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Effects of Three Inorganic Fertilizers on the Biology and Histopathology of infected *Biomphalaria alexandrina* snails

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ABSTRACT

The present work was carried out to calculate the half lethal concentrations (LC_{50}) of three inorganic fertilizers (balanced, high phosphorus and high nitrogen fertilizers) on *Biomphalaria alexandrina* snails and to demonstrate measurable effects on infected *B. alexandrina* with *Schistosoma mansoni* miracidia that were exposed for one week to ¼ LC_{50} (126.4, 400 and 2375 ppm) separately of (balanced, high phosphorus and high nitrogen) fertilizers, respectively. The obtained results showed that the survival of all treated infected snail groups at the 1st shedding were significantly less than the control group. Moreover, results revealed a marked reduction in the infectivity of the infected snails especially those exposed to sublethal concentration of high nitrogen fertilizer when compared to control. In addition, the cercarial production of all treated groups was completely suppressed. These infected snails exhibited histopathological alterations, which is associated with severe damage in the digestive gland cells as well as the mother sporocysts of treated infected snails. Our study revealed the biological and histopathological effects of three inorganic fertilizers on infected *Biomphalaria alexandrina* snails

Keywords: *Biomphalaria alexandrina,* infection, inorganic fertilizers, histopathology

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INTRODUCTION

The dynamic interaction between mollusk and their trematode parasites leads either to a state of co-existence, in which the trematode thrives and produces subsequent stages of its life – cycle, or to incompatibility, where the trematode is either destroyed and eliminated by the host snail defense responses or fails to develop because the host is physiologically unsuitable [1&2]. Successful colonization of a compatible snail host by a digenetic trematode miracidium initiates a complex proliferative development program requiring weeks to reach culmination in the form of production of cercariae, which, once started, may persist for the remainder of the life span of the infected snail [3].

Chemical fertilizers may reach water sources during agriculture activities and may kill snails or make their environmental conditions unsuitable for their life [4-6]. Many researchers tried to cutoff the compatibility between Schistosoma trematodes and their intermediate hosts using molluscicides either chemical or natural materials, where molluscicides interrupt the life cycle of the parasite and help for eliminating the disease [7].

The shedding of *S. mansoni* cercariae from infected *B. alexandrina* snails stopped at 1 ppm of Myrrh treatment and suppressed at 0.8 ppm and the emerged cercariae from treated snails incapable of infecting humans [8]. While [9] observed that the treatment of infected *B. alexandrina* snails with sublethal concentrations of *Furcraea selloa marginata* and *Bacillus thuringiensis* for 24 hours either pre, during or post exposure of snails to *S. mansoni* miracidia caused a marked reduction in the infection rate and the mean total number of shedding cercariae/snail.

Also, the cercarial production of *S. mansoni* infected *B. alexandrina* snails were completely stopped under the effect of sublethal concentration of Mirazid [10]. The suppression of cercarial production were noticed by [11] in case of Regent and Mimic insecticides, [12] in case of Topas fungicide and [13] in case of Match and Vertimec pesticides against infection of *B. alexandrina* snails with *S. mansoni.* In addition, the infectivity of *S. haematobium* miracidia to *B. truncatus* was greatly reduced by the tested sublethal concentrations of methanol extract of *Sesbania sesban* plant, the sublethal concentrations caused considerable reduction in cercarial production and the period of cercarial shedding [14].

The present work investigated the effects of (¼ LC_{50}) sublethal concentration of three separate inorganic fertilizers on survival and infection rate of the infected *Biomphalaria alexandrina* snails with *S. mansoni* miracidia, the cercarial production of these snails was evaluated, as well as, the histopathological alterations in the digestive glands of *B. alexandrina* infected snails, associated to this treatment.

MATERIALS AND METHODS

Experimental Materials

Fertilizers are purchased from Misr el dawliya for Agricultural and Industrial Development Company. Three types of complex mineral nitrogen, phosphorus and potassium (N:P:K) fertilizers as following: balanced content fertilizers (N:P:K, 20:20:20); high phosphorus content fertilizers (N:P:K, 5:40:5) and high nitrogen content fertilizers (N:P:K, 35:5: 5).

Experimental Animals

Biomphalaria alexandrina snails (8 – 10 mm shell diameter) used in this work were obtained from Schistosome Biological Supply Program (SBSP) in Theodor Bilharz Research Institute (TBRI), Giza, Egypt. They maintained in aquaria filled with dechlorinated water under laboratory conditions (temperature 25° ±2°C) and (pH 7-7.7) for three weeks [15]. The snails were provided daily with fresh lettuce leaves as a food source.

