (Fig. 2C and D). These unstructured aggregations may later be encapsulated by other haemocytes, or by cells that may be released from the aggregations.
- Plasmatocytes synthesized numerous desmosomes (De) and contained large amounts of microtubule in order to form capsule and nodule (Fig. 2E).
- Granular haemocytes released their granular content to come into contact with a foreign body at the beginning of capsule/nodule formation. When in contact with the foreign body (Fig. 2F).

In the late stage of *S. carpocapsae* infection (After 18 h.); the infected haemocyte showed enlarged cytoplasmic granules. Also, the cell membrane was completely lysed (Lm) and nucleus was severely distorted (DN) (Fig. 3A–C).

More destructive effects were observed in the haemocytes of *G. mellonella* infected with irradiated *S. carpocapsae* (Fig. 3D–F).

4. Discussion

Entomopathogenic nematodes (EPN) are a ubiquitous group of obligate and lethal parasites of insects. They are widely used as biological control agents of many insect pests (Kaya & Gaugler, 1993).

In this study, the results revealed that after exposure of *S. carpocapsae* to 2 Gy, the pathogenicity increased (for 1 week of irradiation); showing reduction in time needed to give 100% mortality of *G. mellonella*, *Corcyra cephalonica* and *Ephestia kuehniella* larvae. This result coincides with Yussef (2006) who stated that regarding the susceptibility of *Callosobruchus maculatus* to *S. carpocapsae* and gamma irradiation, the lowest

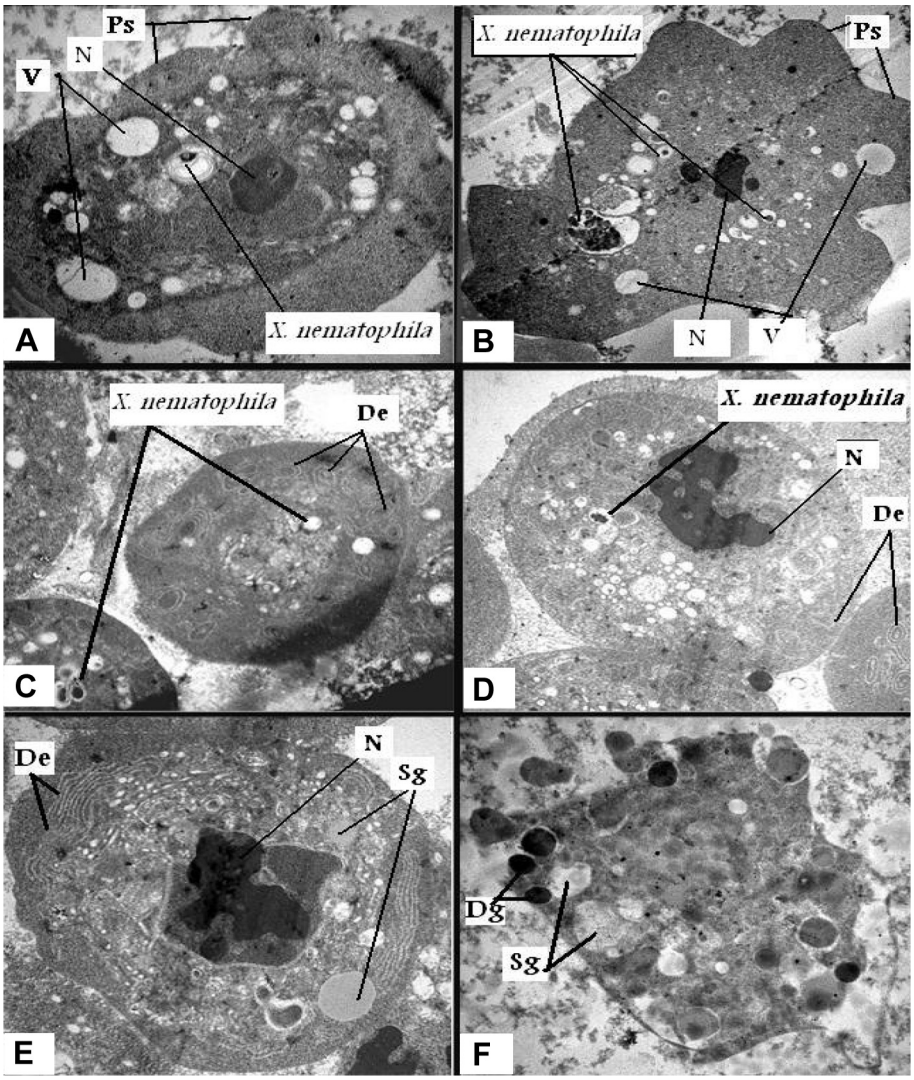

Fig. 2 – Ultrastructure of 5th larval instar of *G. mellonella* haemocytes after 12 h. infection with *S. carpocapsae* (TEM mag. = 12 Kx, bar = A, E and F = 500 nm. B, C and D = 2 microns).

doses (2.5, 5 and 10 Gy) were more effective than the higher ones. The reason of increasing the pathogenicity may be attributed to the effect of low doses of gamma radiation in increasing the *X. nematophila* toxins.

