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Biochemical and histopathological responses of *Biomphalaria alexandrina* to RIPEX (plant growth regulator)

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Abstract

Background Plant growth regulators are widely used in agriculture for increasing the growth and ripening of plants, but they endanger the aquatic ecosystem. The current study assessed the effect of sublethal exposure to RIPEX 48% EC concentrations (8 and 16 µL/L) on oxidative stress parameters, sex hormones, immune potential enzymes, differential hemocyte counts, and the histopathology of digestive glands and ovotestis in *Biomphalaria alexandrina* snails.

Results RIPEX exposure caused an overall increase in Superoxide dismutase and Glutathione-S-Transferase activities in *B. alexandrina*. However, extreme RIPEX exposure inhibits SOD activity in snails. Malondialdehyde activity showed an increase in *B. alexandrina* exposed to both concentrations after all exposure periods. RIPEX also caused a significant increase in testosterone in snails exposed to 16 µL/L, it did, however, reduce hormone levels in snails exposed to 8 µL/L at 7 days. Regarding estradiol, there was a significant increase after 3 days of exposure to 16 µl/L and 7 days of exposure to 8 µL/L. RIPEX exposure also increased the activities of Myeloperoxidase and Adenosine deaminase enzymes in the digestive glands of snails. It increased the total hemocyte count of exposed snails as well as the number of granulocytes. Snail digestive glands and ovotestis showed pathological alterations after 7 days of RIPEX exposure.

Conclusions These findings suggest that RIPEX is toxic to *B. alexandrina* and that this snail can be used as a bioindicator for environmental contamination with plant growth regulators.

Keywords *Biomphalaria alexandrina*, RIPEX, Testosterone, Estradiol, Myeloperoxidase, Adenosine deaminase, Histopathology

1 Background

Agrochemical pollution of surface waters is a growing global environmental concern, particularly in areas where agriculture is rapidly expanding and intensifying. Various synthetic chemicals, such as pesticides or fertilizers, are used to modify crop quantity and quality to satisfy customers' needs [7, 40]. Plant growth promoters are common agrochemicals in many countries [18]. When these chemicals are directly inhaled or consumed with contaminated vegetables, they have a negative impact on human health [39]. The organophosphorus pesticide, ethephon (2-chloroethylphosphonic acid) is an ethylene releasing synthetic chemical that is commonly used as a plant growth regulator (PGR) to promote flower induction, fruit ripening, and other physiological reactions. It is approved for use on a variety of food, feed, non-food, greenhouse nursery stock, and ornamental plants grown outdoors [3, 50].

Laboratory assessment of ethephon toxicity demonstrated that ethephon induced morphometric

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malformations of second antenna, sensory bristles, tail spine, and rostrum of *Daphnia magna* which led to loss function and death [54]. Abd-El Azeem [1] recorded reduction of growth rate, DNA damage and apoptotic effects in digestive gland cells of the slug *Deroceras reticulatum* after exposure to ethephon. Many authors approved the toxicity of Ethephon on rats and birds [2, 17, 55]. Furthermore, ethephon exposure caused genotoxicity, oxidative stress, and reproductive toxicity in mice [16]. Teratogenicity and gonadal system dysfunction in mice have also been linked to ethephon exposure [2, 16]. Ethephon has also been shown to be immunotoxic, have mutagenic effects [26], increase oxidative stress, and lower deoxyribonucleic and Ribonucleic acids concentrations [4].

Ethephon in water bodies may endanger aquatic fauna; however, data on its impact on aquatic organisms are scarce. Several aquatic species have been used to detect plant growth hormones in aquatic systems. However, some of these organisms are challenging to manipulate and maintain in the lab, making it more difficult to use in ecotoxicological evaluations [54]. Aquatic mollusks, including snails, have recently gained attention as a test organism due to the need for developing invertebrate models for environmental monitoring of aquatic contaminants [21, 45]. Snails are the most abundant mollusk group in aquatic systems. Snails present a less exploitative alternative to higher vertebrates as bioindicators because they can be used in a variety of experiments without seeking ethical approval, and their acquisition and maintenance are inexpensive [13]. In this respect, *Biomphalaria alexandrina* snail has been widely used as an indicator organism of freshwater pollution with pesticides [29, 30]. Beyond its potential as a bioindicator, this snail acts as an intermediate host for the transmission of *Schistosoma mansoni* [35]. Therefore, agrochemical concentrations in the environment can influence schistosomiasis transmission through direct and indirect effects on intermediate host and parasite densities [27].

The present investigation aimed at investigating the impact of sublethal exposure to the plant growth regulator, RIPEX 48% EC (the trade name of ethephon, 2-chloroethylphosphonic acid) on various biochemical, immunological, and histological aspects of *B. alexandrina* snails as a bioindicator organism.

2 Methods

2.1 Test organism

Biomphalaria alexandrina snails were collected from Lake Manzalah located in El-Matareya, Dakahlia Governorate, Egypt (31° 11′ N 32° 2′ E) and maintained in well-aerated tap water at a constant temperature of 20 °C. They were fed fresh lettuce leaves daily. After maintenance for 4 weeks under the laboratory conditions, healthy mature snails (10–12 mm in shell diameter) were used in the subsequent experiments.

2.2 Experimental material and exposure conditions

The plant growth regulator, RIPEX (a solution form containing 48% ethephon [2- chloroethylphosphonic acid ($C_2H_6ClO_3P$)] and 52% inert ingredients) was purchased from Chema Industries (New Nubaria city, Behira, Egypt; registry license No. 1840). A series of concentrations were prepared. Three replicas with ten snails each were exposed. After 1-day of exposure, dead snails were counted, removed and LC_{50} and LC_{90} were computed by statistical software of social sciences (SPSS; IBM Corp. Armonk, NY, USA).

