Hemocytic studies on the synergistic effect of the entomopathogenic nematode species, *Steinernema carpocapsae* and gamma radiation on the greater wax moth, *Galleria mellonella* (L.) larvae

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**Abstract**

Combined effect of substerilizing doses of gamma radiation (40 and 100 Gy) and different concentrations of the entomopathogenic nematode, *Steinernema carpocapsae* BA2 (20 and 40 IJs/ml) on the hemocyte count of the greater wax moth, *Galleria mellonella* (L.) larvae was studied. Eight types of hemocytes were described in the hemolymph of the normal larvae of *G. mellonella*. Prohemocytes were the predominant type, while the cystocytes were the rare ones. Morphological malformations and changes in the number of each hemocyte type were observed in F1 larvae (of irradiated male parent pupae with 40 or 100 Gy) or larvae treated with different concentrations of the *S. carpocapsae* BA2 (normal or F1 larvae); these alterations were increased by increasing the radiation dose or the nematode concentration that led to increase the susceptibility of the larvae to the nematode. Therefore, it could be concluded that integration of entomopathogenic nematodes and gamma radiation may serve as integrated control program for *G. mellonella*.

**Keywords:** *Steinernema carpocapsae*, Gamma radiation, *Galleria mellonella*, Hemocytes

**Background**

Entomopathogenic nematodes (EPNs) are a welcome addition to the natural-enemy arsenal. They are generally specific on insects, not environmentally hazardous, compatible with other biological and chemical agents, can seek pests in cryptic habitats, and can be commercially produced in large quantities (Gaugler and Kaya 1990, Laznik et al. 2012, Laznik and Trdan 2017).

Many non-classic control methods are currently used, such as physical and/or biological control of pests in field and store. The use of irradiation technique as a physical control method is cheaper, safer, and more reliable than chemical methods. Parasitism by nematodes has variable deleterious effects on their insect hosts including the following: sterility, reduced fecundity, longevity, flight activity, delayed development, and other behavioral, physiological, and morphological changes (Gaugler 2002).

Combining EPNs with gamma radiation has resulted in successful control strategies (Salem et al. 2008; Sayed 2008, 2011; and Sayed et al. 2015). The hemocytes of insects have been reported to play a role in cellular defense against foreign materials by phagocytosis or encapsulation; they may be concerned in the formation of connective tissue and activation of prothoracic gland before molting (Charles 1971).
The high interest in biological means of controlling insects intensifies the need for investigating the response of insects to diseased organisms. Much work has been done on changes in the hemocytes picture, following injury, and hemorrhage in insects attacked by parasites (Salt 1970).

The greater wax moth, *Galleria mellonella* (L.) larvae, were recorded to be a good model of organisms for studying host response to pathogens due to their rapid growth, high fertility, size, and short life cycle (Mukherjee et al. 2010).

The aim of this work was to investigate the immune response of *G. mellonella* larvae to gamma radiation and the EPNs by estimating the differential hemocyte counts in larvae.

### Materials and methods

#### Insect and entomopathogenic nematodes

*Galleria mellonella* (L.) larvae were obtained from the infested hives and reared in the laboratory at 28 ± 2 °C and 65 ± 5% RH% as described by Glazer and Lewis (2000). *Steinernema carpocapsae* BA2 was originally obtained from the National Research Center (NRC), Pests & plant Protection Department, Giza, Egypt.

#### Irradiation technique and bioassay experiments

##### Irradiation technique

Full-grown pupae were irradiated by 40 and 100 Gy, using the Gamma cell irradiation unit (*60*Co source), located at the National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt. The dose rate was 6.6 kGy/h.