Miracidia

Schistosoma mansoni ova used in this study were obtained from Schistosome Biological Supply Program (SBSP) in Theodor Bilharz Research Institute (TBRI), Giza, Egypt. The ova were allowed to hatch in small amount of dechlorinated water for about 15 minutes under direct light to give miracidia to use in the infection experiment.

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Bioassay tests

Molluscicidal Activity

A stock solution of 1000 ppm was prepared from each fertilizer on the basis of w/v using dechlorinated water (pH 7.0-7.7). A series of concentrations that would permit the computation of LC50 and LC90 were prepared. Three replicates were used, each of ten snails (6-8 mm)/L. Another 3 replicates in dechlorinated water were used as control. Exposure and recovery periods were 24 hours each at 25°C ± 1°C. Then, snails' mortality was recorded [6, 16]. The determination of LC_{50} and LC_{90} values and slope function carried out by using the statistical program SPSS version 17 (SPSS, Inc., Chicago, IL) [17] for windows.

Exposure of Snails to Miracidia

Adult snails (8-10 mm shell diameter) were exposed individually to 4-5 miracidia per snail in glass test tubes filled with 2ml dechlorinated tap water, exposure period was 24 hrs under fluorescent light and temperature (24˚ ±1˚C) as described by [18]. On the next day, snails were divided into four groups, each group contains thirty snails, three groups exposed to ¼ LC_{50} (126.4, 400 and 2375 ppm) separately of (balanced, high phosphorus and high nitrogen) fertilizers, respectively for one week. The fourth group of snails post exposed to miracidia maintained as control group without fertilizers treatment. Examination of snails for cercarial shedding was carried out twice weekly, 21 days post exposure, the cercarial suspension was poured in a graduated petri dish, then few drops of Bouin's fluid were added and all cercariae were counted, using a dissecting microscope. Shedding snails were then isolated and kept in special aquaria in complete darkness.

Histopathological Examination

Infected snails exposed to ¼ LC_{50} (126.4, 400 and 2375 ppm) separately of (balanced, high phosphorus and high nitrogen) fertilizers, respectively for one week. Untreated infected group maintained as control group. Then the control and exposed snails maintained for another week for recovery period. The digestive glands of all groups were removed from their shells, fixed in Bruin's fluid for 5 hrs and then transferred to 70% alcohol. Further procedures included dehydration in 100% alcohol, clearing in xylol and paraffin embedding were followed, finally sectioned at 6µ. Sections were stained with Hematoxylin and Eosin stain (H&E), dried then microscopically examined and photographed by a Zeiss Video camera,Germany [19].

Statistical Analysis

The survival rate and infection rate were analyzed by Chi-square values of contingency tables were conducted using the statistical program SPSS version 17.0 (SPSS, Inc., Chicago, IL) [17] for windows.

RESULTS

The calculated half lethal concentrations (LC_{50}) values were 505.7, 1600 and 9500 ppm for balanced, high phosphorus and high nitrogen composite fertilizers (N:P:K), respectively after 24 hours exposure (Table: 1)

Table (1): Probit analysis of toxic effect of three composite fertilizers (N:P:K) on adult *Biomphalaria alexandrina* snails after 24 hours of exposure.

Tested Fertilizers	LC_{50} (ppm)	Confidence limit of LC_{50} ppm	LC_{90} (ppm)	Slope	$^1/_4$ LC_{50} (ppm)
N:P:K 20:20:20 Balanced	505.7	390.31 - 625.33	851.2	1.89	126.4
N:P:K 5:40:5 High phosphorus	1600	1356.56 - 1843.4	2309.3	1.45	400
N:P:K 35:5:5 High nitrogen	9500	8891.4 - 10108.5	11273.3	1.16	2375

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Table (2): Effect of sublethal concentration (¼ LC_{50}) of each tested fertilizer on infected *B. alexandrina* snails with *Schistosoma mansoni* miracidia.

¼ LC_{50} concentration of tested fertilizers	Total exposed snails	survival snails at 1st shedding		Infection rate		Prepatent period (days)		Cercarial production (number of cercariae/ snails)	
		No.	%	No.	%	range	mean± SD.	range	mean± SD.
Balanced (126.4 ppm)	30	13	43.3	9	69.2	ND	--	ND	--
High phosphorus (400 ppm)	30	9	30.0 **	5	55.5 *	ND	--	ND	--
High nitrogen (2375 ppm)	30	8	26.6 **	4	50.0 **	ND	--	ND	--
Control	30	15	50.0	11	73.3	24-28	25.81±1.4	428-1232	676.72± 274.2

ND = prepatent period and cercarial production was not detected. Prepatent period is the developmental time of Schistosmoa mansoni in snails (Pflüger, 1980)

** Significant between control and each dose of tested fertilizer at (P<0.01).

Results in table (2) reflected the significant effect of sublethal concentration (¼ LC_{50}) of each tested fertilizers on the infected snails, where the ratio of survival rate of infected snails that were treated with (126.4 ppm) of balanced fertilizer was decreased and reached 43.3%. Moreover, the infected snails that were exposed to (400 and 2375 ppm) of high phosphorus and high nitrogen fertilizers, their ratio of survival rates was markedly depressed reaching 30.0 and 26.6 %, respectively with significant difference at (P< 0.01) from the control group 50% at the 1st shedding (Fig. 1).