2.3 Experimental design

Two sublethal concentrations representing LC_{10} and LC_{20} were used (8 and 16 µL/L). 90 snails were divided into three groups, each group with three replicates (ten snails/replicate): control group, the 8 µL/L-exposed group, and the 16 µL/L-exposed group. At 1-, 3-, and 7-day intervals, oxidative stress biomarkers and immune potential enzymes were assessed in the digestive gland. steroid hormones were assessed in ovotestis. Differential hemocyte count, and histology of the digestive glands and ovotestis were assessed. For biochemical investigations, 0.5 g of tissue (digestive glands or ovotestis) from each group was homogenized in 0.5 mL phosphate buffer (pH 6.5) using a glass homogenizer and centrifuged at 10,000×*g*, 4 °C for 10 min. The resulting supernatants were used for subsequent assays.

2.4 Determination of oxidative stress markers

2.4.1 Superoxide dismutase (SOD, EC 1.15.1.1)

SOD activity was determined using the EnzyChrom™ Superoxide Dismutase Assay Kit (Cat. No: ESOD-100; BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. The tissue homogenate using cold lysis buffer (50 mM potassium phosphate, 0.1 mM EDTA and 0.5% Triton X-100) was centrifuged at 12,000*g* for 5 min at 4 °C, supernatant was collected for determination of SOD [43]. The activity of SOD was measured at the optical density (OD) at 440 nm.

2.4.2 Glutathione-S-Transferase (GST, EC 2.5.1.18)

This enzyme catalyzes the GSH conjugation with 1-chloro-2,4-dinitrobenzene (CDNB). It is measured spectrophotometrically at 340 nm. The reaction mixture contains Potassium phosphate buffer 0.1 M, pH 6.5, GSH dissolved in the potassium phosphate buffer (140 mg/100 mL) and CDNB 4 mg/ml in ethyl alcohol. One unit of enzyme activity is defined as the amount

of enzyme which catalyse the formation of 1 µmole of S-conjugate per minute [11].

2.4.3 Malondialdehyde (MDA)

Malondialdehyde in whole homogenates was determined as thiobarbituric acid reactive substances (TBARS) according to the standard method of Ohkawa et al. [37]. The reaction mixture included 0.1 mL of the tissue homogenate, an equal volume of Sodium Dodecyl Sulfate solution, 0.75 mL acetic acid, 0.75 mL thiobarbituric acid, and 0.3 mL distilled water. These components were mixed in a vortex and incubated in a boiling water bath for 1 h then cooled to room temperature. Then 0.5 mL of distilled water and 2.5 mL n-butanol were added to each tube and vigorously mixed with a vortex then rotated in a centrifuge at 2,500 × g for 10 min. Absorbance was read at 532 nm.

2.5 Hormones determination

2.5.1 Testosterone

Testosterone was determined using a testosterone Enzyme-linked immunosorbent assay (ELISA) kit (Cat. No: ADI-900-065; Enzo Life Sciences, NY, USA),

Testosterone is the main androgen which effects both primary and secondary sexual development as sex drive. 100 µL of standard diluent (assay buffer 3 or tissue culture media) was pipetted into the non- specific binding and the maximum binding (0 pg/mL standard) wells. After that 100 µL of standard #1 through #5 into the appropriate wells. The sample (100 µL) was pipetted into the appropriate wells and 50 µL of assay buffer 3 into the non- specific binding wells. Also, 50 µL of yellow antibody were added into each well, except the blank, total activity and non- specific binding wells. The incubation of the plate at room temperature on a plate shaker for 1 h at ~ 500 rpm. After that, 50 µL of blue conjugate was added into each well, except total activity and blank wells. After emptying of the wells from the contents, washing was done by adding 400 µL of wash solution to every well for 3 times. Blue conjugate (5 µL) was added to the total activity wells and 200 µL of a solution of p-nitrophenyl phosphate was added to every well and incubated at 37 °C for 1 h without shaking. Reading of the plate at 405 nm was carried out after adding 50 µL of stop solution to every well [12, 48].

2.5.2 Estradiol

Estradiol was determined using an estradiol ELIZA kit (Cat. No: 501890; Cayman Chemical, Michigan, USA) according to the manufacturers' protocols. The principle of this reaction based on that concentration of estradiol acetylcholinesterase (AChE) tracer is constant, but the native estradiol concentration varies therefore the amount of estradiol acetylcholinesterase (AChE) tracer which bind to estradiol antiserum is inversely proportional to the concentration of native estradiol in the well. 100 µL ELISA buffer was added to non- specific binding and maximum binding wells. Addition of 50 µL from tube #8 to both lowest standard wells (Standard 8) and 50 µL from tube #7 to each of the next standard wells (Standard 7). Sample (50 µL) was added to each well. Estradiol AChE Tracer (50 µL) was added to each total activity and blank wells. After that Estradiol ELISA antiserum (50 µL) was added to each well except total activity, non- specific binding and blank wells. After orbital shaker incubation (2 h at room temperature), absorbance was measured at 414 nm [52].

2.6 Determination of immune potential enzymes

2.6.1 Myeloperoxidase (MPO, EC 1.11.2.2)

One unit of MPO activity is defined as the amount of enzyme that hydrolyzes the substrate and generates taurine chloramine to consume 1.0 µmole of TNB per minute at 25 °C. MPO was determined using a myeloperoxidase colorimetric activity assay kit (Cat. No: MAK068; Sigma-Aldrich, MO, USA). The absorbance was measured at 412 nm.

2.6.2 Adenosine deaminase (ADA, EC 3.5.4.4)

ADA is an enzyme that catalyzes the conversion of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine. ADA was determined according to the principles of the technical bulletin adenosine deaminase activity assay kit (Cat. No: MAK400; Merck KGaA, Darmstadt, Germany). One unit of adenosine deaminase is the amount of enzyme that hydrolyzes adenosine to yield 1.0 µmol of inosine per minute at 37 °C. The reagents are 1 × ADA assay buffer (41 µL), ADA Convertor (2 µL), ADA developer (2 µL) and ADA substrate (5 µL).