##### Bioassay experiments

Normal 4th instar larvae and the larvae resulted from irradiated male parent pupae at doses 40 and 100 Gy (F1

### Table 1

Total hemocyte count/mm³ of blood from *Galleria mellonella* larvae treated with gamma radiation alone or combined with the entomopathogenic nematode, *Steinernema carpocapsae* BA2

| Treatment | Radiation doses (Gy) | THC/mm³ × 10³ | *S. carpocapsae* BA2 |
|-----------|----------------------|---------------|---------------------|
|           | Gamma rays           |               |                     |
| Control   | 20 IJ                 | 32.08 ± 0.54ᵃ  | 13.13 ± 0.34ᵃ       | 10.91 ± 0.75ᵃ       |
| 40        | 21.75 ± 0.22ᵇ        | 10.4 ± 0.32ᵇ  | 9.2 ± 0.53ᵇ         |
| 100       | 20.56 ± 0.29ᵇ        | 8.73 ± 0.21ᶜ  | 5.61 ± 0.2ᶜ         |

Letters indicate the variance between the means (Duncan’s multiple range test). Values represent the mean ± S.E. of 3 replicates for each group.
larvae) were treated by the nematode at 2 concentrations: 20 and 40 IJs/ml. Untreated larvae were used as control. Treated and control larvae were incubated at 25 ± 1 °C.

Preparation of blood film
To determine the differential hemocytes and the pathological changes resulted from radiation and nematode treatments, hemolymph smears were taken from 4th instar larvae. A drop of blood was spread as a thin film by the aid of a cover slip, air dried, fixed in absolute methanol for 5 min, stained in Giemsa stain for 35 min and the stain was flushed off by distilled water (Abdel-Rahman 1978).

Counting method
The proleg on the abdominal segment was cut by a fine pair of scissors, and blood was allowed to ooze on a clean, grease free, glass microscopic slide. The hemolymph was quickly drawn up to the 0.5 mark in a Thoma white blood cell dilution pipette and immediately diluted to 11 mark with Turek solution (24%) glacial acetic acid, slightly colored with gentian violet. The first two or three drops of this solution were discarded; the hemocytes were counted at the 4 corners and multiplied by a factor of 50 to get the number of cells per cubic millimeter. If the cells were clumped or unevenly distributed in the chamber, the preparation was discarded, as described by Rizk (1991).

Statistical analysis
The data were statistically analyzed by analysis of variance (F), followed by Duncan’s multiple range test to examine the significant differences between treatments. The 5% level of probability was used in all statistical tests, using COSTAT computer program.

Results and discussion
Total hemocyte count (THC)
Total number of hemocytes of G. mellonella larvae decreased gradually by increasing the dose of radiation applied to parent male pupae as compared to the control (Table 1). After nematode treatments (20 and 40 IJs/ml), the total hemocyte counts decreased significantly and more decreases occurred when they applied to F1 larvae of irradiated parent male pupae (40 and 100 Gy).

The present study showed that increasing parental radiation dose or treatment of F1 larvae with S. carpocapsae BA2 significantly decreased the THC of 4th instar larvae or F1 of G. mellonella larvae. These investigations

Fig. 2 Morphological changes in prohemocytes (x = 1600). a Normal: small, round to slightly ovoid prohemocyte cell. b 40 Gy, c 100 Gy, d BA2 40 IJs/ml, e 40 Gy + BA2 20 IJs/ml, f 100 Gy + BA2 20 IJs/ml, g 40 Gy + BA2 40 IJs/ml, h 100 Gy + BA2 20 IJs/ml, and i 100 Gy + BA2 40 IJs/ml: destruction of the cell membrane and presence of vacuoles (arrow)
Fig. 3 Abnormalities in plasmatocytes ($x = 1600$). a Normal: round with central nucleus plasmatocytes. b 40 Gy, c 100 Gy, and d BA2 20 IJs/ml: vacuolation of cytoplasm (arrow). e BA2 40 IJs/ml, f 40 Gy + BA2 20 IJs/ml, g 40 Gy + BA2 40 IJs/ml, h 100 Gy + BA2 20 IJs/ml, and i 100 Gy + BA2 40 IJs/ml: the cell takes an oval shape with acentric nucleus (arrow). nu indicates the hemocyte nucleus.