In the present study, six types of haemocytes were identified in 5th larval instar of *G. mellonella*; Prohaemocytes, Plasmatocytes, Granulocytes, Oenocytoids and Spherulocytes.

The prohaemocytes are specialized for division plasmatocytes are specialized for phagocytosis. Granulocytes, spherulocytes and oenocytoid are specialized for secretions and storage; and coagulocytes, specialized for clotting (Brehélin & Zachary, 1986). There is an inherent variability of haemocytes within species depending on the developmental and physiological stages (Beetz, Holthusen, Koolman, & Trenczek, 2008; Sanjayan, Ravikumar, & Albert, 1996).

Most researches agree that plasmatocytes form a bulk of capsules around foreign bodies too large to be phagocytosed, or nodules around masses of bacteria and necrotic melanised material, *in vivo*. Capsule and nodule formations look identical at the cytological level (Lavine & Strand, 2002; Ratcliffe & Gagen, 1977). In these formations, plasmatocytes synthesize numerous desmosomes and contain large amounts of microtubules in their cytoplasm (Götz, 1986). The role of plasmatocytes in phagocytosis is disputed.

Granular haemocytes have also been shown to be the first cells to come into contact, in small numbers, with a foreign body at the beginning of capsule/nodule formation. When in contact with the foreign body, they release their granular content (Ratcliffe & Gagen, 1977; Schmit & Ratcliffe, 1977). According to most authors, this exocytosis of typical inclusions by granular haemocytes serves to attract plasmatocytes (Gillespie, Kanost, & Trenczek, 1997) or at least helps plasmatocytes to build the capsule or nodule (Pech & Strand, 1996). This exocytosis of opsonin-like material is another main function of granular haemocytes.

The infection of *G. mellonella* with *S. carpocapsae* induced several pathological detritions. During infection, the haemocytes undergo considerable structural changes. The contents of the granules seem to swell giving the cells an extremely vacuolated appearance. Haemocytes that have phagocytized