2.7 Differential hemocyte count

The hemolymph was collected from the snail (control, exposed) according to Sminia [47]. After being touched on the head-foot by a Pasteur pipette, the snail was forced to retract deeply into its shell and extrude hemolymph, which was collected for a differential hemocyte count. Hemolymph was smeared on the microscope slide for spreading. After fixation in 99.8% methanol for 5 min, the slide was turned at a 45° angle for drying at room temperature and stained with Giemsa stain for 20 min. The differentiation of hemocytes was determined to be proportional to counting 100.

2.8 Histological investigations

Histological examinations were carried out after 7 days of exposure to 8 and 16 µL/L RIPEX. The digestive

glands and ovotestis were dissected out and immediately fixed in Bouin's fluid. After 1 day of fixation, samples were then dehydrated through a series of alcohols, and cleared in xylene. Paraffin wax blocks were made [42]. Then Sects. (5–7 µm thickness) were cut and stained with hematoxylin and eosin (Mayer's H and E). Staining was followed by a good wash with tap water. Histological sections were photographed using a photo-automated camera (Optika, Italy).

2.9 Statistical analysis

Data were expressed as the mean ± standard deviation and analyzed by statistical software of social sciences (SPSS; IBM Corp. Armonk, NY, USA). Two-way ANOVA analysis of variance was used to identify differences between the control and exposed groups, between the different concentrations and the time points of exposure. The level of significance was set at $p \leq 0.05$.

Table 1 Toxicity of plant growth regulator, RIPEX against *Biomphalaria alexandrina* after 1 day. post exposure

Concentration	LC_{10}	LC_{20}	LC_{50}	LC_{90}	Slope
B. alexandrina	8	16	39.9	73.6	1.6

3 Results

3.1 RIPEX toxicity

The toxicity results of RIPEX to *Biomphalaria alexandrina* after 1 day indicated that the values of LC_{50} and LC_{90} were 39.9 5 and 73.6 µL/L, respectively. The LC_{10} and LC_{20} sublethal concentrations were 8 and 16 µL/L, respectively (Table 1 and Fig. 1).

3.2 Effect of RIPEX on the oxidative stress markers

The exposure to RIPEX affected the activities of SOD and GST and the levels of MDA in the digestive gland of *B. alexandrina* (Table 2). Snails exposed to RIPEX at 8 µL/L showed an insignificant increase ($p = 0.6$) in the activity of SOD compared to the control snails. However, *B. alexandrina* snails exposed to 16 µL/L for 7 days exhibited a significant reduction in SOD activity ($p = 0.05$). GST activity significantly increased ($p \leq 0.001$) after exposure to both concentrations. The highest increase was noted in snails exposed to 16 µL/L for 1 day (39.7 ± 3.2 U/mg) and the highest value at the concentration 8 µL/L was (33.7 ± 1.7 U/mg) at 7 days, compared with the controls at 17.1 ± 3.6 U/mg (after 1 day) and 22.6 ± 1.3 U/mg (after 7 days). Dependent on time intervals, exposure for 7 days caused significant increase ($p = 0.003$). The exposure to different concentrations of RIPEX caused a significant increase ($p \leq 0.04$) in the levels of MDA. The concentration 8 µL/L increased the MDA level (45.06 ± 4.1) compared to control (16.4 ± 2.1). Dependent

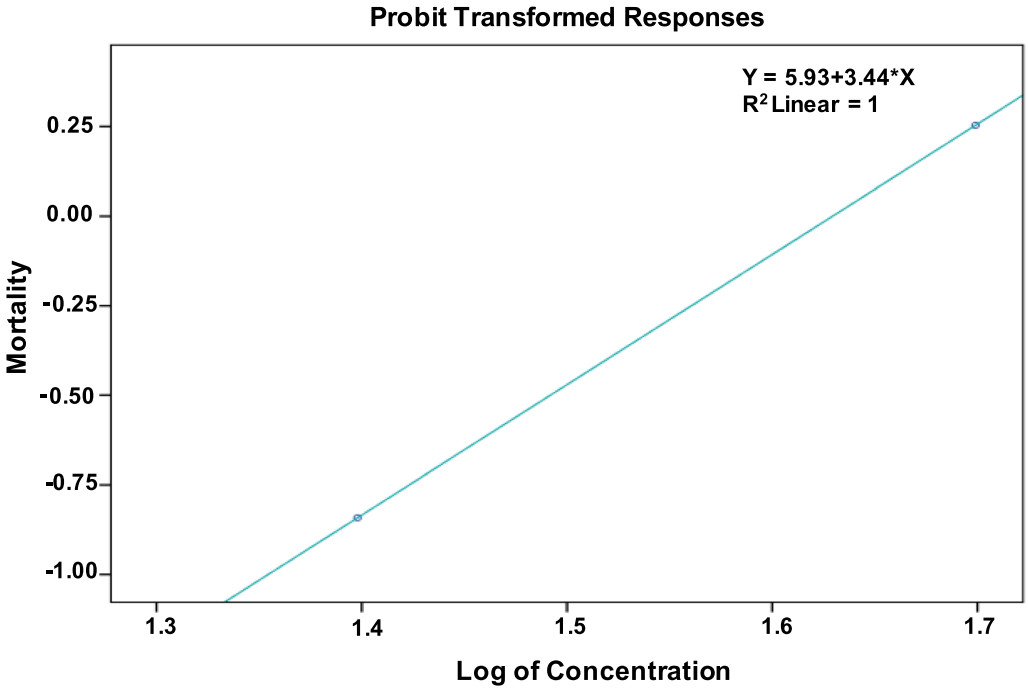

Fig. 1 Concentrations-mortality relationship (probit transformed responses) of RIPEX 48% EC against *B. alexandrina* snails after 1 day of exposure

