Physiological and Ultrastructural Alterations in the Crayfish Procambarus clarkii Treated with Spinosad (Bacterial Derived Insecticide)

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Abstract

Physiological and ultrastructural investigations have been carried out on Procambarus clarkii exposed to sublethal concentrations of Spinosad insecticide. The study showed that the highest mortality percentage was 88% for males and 70% for female occurred at 64 × 10⁴ ppm Spinosad. The lethal toxicity (LC₅₀) was 24.1 g/l and 29.8 g/l for males and females respectively. Exposure to 1/2 LC₅₀ (12.07 g/l). Spinosad for 7 days caused many physiological disorders including decrease the haemolymph glucose levels and elevated uric acid levels. 12.07 g/l Spinosad resulted in a significant decrease in the total proteins of haemolymph, hepatopancreas and muscles in both males and females. The total cholesterol and triglycerides levels were significantly increased in both haemolymph and hepatopancreas and decreased in muscles of males. On the other hand, cholesterol and triglycerides levels were significantly decreased in haemolymph, hepatopancreas and muscles of females. TEM examinations of male hepatopancreas exposed to 12.07 g/l Spinosad for 7 days revealed ruptured microvilli of absorptive cells, deformed mitochondria, destructed rough endoplasmic reticulum, vacuolated cytoplasm, pyknotic nuclei and appearance of vesicles containing small dark granules.

Keywords: Physiological; Ultrastructure alterations; P. clarkii spinosad

Materials and Methods

Collection and acclimation of specimens

Adult P. clarkii were collected by using 0.7 cm diagonal net size from Bany Helal irrigation canal at Sharkia Governorate during April 2017. The collected specimens were transferred alive to the laboratory, where they maintained in glass aquaria (17.5 h × 38.5 l × 23 w cm). The conditions were maintained at 25°C and a 12:12 h light-dark regime. Water was changed every four days. Animals were fed with carrot.

Determination of Spinosad LC₅₀: Stock solutions of Spinosad were prepared by using distilled water as a solvent to give 4 × 10³, 8 × 10³, 16 × 10³, 32 × 10³ ppm. Three replicates per each concentration were used to determine the LC₅₀. Ten full mature animals either males and females (9-12 cm) were placed in each aquarium. An equal number from each sex were left without treatment as a control. Experiments were checked at 24 h intervals up to 96 h. The dead crayfish were counted and reported. LC₅₀ was determined according to Finney [18] by the graphic method of the curve dose-effect, using the profit analysis.

Chronic exposure

In the long-term exposure, 1/4 and 1/2 of 96 h LC₅₀ of the tested pesticides was used and redosed every 4 days in a static renewal manner. Living animals, surviving the effect of the tested pesticide were sacrificed after 7 days of exposure.

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Haemolymph and tissue sampling

**Haemolymph sampling:** Haemolymph was obtained from conscious *P. clarkii* by direct puncture of the heart using a syringe containing EDTA as anticoagulant for subsequent analysis.

**Tissue sampling:** After decapitation of *P. clarkii*, pieces of muscle and hepatopancreas were taken for physiological and ultrastructural studies.

Physiological studies

Haemolymph glucose and uric acid levels were measured according to GOD-PAP method [19]. Haemolymph, hepatopancreatic and muscle total proteins were determined according to the method of the Biuret method [20].

Triglycerides and total cholesterol in haemolymph, hepatopancreas and muscle were measured according to the method of Fernandez et al. [21] (Tables 1-4).

Transmission electron microscopy preparations

The dissected hepatopancreas of crayfish exposed to either clean water or 1/2 LC₅₀ of Spinosad for 7 days was fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing 0.15 sucrose at pH 7.2 for 2 h. Ultra-thin sections stained with aqueous urinyl acetate and lead citrate and then examined using a JOEL Transmission Electron Microscopic at the regional center for Mycology and Biotechnology in El-Azhar University, Nasr city, Cairo, Egypt.

Statistical analysis

The statistical analysis was performed using SPSS 14.00. The mean values obtained in the different groups were compared by unpaired student’s t-test.

Results

**Mortality tests of Spinosad on *P. clarkii***

Effect of different concentrations of Spinosad on mortality percentages of adult males and females *P. clarkii* at different exposure periods (Figure 1).

**Lethal toxicities (LC₅₀) of Spinosad pesticide**

The different LC₅₀ values of male and female *P. clarkii* exposed to Spinosad were illustrated in Table 1.