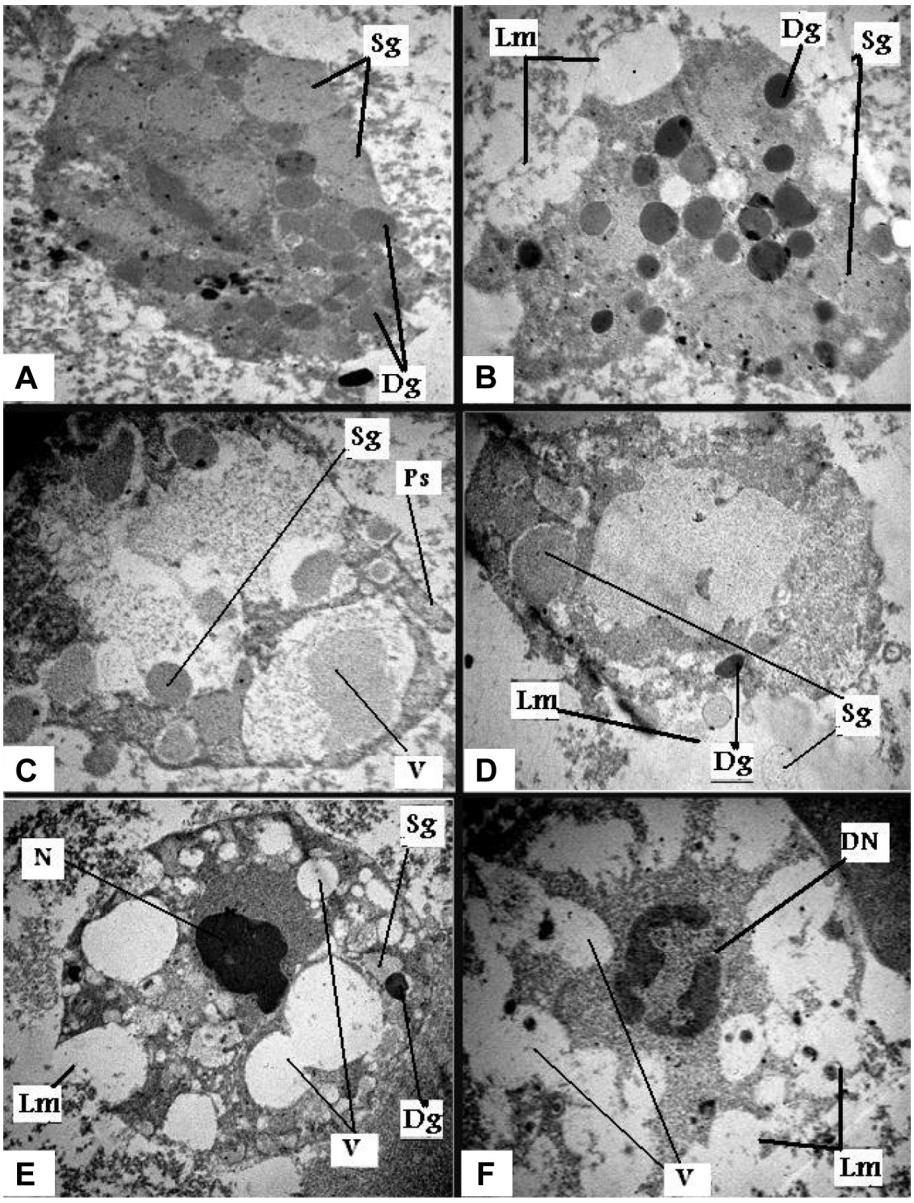

Fig. 3 – Ultrastructure of 5th larval instar of *G. mellonella* haemocytes after 18 h. infection with *S. carpocapsae* (TEM mag. = 12 Kx, bar: A and E = 2 microns. B, C, D and F = 500 nm).

bacteria and/or other foreign particles tend to adhere to one another to contact, and to form aggregations. These unstructured aggregations may later be encapsulated by other haemocytes, or by cells that may be released from the aggregations.

The late stage of bacterial infection showed infected haemocyte with enlarged cytoplasmic granules having premelanosome-like structure in granulocyte. Also, cell membrane is completely lysed and nucleus is severely distorted. Phagocytosing haemocytes contained intracellular *X. nematophila* and attached bacteria were also observed.

Similar observations were reported by Brayner et al. (2007) who found that GRs, PLs and OEs presented morphological alterations indicative of innate immunological activation in mosquitoes infected with *Wuchereria bancrofti*. Similarly, Faraldo, Gregório, and Lello (2008) reported that at 24-h post-injection of *Saccharomyces cerevisae* yeast cells to *Chrysomya megacephala* cell debris and some free yeast cells were surrounded by granules and electron dense PLs which were probably initiating the nodulation process. Abd El-Aziz and Awad (2010) reported that ultrastructural alterations and malformations have been observed in circulating haemocytes of *Agrotis ipsilon* larvae treated with *Bacillus thuringiensis*.

Also, Parka, Yonghwa, and Yonggyun (2005) reported that morphological changes of the haemocytes after the bacterial infection similar to cell changes during apoptosis (hemolymph septicemia due to the induction of the programmed cell death). At 4–8 h post-infection, the cell membrane blebbing and apoptotic vesicles were observed and the nuclear membrane was broken apart. At 12 h. post-infection, the

overall cell shape was lost externally. Also, vacuolation of the endoplasmic reticulum, cell swelling, and cell death by colloid-osmotic lyses. This leads to pores created by toxin on macrophage and blood cell plasma membrane increases with toxin concentration, which leads to a rapid cell lyses (Ribeiro, Vignes, & Brehélin, 2003).

The reason of haemocytes vacuolations was explained by Ribeiro et al. (2003) who reported that *X. nematophila* exhibits different cytotoxic activities on insect (*Spodoptera littoralis*) haemocytes. They purified a cytotoxin and called it (α_Xenorhabdolysin, αX). Also, they showed that plasma membrane of insect haemocytes was the first target of this toxin. Electrophysiological and pharmacological approaches indicate that the initial effect of αX on macrophage plasma membrane is an increase of monovalent cation permeability, sensitive to potassium channel blockers. As a consequence, several events can occur intracellularly, such as selective vacuolation of the endoplasmic reticulum, cell swelling, and cell death by colloid-osmotic lysis.

5. Conclusion

In general, it may be concluded that gamma irradiated (2Gy) of *S. carpocapsae* may be attributed as a major control method of *G. mellonella*. Also, it appears that infection of *G. mellonella* with B gamma irradiated of *S. carpocapsae* is characterized by specific haemocyte composition to meet the immune response needs. In other words, it can be stated that the results presented above are of interest to describe a reaction which may be of importance in the cellular immune response of insects to foreign substances.

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