Table 2 Effect of plant growth regulator RIPEX on the activities of SOD and GST and the level of MDA in the digestive gland of the snail *Biomphalaria alexandrina*

Intervals	SOD (U/mg protein)			GST (U/mg protein)			MDA (nmol/gm tissue)		
	Control	8 µL/L	16 µL/L	Control	8 µL/L	16 µL/L	Control	8 µL/L	16 µL/L
1 day	6.1 ± 0.4	5.1 ± 0.2	8.1 ± 0.2	17.1 ± 3.6	16.4 ± 1.1	39.7 ± 3.2$^{*\$}$	14.03 ± 2.7	22.3 ± 1	30 ± 1.4
3 days	6.8 ± 0.8	7.4 ± 0.2	4.9 ± 0.4	17.7 ± 1.04	27.7 ± 7.5	36.3 ± 1.5*	16.4 ± 2.1	33.6 ± 1.9	41.6 ± 1.5*
7 days	8 ± 0.01	9.4 ± 0.2	3.03 ± 0.2*	22.6 ± 1.3	33.7 ± 1.7*	36 ± 0.8*	13.5 ± 0.7	45.06 ± 4.1*	56.1 ± 0.7*$^{\$}$

Data were expressed as mean ± S.D., n = 3 * Represented significant difference between control and exposed groups; $ between time points of exposure when $P \leq 0.05$

on time of exposure, MDA levels were 45.06 ± 4.1and 56.1 ± 0.7 nmol/g at the concentrations, 8 and 16 µL/L, respectively, compared to 13.5 ± 0.7 nmol/g for the control (Table 2).

3.3 Effect of RIPEX on the activity of steroid hormones

Exposure to 16 µL/L caused a significant increase in testosterone concentration (52.6 ± 2.5 nmol/L) ($p = 0.002$) compared with control (34.6 ± 2.3 nmol/L). While the concentration 8 µL/L RIPEX caused a significant increase ($p = 0.005$) in testosterone after 3 days of exposure compared with 16 µL/L. Dependent on the time intervals, there is no any significance except the significant decrease in testosterone concentration which occurred at the 7 days (19.6 ± 2 nmol/L) of exposure ($p = 0.003$) compared with 3 days (47.3 ± 6 nmol/L).

Regarding estradiol, there was a significant increase ($p = 0.003$) in the hormone concentration after 3 days of exposure to 16 µl/L (73.6 ± 3.5 pg/mL compared with the control (43.3 pg/mL) and 7 days of exposure to 8 µL/L (56.3 ± 1.5 pg/mL compared with the control (50.3 ± 5.6 pg/mL) (Fig. 2, b).

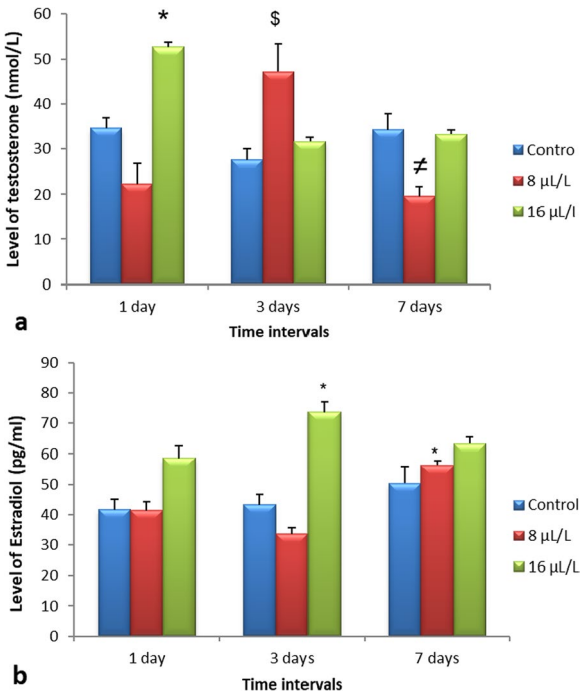

Fig. 2 The effect of different concentrations of RIPEX on steroid hormones in ovotestis of *Biomphalaria alexandrina*. **a** Testosterone, **b** estradiol. Values are expressed as mean ± SD. Significance difference as compared to control (*); (≠) between the concentrations and ($) between the time intervals when $p \leq 0.05$

3.4 Effect of RIPEX on immune potential enzymes

The exposure of *B. alexandrina* to two concentrations of RIPEX caused a significant increase in the activity of MPO enzyme (Fig. 3a) dependent on the time of exposure at 3 days ($p = 0.004$) and at 7 days ($p = 0.01$). Exposure to 8 µL/L RIPEX, significantly increased the activity of MPO (55.3 ± 4.8 mU/mg protein ($p = 0.004$) compared to 15.4 ± 1.2 mU/mg for the control. While 16 µL/L exposed snails the activity was (53.03 ± 2.3 mU/mg protein and 73.9 ± 2.8 mU/mg protein, $p \leq 0.05$). Also, the concentration 16 µL/L caused a significant increase ($p = 0.05$) of the activity when compared with 8 µL.

Both concentrations (8 and 16 µL/L) significantly increased ($p \leq 0.009$) the activity of ADA when compared with the control (Fig. 3b) at all time points. The increasing of the activity at 16 µL/L concentration. Dependent on the time intervals, the exposure for 7 days recorded significant increase when compared with 1 day and 3 days ($p \leq 0.05$). The values of activity at7 days were 27.03 ± 14 and 74.3 ± 1.9 U/mg protein.