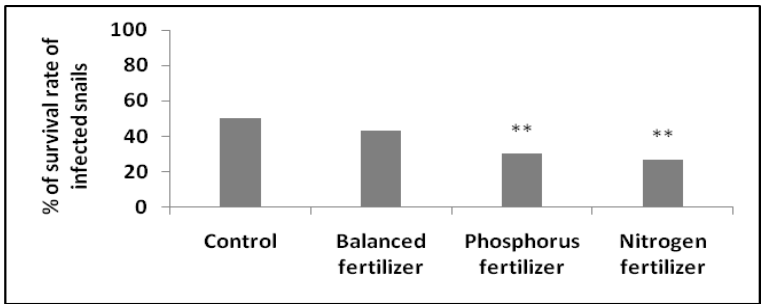

Fig (1): Effect of sublethal concentration (¼ LC_{50}) of each tested fertilizer on survival rate at 1st shedding of *Biomphalaria alexandrina* exposed to *Schistosoma mansoni* miracidia.

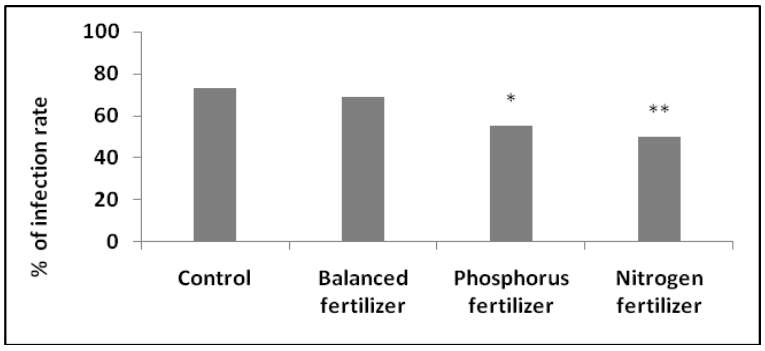

Fig (2): Effect of sublethal concentration (¼ LC_{50}) of each tested fertilizer on infection rate of *Biomphalaria alexandrina* exposed to *Schistosoma mansoni* miracidia.

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Positive dead infected snails/week during the experiment helped us to calculate the infection rate for snails that were treated with fertilizers. Data revealed that infection rate was decreased reaching (69.2, 55.5 and 50.0%) for balanced, high phosphorus and high nitrogen fertilizers compared to 73.3 % of untreated infected group (Fig. 2). While prepatent period could not be determined in all treated groups because they were dead without production of cercariae, prepatent period of control group range was (24-28 days) and cercarial production range was (428- 1232 cercariae/ snails) as shown in table (2).