Fig. 4 Morphological changes in granulated cells ($x = 1600$). a Normal granulocytes: slightly round, with undifferentiated cytoplasm and nucleus granulocyte. b 40 Gy c 100 Gy and d BA2 20 IJs/ml and e BA2 40 IJs/ml treatments showing granulocyte in oval shape with cytoplasm vacuolation.
were in agreement with those obtained by Souka et al. (1993), Ayaad et al. (2001), Sayed (2008), and El-Sonbaty et al. (2016).

**Galleria mellonella** hemocyte type

Hemocytes were distinguished based on their morphological characteristics and staining affinity. Eight types were found in 4th instar larvae of *G. mellonella* (Fig. 1): (a) prohemocytes: were small, round to slightly ovoid cells, with undifferentiated cytoplasm. The single, round nucleus with dense, homogenous chromatin is usually enlarged in size leading to formation of macronucleus, which in turn sparing the cellular cytoplasm; (b) plasmatocytes: characterized by their round shape. The nucleus with dark stains, round with punctuate, or granular chromatin and usually central in position; (c) granulated cells: were slightly round, characterized by the undifferentiated cytoplasm and nucleus; (d) spindle cells: spindle-shaped cells distinguished by the presence of very large, distinct, usually spindle nucleus (nu), mostly

![Fig. 5 Morphological changes in spindle cells (x = 1600). a Normal: spindle shaped cells with spindle nucleus (arrow). b 40 Gy, c 100 Gy, d BA2 20 l/s/ml, e BA2 40 l/s/ml, and f 40 Gy + BA2 20 l/s/ml: the spindle cell takes irregular shape approximately round nucleus (arrow). nu indicates the hemocyte nucleus.](image)

![Fig. 6 Morphological changes in spherule cells (x = 1600). a Normal cells: round with undistinguished nucleus b 40 Gy and c 100 Gy: increased the cell size. d BA2 20 l/s/ml induced cytoplasmic vacuolation (arrow) and e BA2 40 l/s/ml: irregular shape of the sphere cell. f 40 Gy + BA2 20 l/s/ml and g 40 Gy + BA2 40 l/s/ml: increased the cell size with cytoplasm vacuolation (arrow).](image)
appearing as a solid purple structure; (e) spherule cells: were round with undistinguished nucleus. After Giemsa stain, they appeared in dark pink color; (f) adipohemocytes: were rounds containing variable amounts of refringent fat droplets and several other non-lipid inclusions; (g) oenocytoids: were slightly round characterized by round, large, and approximately centric nucleus with homogenous chromatin. The cytoplasm contained numerous granular inclusions and filled with intricated canaliculi; and (h) cystocytes: were extremely fragile cells with a single, small, irregular nucleus in envelope containing distinct, round, acidophilic inclusions.

**Morphological effects of gamma radiation and/or EPNs on Galleria mellonella hemocytes**

1- Prohemocytes: prohemocytes from F1 larvae (resulted from irradiated male pupae mated with normal female) and F1 larvae treated with S. carpocapsae exposed elongation in the shape, destruction of the cell membrane, and evidence of vacuoles (Fig. 2).

2- Plasmatocytes: morphological changes were observed in plasmatocytes in F1 larvae after different treatments. Generally, radiation with 40 Gy and 100 Gy to the parent male pupae caused the cell to be enlarged or took slightly oval shape. The nucleus lost its central position and moved to the cell wall (Fig. 3 b and c). *S. carpocapsae* affects plasmatocytes in the same way of gamma radiation inducing vacuolation of cytoplasm. The combined effect of nematodes and radiation would be more destructive to cells than using each of them only as the cells become irregular in shape (Fig. 3).