3.5 Differential hemocyte count

Examination of hemolymph samples from *B. alexandrina* snails indicated three morphologically distinct types of hemocytes; hyalinocytes, round small and spreading hemocytes. Hyalinocytes are characterized by their circular shape, clear, dense cell membrane, and large nucleus to cytoplasm ratio. It represented by a high count in control (56.3 ± 0.5, 1 day). Small round cells are intermediate cells with few cytoplasmic granules and no pseudopodia. Spreading hemocytes have

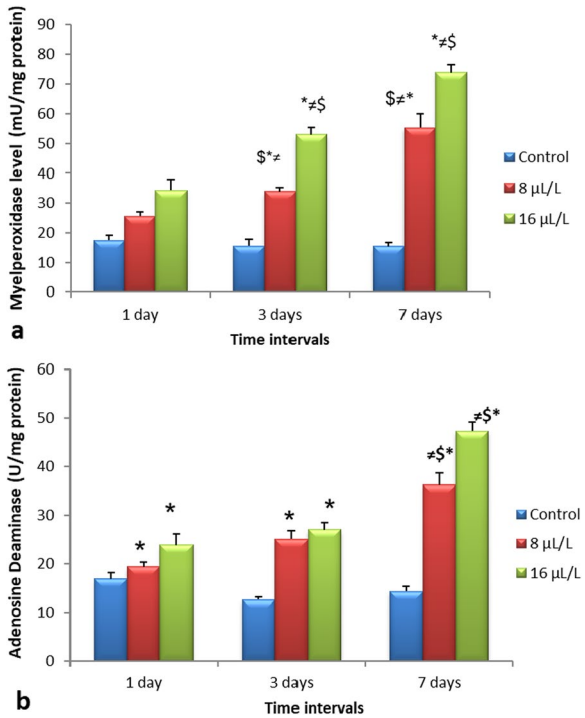

Fig. 3 The effect of different concentrations of RIPEX on immune potential enzymes in digestive glands of *Biomphalaria alexandrina*. **a**: myeloperoxidase (MPO) activity. **b**: adenosine deaminase (ADA) activity. Values are expressed as mean ± SD. Significance difference as compared to control (*) and between different concentrations ($^{\neq}$) and ($^{\$}$) between the time intervals, $p \leq 0.05$

different sizes, with a small nucleus and large cytoplasmic granules. They are phagocytic cells because they can form few and short spreading pseudopodia. The exposure to the two concentrations of RIPEX increased the hemocyte count, and a significant increase was observed in the granulocytes of snails exposed to 8 µL/L ($p = 0.02$) and 16 µL/L ($p = 0.006$) for 1, 3, and 7 days compared to the control. (Table 3). The treatment caused some morphological abnormalities in hemocytes such as hyalinocytes with filopodial elongation, deformed granulocytes with vacuoles and spreading filopodia (Fig. 4).

3.6 Effect of RIPEX on the histopathology of the digestive glands and ovotestis

The digestive glands of *B. alexandrina* are composed of tubules, and each tubule consists of one layer of columnar epithelial cells differentiated into digestive and secretory cells, which are arranged around a central lumen (Fig. 5a). The tubules are linked together by connective tissue. RIPEX exposure caused some alterations, such as a degeneration in the connective tissue. Snails exposed to 8 µL/L showed the presence of cellular blebs, which indicate the death of cells and clogging of the lumen. Undigested food was observed (Fig. 5b–d). Snails exposed to 16 µL/L, exhibited degeneration of the lined cells with some pathological signs such as vacuolation and dilation of the lumen. The tubule became damaged, deformed, and necrotic (Fig. 5e, f).

Ovotestis of control *B. alexandrina* snails consist of several acini joined by connective tissue. Each acinus is lined with germinal epithelium composed of different stages of spermatogonia and oogonia. They differentiate into primary, secondary, and one or two mature oocytes (oogonia). Matured sperms are found in groups inside the acinus (Fig. 6a). The histological structure of the ovotestis of exposed snails changed after 7 days of exposure to RIPEX. In snails exposed to 8 µL/L, an increasing number of sperms, necrotic sperms, and atrophy were observed. Deformation of male or female gametocytes and degeneration of acini were also abundant. Also, the exposure to 16 µL/L made the acini more degenerated and necrosis was increased (Fig. 6b–e).

4 Discussion

There is extensive use of PGRs in agricultural practices. These substances have numerous deleterious effects on living organisms. The present study aimed at assessing the toxicological impacts of RIPEX (an organophosphorus pesticide containing ethephon containing 48% ethephon as the active ingredient plus 52% inert ingredients). Toxicity assays of RIPEX with *B. alexandrina* indicated that the 1 day LC_{50} and -LC_{90} values were 39.95 and 73.6 µl/L, respectively. These results were supported by [54] who demonstrated the EC_{50} value for ethephon against *Daphnia magna* embryos (125 mg/L after 48 h

Table 3 Effect of plant growth regulator RIPEX on differential hemocytes count of the snail *Biomophlaria alexandrina*

Intervals	Hyalinocyte			Round small			SP (Granulocytes)		
	Control	8 µL/L	16 µL/L	Control	8 µL/L	16 µL/L	Control	8 µL/L	16 µL/L
1 day	56.3 ± 0.5	61.3 ± 0.9	67 ± 1	21.6 ± 1.5	19.6 ± 2	19.6 ± 2.5	15.3 ± 1.5	35 ± 2*	36.6 ± 1.5*
3 days	57 ± 1	58.6 ± 0.9	65.6 ± 0.5	21.6 ± 0.5	20.6 ± 2	20.3 ± 1.5	15.6 ± 0.5	37 ± 1*	40 ± 1*
7 days	57.3 ± 1.5	56.6 ± 0.4	62.3 ± 0.5	23 ± 1	17.3 ± 2.5	21.3 ± 4.7	15.3 ± 0.5	38.6 ± 0.5*	43.3 ± 1.5*

Data were expressed as mean ± S. D, significant difference indicated by (*) from control when ($P \leq 0.05$)