Histopathological Examinations

Mother Sporocysts of Untreated Infected B. alexandrina Snails

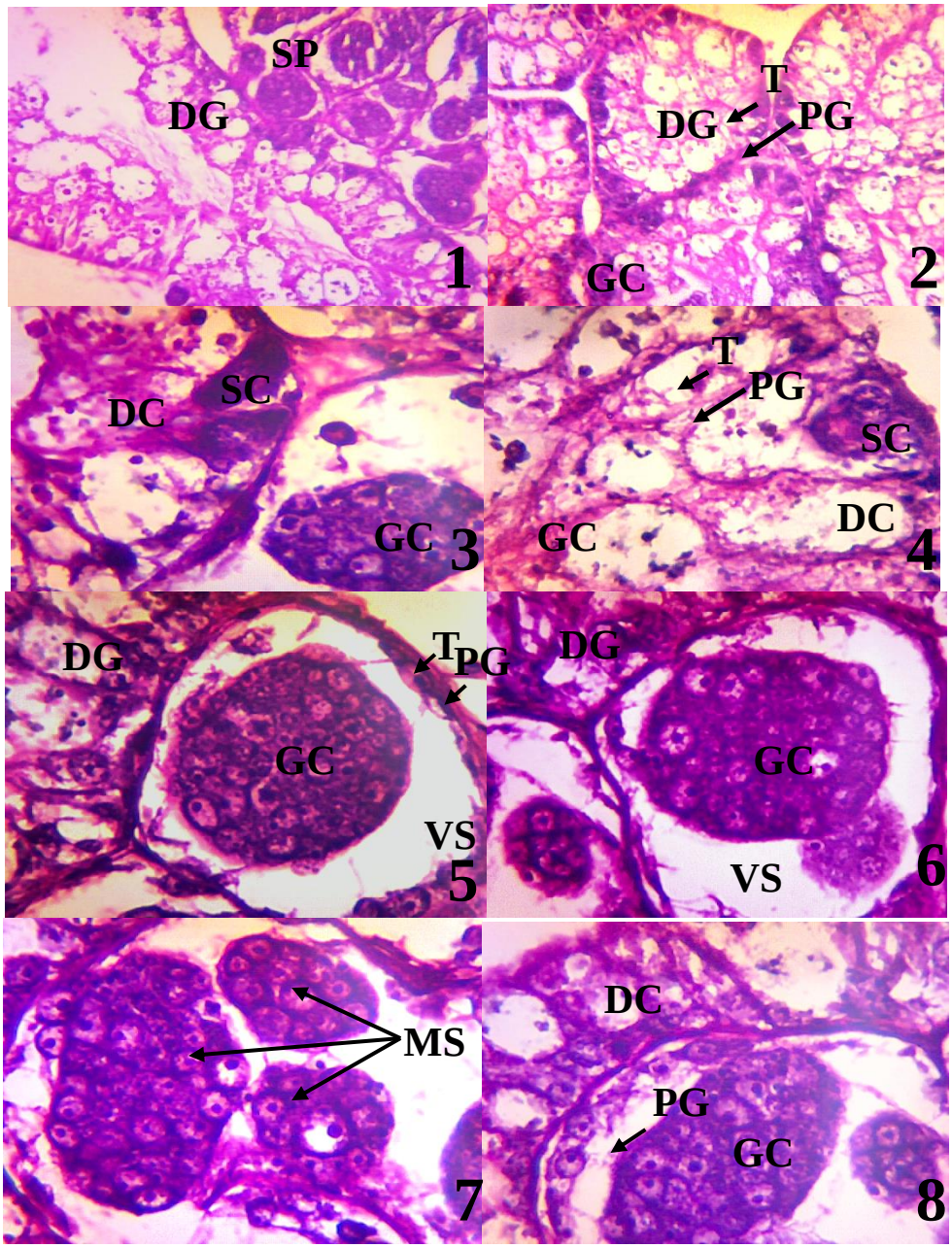

Plate (A): Section in the digestive gland of untreated infected *Biomphalaria alexandrina* snails (Hematoxylin and Eosin stained).

1 and 2 = x 100 3 to 8 = x 400

DG: Digestive gland, SP: Sporocyst, SC: Secretory cell, DC: Digestive cell, GC: Germinal cells, T: Tegument, PG: Penetration gland, VS: Vacant space, MSP: Multiple sporocysts.

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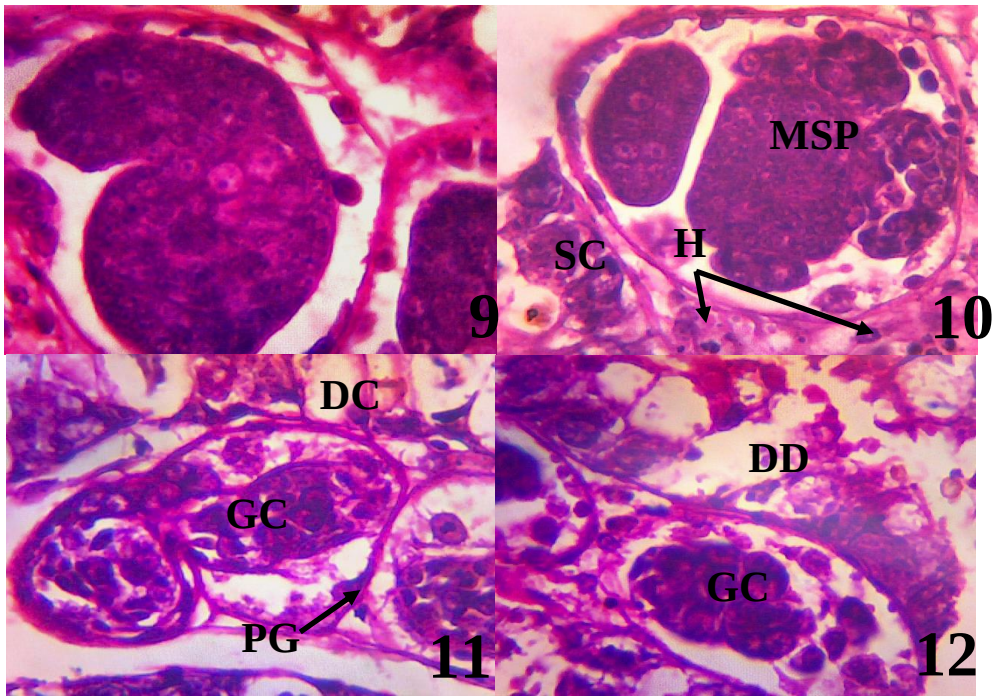
After about 14 days of infection the mother sporocysts become stable in the digestive gland of snails, the digestive gland consists of two types of cells; digestive cells which are columnar and including granulated acidophilic cytoplasm, with spherical nuclei located at the basal region and secretory cells which are pyramidal in shape, with large spherical nuclei laying in the basal part of the cells (Plate A: sections 1, 2, 3 & 4).