Analytical studies

**Transmission Electron Microscopy examinations of hepatopancreas of *P. clarkii***:

Normal hepatopancreas: Transmission electron microscopy examinations have been revealed the presence of three distinct cell types in the hepatopancreas of *P. clarkii*. These three types are the absorptive cells (Ac), secretory cells (Sc) and fibrillar cells (Fc). The absorptive cell is large columnar with a centrally located nucleus (N). The apical surface of absorptive cell has numerous microvilli (MV). The cytoplasm contains a number of irregularly shaped mitochondria (M) and parallel tubules of rough endoplasmic reticulum (RER) (plate I: a and b) (Figure 2). The secretory cell is the largest cell type and is characterized by the presence of a basely located nucleus (N) with small nucleolus (NU). The cytoplasm of this cell contain very thin layer of rough endoplasmic reticulum (RER) found in a perinuclear position and small numbers of mitochondria (plate I: c). The fibrillar cell (FC) has a large nucleus (N) with prominent nucleolus (NU) and massive rough endoplasmic reticulum (RER) (plate I: d).

**Effect of spinosad**

Plate II (Figures 3a-3d) shows electron micrographs of hepatopancreatic cells of *P. clarkii* after exposure to 1/2 LC₅₀ (12.07 g/l) Spinosad for 7 days. Microvilli of the absorptive cells become ruptured.

| Pesticide | Exposure period | 96 h LC₅₀ | ½ LC₅₀ for 15 days |
|-----------|----------------|-----------|--------------------|
|           | Male | Female | Male | Female |
| Spinosad (g/l) | 24.1 | 29.8 | 12.07 | 14.9 |

Table 1: (LC₅₀) values and sub lethal concentrations of both male and female *P. clarkii* exposed to different Spinosad concentrations under laboratory conditions.

![Figure 1](image-url)
Figure 2: Plate I (a) Electron micrograph showing the apical portion of control hepatopancreatic absorptive cell of *P. clarkii*. F: Filaments; M: Mitochondria; MI: Microvilli (original mag. X=16000). (b) The basal portion of control hepatopancreatic absorptive cell, CM: Chromatin Materials; GC: Golgi Complex; M: Mitochondria; N: Nucleus; RER: Rough Endoplasmic Reticulum; V: Vacuole (original mag. X=8000). (c) Secretory cell, CM: Chromatin Material; M: Mitochondria; N: Nucleus; NU: Nucleolus; NM: Nuclear Membrane; RER: Rough Endoplasmic Reticulum (original mag. X=20000). (d) Fibrillar cell, CM: Chromatin Materials; NU: Nucleolus; N: Nucleus; RER: Rough Endoplasmic Reticulum; V: Vacuole (original mag. X=20000).

Figure 3: Plate II (a) Electron micrograph showing the absorptive cell of *P. clarkii* after exposure to 12.07 g/l Spinosad. RMI: Ruptured Microvilli; RER: Concentric Whorls of Rough Endoplasmic Reticulum; V: Vacuole containing Small Granules (X=8000). (b) Electron micrograph showing the basal region of an absorptive cell of *P. clarkii* after exposure to 12.07 g/l Spinosad. M: Mitochondria; GC: Granular Cytoplasm; VA: Vacuolated Cytoplasm; V: Small Vesicles Encapsulating Cellular Debris (X=25000). (c) Electron micrograph showing the basal region of an absorptive cell of *P. clarkii* after exposure to 12.07 g/l Spinosad. CM: Chromatin Material; GC: Granular Cytoplasm; NM: Nuclear Membrane; PN: Pyknotic Nucleus (X=12000). (d) Electron micrograph showing the secretory cell of *P. clarkii* after exposure to 12.07 g/l Spinosad. CM: Chromatin Material; FRER: Rough Endoplasmic Reticulum; N: Nucleus; NU: Nucleolus (X=15000).
Discussion and Conclusion

The red swamp crayfish, Procambarus clarkii, was introduced worldwide and has become the dominant freshwater crayfish in almost all areas it occupies [22]. P. clarkii greatly spread all over the River Nile [10]. Considerable efforts have been paid to control its dispersal with pesticides [23,24]. Spinosad may be used to control agricultural pests. In the present study, toxicological effects of Spinosad on P. clarkii were determined. This study indicated that there is no mortality in males and females P. clarkii at 8 × 10³ ppm Spinosad. The highest mortality occurs at concentration 64 × 10³ ppm while the lowest mortality at concentration 16 × 10³ ppm for both male and females. It is also clear that adult females are more tolerant to toxicity than adult males of P. clarkii. Lc₅₀ of Spinosad for 96 h. for P. clarkii has been determined to be 24.1 and 29.8 g/l for adults male and female, respectively. This result disagree with Abdel-Kader [25] who calculated Lc₅₀ of Spinosad for 5 days to be 858 and 4338 ppm for adults male and female P. clarkii, respectively.