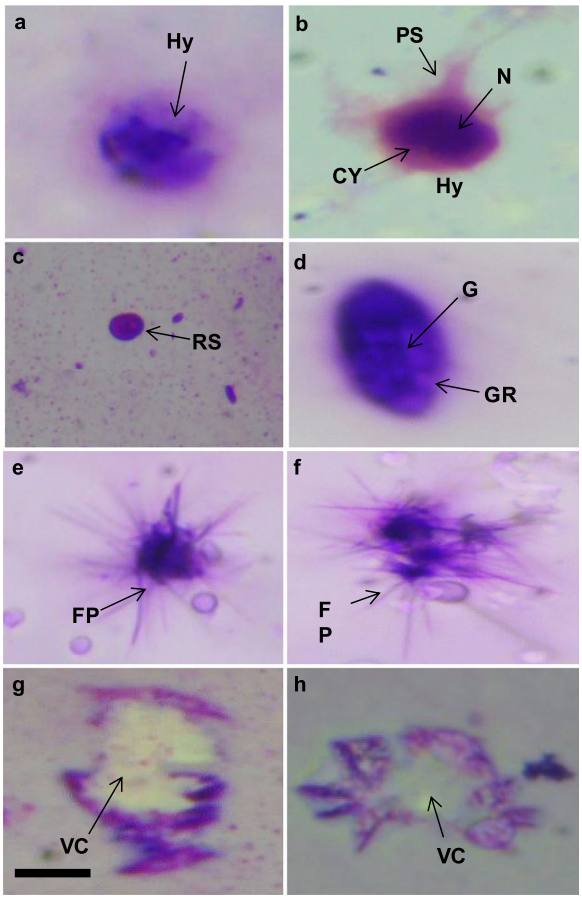

Fig. 4 Light micrographs showing different types of normal and abnormal hemocytes of *Biomphalaria alexandrina* after exposure to RIPEX 48% EC. **a**–**d**: normal hemocytes; **a**, **b**: Hyalinocyte, **c** Round small cell. **e**, **f** spreading hemocytes after exposure to 8 µL/L of ethephon. **g**, **h** Vacuolated hemocytes after exposure 16 µL/L of ethephon. (Hy) Hyalinocyte; (RS) Round small; (GR) Granulocyte (SP) Spreading cell and (VC) Vacuolated cells. PS: Pseudopodia, N: Nucleus, CY: Cytoplasm, G: Granules, FP: Filopodia. Scale bar = 20 mm

of exposure). This pesticide seems more toxic to *Biomphalaria* than other organophosphorus compound formulations such as chlorpyrifos (LC_{50} 9.6 mg/L; [30] and profenofos (LC_{50} 1.10 mg/L; [10]).

A homeostasis exists between the generation of oxygen free radicals and the production of antioxidant molecules under normal physiological conditions [23]. An imbalance between the antioxidant and oxidant systems is known as oxidative stress [32]. In both eukaryotes and prokaryotes, superoxide dismutase (SOD) and glutathione S-transferases are crucial antioxidant enzymes that may remove too much oxygen from free radicals and shield the organism from oxidative damage [34, 38]. They also participate in the detoxification of several xenobiotics [8, 15].

The present study revealed that exposure of *B. alexandrina* snails to sublethal concentrations of RIPEX 48% EC disturbed the antioxidant activity of the exposed snails. Acute exposure (24 h) tends to reduce overall SOD and GST activities in snails, followed by an increase after 3 days of exposure and a sharp inhibition after 7 days of exposure. MDA levels were significantly higher in exposed snails compared to controls after all exposure periods. These results suggest a state of oxidative stress in snails induced by RIPEX exposure. The snails experienced a sudden decrease in enzyme activities after 1 day of exposure and attempted to compensate by overproducing enzymes after 3 days of exposure. However, by the 7^{th} day of exposure, snails were unable to overcome the excess production of reactive oxygen species, as manifested by the increase in MDA levels (lipid peroxidation), leading to an inhibition of antioxidant enzyme production. MDA is among the most major impacts of lipid peroxidation and has the power to disrupt ion exchange in the cell membrane, modify ion permeability, and influence enzyme function [9]. The mechanism of RIPEX 48% EC induced oxidative stress in *Biomphalaria* may differ from other organophosphorus pesticides. It caused enzyme inhibition only after 7 days of exposure. However, chlorpyrifos, for example, caused an increase in SOD activity and a reduction in GST activity in *B. alexandrina* shortly after 1 day of exposure [30].

Testosterone and estradiol play a crucial role in the reproduction of *B. alexandrina* snails [20, 45]. Changes in these hormones can be used as an endpoint of chemical toxicity [14]. RIPEX 48% EC caused a hormonal disruption in *B. alexandrina* snails. Snails exposed to 16 µL/L showed a significant increase in testosterone after 3 days of exposure. However, RIPEX decreased the hormone levels in snails exposed to 8 µL/L after 1 and 3 days of exposure. With regard to estradiol, the hormone tends to increase in exposed snails. However, the most prominent increase was observed in snails exposed to 16 µL/L after 3 days of exposure. RIPEX may therefore have an endocrine disrupting effect on *B. alexandrina* snails. Similar findings were also reported by Ibrahim and Hussein [30], who reported a significant increase in the levels of both testosterone and estradiol after exposure of *B. alexandrina* to sublethal concentrations of chlorpyrifos 48% EC. Other agrochemicals, however, such as the oxyfluorfen (24% EC) herbicide, reduced testosterone and 17-estradiol levels in *B. alexandrina* after two weeks of exposure [31].