Mother sporocysts consist of sac-like organisms; have cluster or masses of germinal cells, which are spherical in shape possess increased amount of cytoplasm and have rounded or oval nucleus with prominent nucleolus. They rely on absorption of its nutriment through its outer cuticle layer or tegument. The sporocyst tegument surface serves as an important interface for molecular communication between the parasite and molluscan intermediate host. Tegument has cells of penetrating glands helping in penetration of snail tissues. The space between cluster and tegument called vacant space (Plate A: section 5). Mother sporocysts, during infection, have four types (single, multiple, mature and migratory). In the present work, results showed two types (single and multiple mother sporocysts) after 14 days of infection (Plate A: sections 6, 7 & 8). Also, it was noticed the lack of tissue reaction towards the parasite in untreated snails.

Histopathological Changes of Treated Infected B. alexandrina Snails

The effect of Balanced Fertilizers (N:P:K, 20:20:20): The mother sporocysts have almost the typical mentioned form of untreated one. Also the two types of single and multiple mother sporocysts are found **(Plate B: sections 9 &10)**. Sublethal concentration (¼ LC_{50}) of balanced fertilizer has no effect on mother sporocyst directly, but it enhanced the tissue response showed in representing some hemocytes inflow around multiple mother sporocysts trying to eliminate them (Plate B: section 10). In little sections damaged tegument of sporocyst was detected (Plate B: section 13). On the other hand, the digestive gland suffered due to the effect of the used sublethal concentration of balanced fertilizer, where it became vacuolated, and missed their nuclei, while the other digestive gland were degenerated and ruptured (Plate B: sections 11, 12, 13 & 14).