3- Granulated cells: gamma irradiation (40 and 100 Gy) of parent pupae significantly increased the size of granulated cells in the hemolymph of F1 *G. mellonella* larvae. Granulated cells enlarged or may take oval shape as a result of *S. carpocapsae* treatment alone (Fig. 4d, e). The combined effect of gamma radiation and *S. carpocapsae* (20 and 40 IJs/ml) led to disintegration and disappearance of granulated cells.

4- Spindle cells: the nucleus of the spindle cells became slightly round (Fig. 5 b and c). Moreover, they showed notable changes in their shape, when *S. carpocapsae* BA2 were applied alone to *G. mellonella* larvae. The combined effect of gamma radiation and *S. carpocapsae* led to disappearance of the spindle cells, except the combination of 40 Gy and 20 IJs/ml of *S. carpocapsae*, the spindle cells became compressed in size with approximately round nucleus (Fig. 5f).

5- Spherule cells: irradiation of parent pupae, *S. carpocapsae*, and their combination (40 Gy with 20 and 40 IJs/ml) affected the shape of the spherule cells of the larvae. The changes were enlargement, losing the round shape and appearance of vacuoles in the cell. The other combinations of gamma radiation (100 Gy) and EPN (20 and 40 IJs/ml) resulted in disappearance of the spherule cells (Fig. 6).

6- Adipohemocytes: gamma radiation of pupae with 40 and 100 Gy and *S. carpocapsae* (20 IJs/ml) treatments increased the size of adipohemocytes in larvae (Fig. 7). Adipohemocytes vanished from the hemolymph of *G. mellonella* larvae under *S. carpocapsae* BA2 (40 IJs/ml) and under the combined effect of gamma radiation (40 and 100 Gy) and EPN (20 and 40 IJs/ml).

7- Oenocytoids: exposure of *G. mellonella* pupae to 40 Gy induced enlargement of the cell size in the hemolymph of F1 larvae. When the pupae exposed to 100
Gy, oenocytoid cell enlarged and the cytoplasm degranulated (Fig. 8). The treatments with S. carpocapsae alone or in F1 G. mellonella larvae lysed oenocytoids.

8- Cystocytes: treatment with gamma radiation to parent pupae with 40 and 100 Gy or larvae treatment S. carpocapsae BA2 caused completely disappearance of cystocytes from the hemolymph of G. mellonella larvae.

In case of nematode’s treatments, a hemocyte’s response appeared as immunocompetent cells. This response was by phagocytosis (Fig. 9 a and b) or by encapsulation of foreign body (Fig. 9 e and f) or by increasing the mitotic division (Fig. 9 c and d).

The observed pathological conditions in the infected hemocytes are characterized as follows: (1) enlargement or elongation of the cell, (2) vacuolization and degeneration of the cytoplasm, and (3) lyses of the cell membrane. The changes were a response of the actions of gamma radiation and the EPNs. Similar results were previously indicated by Rizk (1991), Salem et al. (2014), and El-Sonbaty et al. (2016). The reason of hemocyte vacuolations by the EPN was explained by Ribeiro et al. (2003) who reported intracellularly hemocyte changes, such as selective vacuolation of the endoplasmic reticulum, cell swelling, and cell death by colloid-osmotic lysis in

Table 2 Hemocyte percentage of F1 Galleria mellonella larvae (irradiated parent male pupae with 40 or 100 Gy) alone or combined with Steinernema carpocapsae BA2 (20 and 40 IJs/ml) treatments