Myeloperoxidase (MPO),which converts H_2O_2 to hypoclorous acid (HOCl), a potent antibiotic, is frequently regarded as the cornerstone of ROS-mediated cytotoxicity, and there is strong evidence that molluscan hemocytes have MPO activity comparable to that of

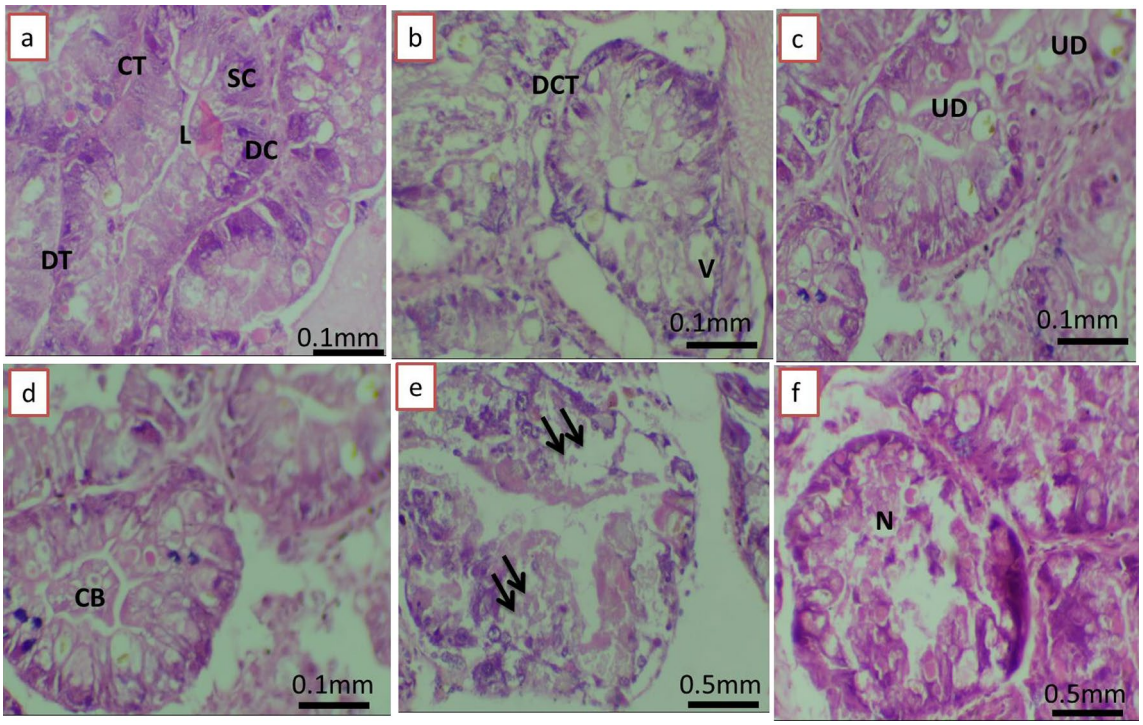

Fig. 5 Light micrographs showing the effect RIPEX 48% EC on digestive glands of *Biomphalaria alexandrina*. **a** Control, digestive tubule (DT), digestive cell (DC.), secretory cell (SC), lumen (L). **b**–**d** Snails exposed to 8 µL/L showing the presence of cellular blebs (CB), vacuolation (V) and undigested food (UD). **e**, **f**: Snails exposed to 16 µL/L showing tissue degeneration (arrows) and necrosis (Ne)

mammalian phagocytes [22, 44, 49]. Adenosine deaminase (ADA) plays an important role in the immune systems of vertebrate and invertebrate animals, including *Biomphalaria* snails [51]. Its deficiency is linked to combined immunodeficiency disease [25]. Exposure of *B. alexandrina* to 8 and 16 µL/L RIPEX caused a significant increase in the activities of MPO and ADA enzymes in the digestive glands of snails after 3 and 7 days of exposure. The increase in enzyme activity seems time- and concertation dependent. The digestive glands are the primary site for the metabolism of chemical contaminants in gastropods [46]. The increase in these enzymes suggests an immunological response of *B. alexandrina* to RIPEX toxicity since MPO, for example, can convert pollutants to free radicals via single electron abstractions [41], which can result in redox-cycling events that produce oxyradicals and possibly initiate lipid peroxidation [36].

Snails primarily rely on cell-mediated cytotoxicity to get rid of invading objects [5, 53]. The most well-studied defensive cells in snails are hemocytes. Hemocytes progress from small, rounded, agranular cells to large, granular, spreading cells via intermediate phases [6, 53]. Granular hemocytes mostly phagocytose foreign substances [28]. The exposure to two concentrations of RIPEX 48% EC increased the hemocyte count. The hyalinocytes appeared with vacuoles in samples collected from exposed snails. A significant increase was noted in granulocytes, indicating an increase in the snail's cellular immune response to challenge RIPEX toxicity. Ibrahim and Hussein [30] showed that exposure of B. alexandrina to chlorpyrifos had negative impacts on the morphology and total hemocyte count of exposed snails. Furthermore, light microscopy revealed that some hyalinocytes had shrunken nuclei, incomplete cell division, and pseudopodia formation. These effects on the hemocyte count and morphology of B. alexandrina have a negative implication on the snails' immunity, as these organisms depend largely on hemocytes for their protection against foreign encounters.

Histopathological investigations of the digestive glands of *B. alexandrina* showed that RIPEX exposure for 7 days caused some pathological alterations such as the degeneration of connective tissue, cellular blebbing, and the degeneration of lined cells in the lumen. The digestive tubules became damaged, deformed, and necrotic. Histopathology is an important endpoint in assessing the pathological changes in aquatic animals exposed to contaminants [33]. The high sensitivity of the digestive glands is directly attributed to its role in homeostasis, contaminant uptake, digestion, metabolism and the detoxification process [24]. The observed effects may be