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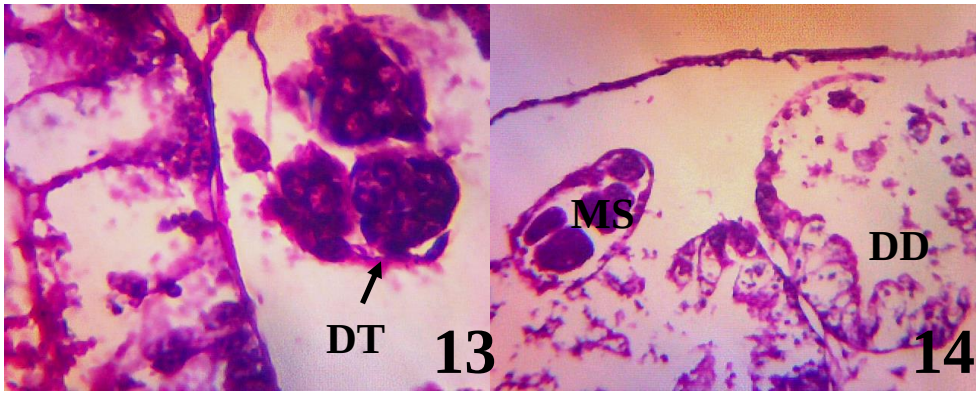
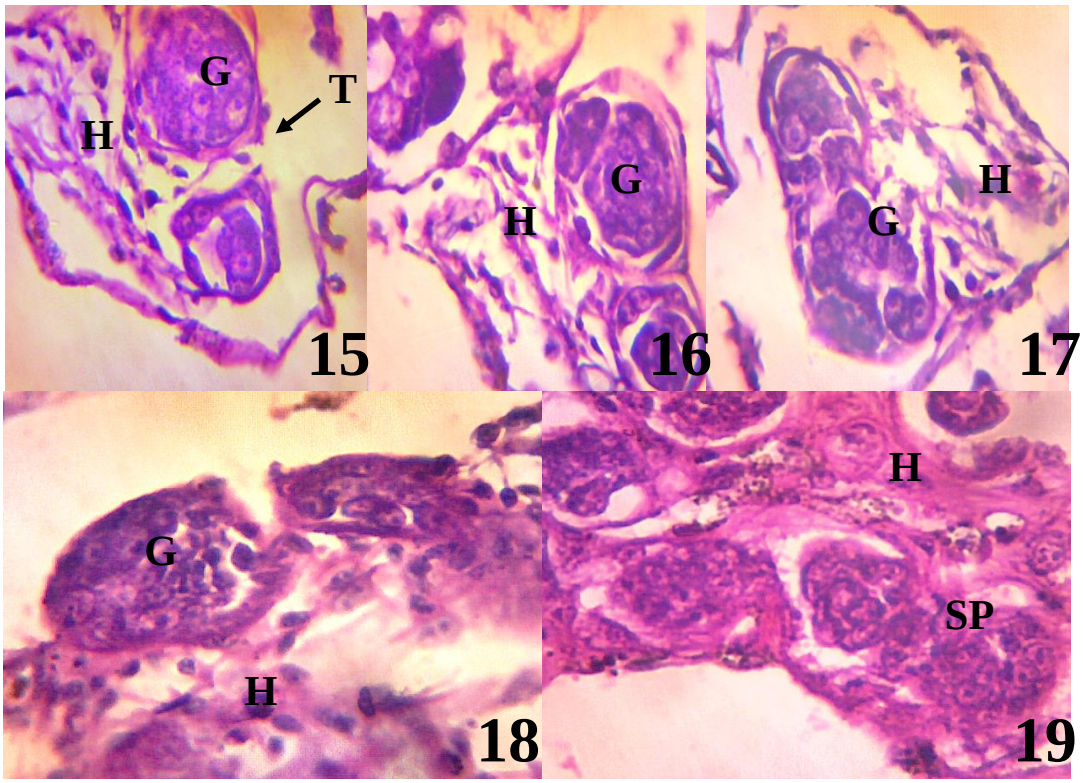


Plate (B): Section in the digestive gland of infected *Biomphalaria alexandrina* snails treated with sublethal concentration (¼ LC_{50}) of balanced fertilizer (Hematoxylin and Eosin stained).
9 to 13 = x 400 14 = x 100
GC: Germinal cells, T: Tegument, PG: Penetration gland, SC: Secretory cell, H: Hemocytes, MSP: Multiple sporocysts, DC: Digestive cell, DDC: Degenerated digestive cell, DT: Damaged tegument, DDG: Degenerated digestive gland.

The effect of High Phosphorus Fertilizers (N:P:K, 5:40:5): Infected snails that were exposed to sublethal concentration (¼ LC_{50}) of high phosphorus fertilizer, showed mother sporocysts from single type with small size of germinal cells, having the typical structure and surrounded by numerous distinct hemocytes that were trying to encapsulate sporocysts (Plate C: sections 15, 16 &17). Also, the valuable cellular reactions in response to eliminating the development of sporocysts observed in the degeneration of the tegument of sporocysts (Plate C: section 18), which underwent encapsulation (Plate C: section 19), while the digestive gland almost appears as normal, except little vacuoles were found in digestive cell and increase in number of secretory cells (Plate C: sections 20 & 21).



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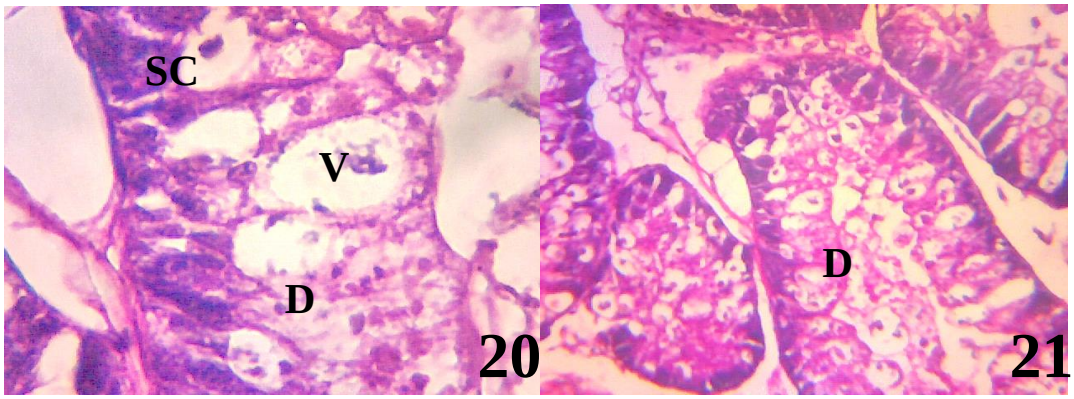

Plate (C): Section in the digestive gland of infected *Biomphalaria alexandrina* snails treated with sublethal concentration (¼ LC_{50}) of high phosphorus fertilizer (Hematoxylin and Eosin stained).
15 to 20 = x 400 21 = x 100
GC: Germinal cells, H: Hemocytes, T: Tegument, SP: Sporocyst, DC: Digestive cell, SC: Secretory cell, V: Vacuole, DG: Digestive gland.