Physiological measurements have been used as indicators of the state of animal health condition and as a biochemical method for assessing the possible mode of action of stressors [26,27]. Analyses of haemolymph constituents have proved to be useful in the detection and diagnosis of metabolic disturbances and disease processes [28]. Blood glucose appeared to be a sensitive and reliable indicator of environmental stress [29]. The present study showed clearly that glucose level in haemolymph of male and female P. clarkii was significantly decreased after treatment with Spinosad. This increase may be due to an increase in haemolymph concentration of catecholamines and corticosetroids [30] as a stress response of animal subjected to environmental alterations. This finding is in agreement with Winkaler et al. [31] reported a high plasma glucose levels in the fish Prochilodus lineatus exposed to neem extract. The present study showed clearly that uric acid in haemolymph of male and female P. clarkii significantly increased after treatment with Spinosad. Low values of uric acid have insignificant meaning but increasing values indicate several disturbances in the kidney [32]. This increase in haemolymph uric acid concentrations may be attributed to the action of pesticides as well as oxygen insufficiency which causes pathological changes of the green glands [33]. Moreover, accumulation of pesticide in the green gland which may cause damage of cells followed by an increase in haemolymph uric acid. This finding is in agreement with Hamdi [34] who showed that there is increase in haemolymph uric acid of crayfish P. clarkii after treatment with Malathion. Proteins are important biochemical components and an assessment of the total protein content in haemolymph, hepatopancreas and muscles of fish has been determined to have 24.1 and 29.8 g/l for adults male and female, respectively. This result disagree with Abdel-Kader [25] who calculated Lc₅₀ of Spinosad for 5 days to be 858 and 4338 ppm for adults male and female P. clarkii, respectively.

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Senthil et al. [37] and Narra et al. [38] reported decreasing in total protein level in haemolymph, hepatopancreas and muscle of freshwater field crabs *Spiralothelphusa hydrodroma* and *Barytelephusaaguerini*, respectively exposed to Chloryprifos.

Cholesterol is a major concern of diet-conscious persons [39]. The present study showed that Spinosad has induced decreasing in total cholesterol and triglyceride concentrations in haemolymph, hepatopancreas and muscle in both sexes. The decrement in the cholesterol and triglyceride may be due to the increased activity levels of lipase, the enzymes responsible for the breakdown of lipid into free fatty acid and cholesterol. This decline may be due to increase hormonal secretions that enhance metabolic rate which in turn reduce the metabolic reserve of the triglyceride [40]. Also, it may be due to the imposition of high energy demands to counter the toxic stress. A decrease in hepaticanptic and muscle total cholesterol and triglyceride has been reported in *P. clarkii* exposed to Malathion [34] and this decrease might be attributed to the alteration of the hepatopancreas which were manifested by vacular degeneration of the hepatocytes. On the other hand, the increase in cholesterol and triglyceride in haemolymph and hepatopancreas of males may be due to inhibition of lipase activity and other enzymes of lipid metabolism.

In Crustacea, there is a correlation between the physiological condition of the organism and the structure and the appearance of hepatopancreas [41]. The hepatopancreas represents a corner stone in the body metabolism and considered to be the most sensitive organ to pollutants and toxicants [42] and the main site of accumulation and detoxification in crayfish bodies [43]. The present study showed clearly that there are three main cell types forming the digestive tubules of *P. clarkii*. These cells were the absorptive, secretory and fibrillar cells. These findings are in agreement with Abdelmonem et al. [5]. The obtained results showed that Spinosad caused ruptured microvilli of the absorptive cells, granular and vacuolated cytoplasm; rough endoplasmic reticulum had the form of concentric whorls and nuclear pyknosis. Heterochromatin condensation and marginalization may be due to progressive inactivation of nuclear component [44]. Electron micrographs showed also the presence of vacuoles containing cytoplasmic debris in the cytoplasm. These vacuoles probably arisen to digest the destructed cellular organelles as a result of treatment with the tested pesticides. Asztalos et al. [45] proposed that the focal development of empty vacuoles might be the starting point of cellular autolysis process. The presences of autophagic vacuole in this study improve the detoxification role of hepatopancreatic cells against pesticides. The fragmentation of RER might be a consequence of final hyperactivity prior to cell necrosis [46].

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