| Treatments | Prohemocytes | Plasmatocytes | Granulocytes | Spindle cells | Spherule cells | Adipohemocytes | Oenocytoids | Cystocytes |
|------------|--------------|---------------|--------------|---------------|---------------|---------------|-------------|------------|
| Control    | 49.56        | 23.16         | 5.62         | 7.9           | 3.05          | 7.15          | 3.27        | 0.28       |
| 40 Gy      | 49.59        | 23.69         | 5.55         | 7.9           | 3.02          | 7.15          | 3.19        | 0.0        |
| 100 Gy     | 50.29        | 25.12         | 4.81         | 7.8           | 2.37          | 7.06          | 3.17        | 0.0        |
| 20 IJs/ml  | 74.41        | 13.84         | 2.64         | 5.588         | 2.2           | 6.39          | 1.32        | 0.0        |
| 40 IJs/ml  | 82.81        | 10.29         | 1.55         | 3.817         | 1.53          | 6.39          | 1.32        | 0.0        |
| 40 Gy + 20 IJs/ml | 85.71    | 8.23          | 0.00         | 4.46          | 1.59          | 6.39          | 1.32        | 0.0        |
| 40 Gy + 40 IJs/ml | 92.49    | 7.51          | 0.00         | 0.00          | 1.00          | 0.00          | 0.00        | 0.00       |
| 40 Gy + 40 IJs/ml | 95.27    | 4.73          | 0.00         | 0.00          | 0.00          | 0.00          | 0.00        | 0.00       |
| 100 Gy + 20 IJs/ml | 96.55    | 3.45          | 0.00         | 0.00          | 0.00          | 0.00          | 0.00        | 0.00       |

Values represent the mean of percentages of 3 replicates for each group
Spodoptera littoralis (Boisd.) infected with X. nematophilus, the symbiotic bacteria of S. carpocapsae.

Differential hemocyte counts (DHCs) in G. mellonella larvae
The percentages of the distinguished hemocytes in normal 4th instar larvae of G. mellonella and after different treatments with gamma radiation or/and S. carpocapsae are presented in Table 2. Results showed that gamma irradiation of G. mellonella male pupae raised the percentage of prohemocytes and plasmatocytes, while decreased the percentages of the other hemocytes in the hemolymph of the 4th instar larvae. S. carpocapsae treatment led to an increase in the prohemocyte percentage and decrease in the percentages of the other hemocyte type. The treatment of F1 larvae with S. carpocapsae resulted in excess in prohemocyte percentage with reduction in plasmatocytes, spindle cells, and spherule cell percentages. Moreover, these treatments caused disappearance of granulocytes, adipohemocytes, oenocytoids, and cystocytes.

The present study on DHCs of normal G. mellonella larvae treated with different radiation doses or the EPN (BA2) showed an increase in the percent of prohemocytes. While the other types decreased with increasing the treatments. Obtained results were in accordance with those of Rizk (1991) and Salem et al. (2008 and 2014).

It is well known that the stimulatory factor resulted from nematode infection stimulates the hemopietic organs to form new hemocyte generations and/or mitosis of the circulating hemocytes (Lackie 1988). Therefore, the increase in prohemocytes is a result of mitosis in response to nematode treatment. As prohemocytes are hypothesized to be stem cells that differentiate into one or more of the aforementioned hemocyte types (Lavine and Strand 2002), this is in accordance with the results of Ayaad et al. (2001). Regarding to the reports of Matha et al. (1990) and El-Maasarawy et al. (1995) that spherulocytes, spindle cell, and plasmatocytes are considered as immunocompetent cells in G. mellonella; therefore, the decrease in plasmatocytes, granulocytes, spindle cells, and spherule cells in this study may reflect their importance in phagocytosis and encapsulation. The sharply decrease in adipohemocytes, oenocytoids, and cystocytes may be due to the toxin released by the symbiotic bacteria (Dunphy and Webster 1988).

The remarkable decrease in DHCs of 4th instar G. mellonella larvae, increased with gamma irradiation doses (40–100 Gy), is in agreement with that of Rizk (1991) and El-Sonbaty et al. (2016) that may be due to the effect of gamma radiation on the physiological condition of the body (Al Khalaf and AbdelBaki 2013).

Conclusion
G. mellonella larvae resulted from irradiated parent at high irradiation doses became more susceptible to the infection with the nematodes. Radiation exposure and nematode infection reduced the immune response of G. mellonella larvae. So, it would be suggested that integration of S. carpocapsae and gamma radiation can be an ideal eco-friendly pest control program.
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