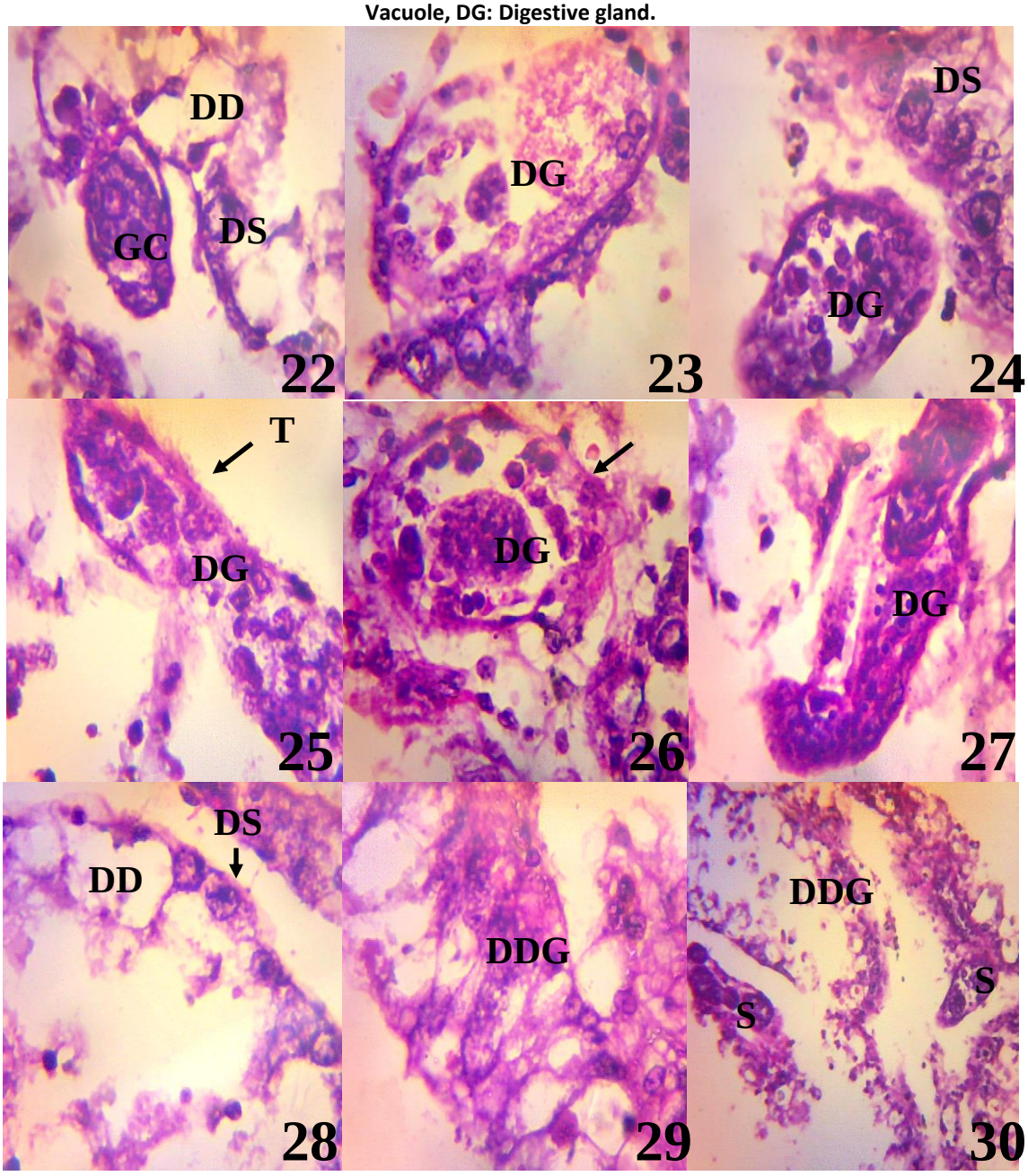

Plate (D): Section in the digestive gland of infected *Biomphalaria alexandrina* snails treated with sublethal concentration (¼ LC_{50}) of high nitrogen fertilizer (Hematoxylin and Eosin stained).
22 to 29 = x 400 30 = x 100
DDC: Degenerated Digestive cell, DSC: Degenerated Secretory cell, GC: Germinal cells, DGC: Damaged Germinal cells, T: Tegument, DT: Damaged Tegument, DDG: Damaged Digestive gland, SP: Sporocyst.