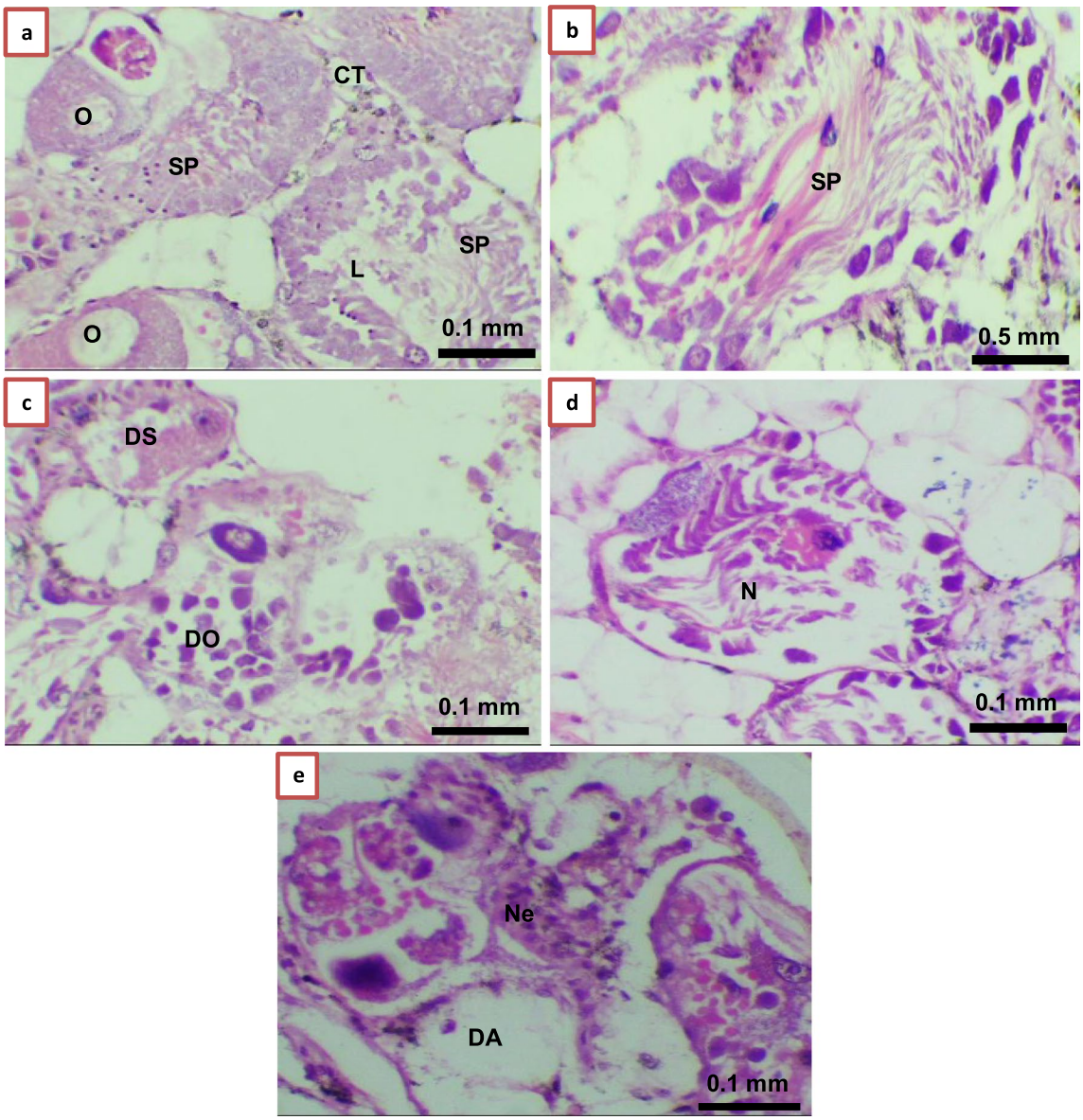

Fig. 6 Light micrographs showing the effect of RIPEX 48% EC on the ovotestis of *Biomphalaria alexandrina*. **a**: Control showing lumen (L), spermatozoa (SP), ova (O), and connective tissue (CT). **b**, **c** Snails exposed to 8 µL/L, showing degenerated spermatozoa (DS) and degenerated ova (DO). **d**, **e** Snails exposed to 16 µL/L show necrosis (Ne) and degenerated acinus (DA)

due to direct accumulation of the RIPEX in the digestive gland cells or indirectly via oxidative damage caused by reactive oxygen species (ROS) production. Indeed, extreme oxidative stress has been linked to tissue damage involving a variety of physiological processes and environmental variables, including necrotic and apoptotic cell death [19]. Moreover, exposure to two different concentrations of RIPEX for 7 days altered the histological structure of ovotestis with an increase in the number of necrotic sperms, atrophy, deformation of male or female gametocytes, and degeneration of the acini. These changes are likely to reduce the reproductive potential of snails and, therefore, their fitness in the aquatic system.

5 Conclusion

Sublethal concentrations of RIPEX 48% EC had a multifaceted effect on *Biomphalaria alexandrina* snails. In snails, chronic exposure to RIPEX reduced the production of the antioxidant enzymes SOD and GST while increasing MDA production, indicating an oxidative stress state. Furthermore, it had an endocrine disrupting effect on snails, as evidenced by an overall increase in steroid hormone levels.

Snail immune system appears to be vulnerable to RIPEX exposure. It caused a significant increase in the activity of MPO and ADA enzymes in snail digestive glands after 3 and 7 days of exposure, as well as a significant increase in granulocytes in exposed snail groups. Pathological abnormalities in exposed snails' digestive glands and ovotestis were also signs of histopathological changes caused by RIPEX exposure. These findings show that RIPEX has a negative impact on aquatic fauna, including *B. alexandrina*. Thus, *Biomphalaria* snails can be used as bioindicators for environmental pollution with plant growth regulators.

Abbreviations

SOD	Superoxide dismutase
GST	Glutathione-S-Transferase
MDA	Malondialdehyde
MPO	Myeloperoxidase
ADA	Adenosine deaminase
PGR	Plant growth regulator
GSH	Glutathione
CDNB	1-Chloro-2,4-dinitrobenzene
TBARS	Thiobarbituric acid reactive substances
ROS	Reactive oxygen species
ELISA	Enzyme-linked immunosorbent assay

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Author contributions
All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Hoda H. Abdel-Azeem] and [Mohamed R. Habib]. The first draft of the manuscript was written by [Hoda H. Abdel-Azeem] and [Mohamed R. Habib] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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