The effect of High Nitrogen Fertilizers (N:P:K, 35:5:5): The most destructive effect in the mother sporocysts was shown with the infected snails that were subjected to sublethal concentration (¼ LC_{50}) of high nitrogen fertilizer. Plate D, (sections 22, 23 & 24) showed a huge degeneration of tegument of sporocysts and germinal cells. Other sporocysts lost their identical shape resulting in dissolution of their tegument and then dispersed germinal cells were observed (Plate D: sections 25, 26 & 27). A marked effect on digestive gland overall was observed in the treated infected snails, where the digestive cell was missing their typical shape, ruptured and degenerated. Furthermore, the secretory cells suffered from the disfiguring of their shapes, further more these cells appeared damaged and hardly detected through some remained nuclei Plate D: sections 28, 29 & 30).

DISCUSSION

The effect of sublethal concentration (¼ LC_{50}) of the three tested fertilizers types on the infected *B. alexandrina* snails for one week exposure period showed that the survival rates of snails at 1st shedding were significantly less than their corresponding control group. These results were in accordance with the previous studies for *B. alexandrina* snails post their exposure to the fungicide Topas [12] ; the pesticides Match and Vertimec after 3 weeks of snails exposure to *S. mansoni* miracidia [13] and the pesticides Basudin and Selecron and the phytoalkaloid Colchicine against infected *B. alexandrina* snails [20].

Moreover, results revealed a marked reduction in the infection rates of the infected snails especially that exposed to sublethal concentration of high nitrogen fertilizers when compared to the control. These results were in agreement with [6] who observed that chelated copper fertilizers caused higher reduction in snail infection rate than chelated zinc.

However, the cercarial production of all treated snails was completely suppressed. This suppression might be due to the accumulation of the tested compounds (fertilizers) in the snails. This was recorded by [11] in case of the insecticides Regent and Mimic, [12] in case of the fungicide Topas and [13] in case of the pesticides Match and Vertimec against infection of *B. alexandrina* snails with *S. mansoni.* Similar observation was recorded on infected *B. truncatus* with *S. haematobium* post exposed to the pesticides Chlorpyrifos and profenofos [21]. Moreover, possible deterioration in the physiological parameters of treated snails resulted in lowering the developmental rate of the parasite within these snails, shortening the duration of cercarial shedding and reducing the cercarial production/ infected snail [22].

In the present work, all tested fertilizers induced histopathological changes of both digestive gland and mother sporocysts development. The most prominent severe damage was clear in case of high nitrogen fertilizer treatment, where the digestive cells missed their typical shape, ruptured and degenerated. Furthermore, the secretory cells suffered from disfiguring their shape, damaged and barely distinguished through some remnants nuclei. Moreover, the most destructive effect in the mother sporocysts was shown as huge degeneration of tegument of sporocysts and germinal cells. Other sporocysts lost their identical shape resulting in dissolution of their tegument and then dispersed germinal cells were observed. This result was in agreement with [10] who stated that exposure to Mirazid combined with *S. mansoni* infection led to degeneration of germinal epithelial layer, disappearance of central lumen, appearance of immature sporocysts and vacuoles.

On the other hand, digestive glands of infected snails exposed to the sublethal concentration of balanced fertilizer, lost their nuclei and appeared vacuolated, while others were degenerated and ruptured. Meanwhile, the sublethal concentration of balanced fertilizer has no effect on mother sporocyst directly, but it was found enhancement of the tissue response represented by some hemocytes inflow around multiple mother sporocysts trying to eliminate them. In little sections damaged tegument of sporocyst was detected.

On the contrary, the digestive gland of infected snails that were exposed to sublethal concentration of high phosphorus fertilizer showed a moderate effect, almost appeared normal, except for little vacuoles found in digestive cells and the number of secretory cells increased. Also, single type of mother sporocysts appeared with small size of germinal cells, having the typical structure and surrounded by numerous distinct hemocytes that trying to encapsulate sporocysts. The effective cellular reactions which were responsible for eliminating the development of sporocysts were observed in the degeneration of the tegument of sporocysts, which underwent encapsulation.

So, it could be concluded that the success or failure of snails' infection with trematodes depends on snails' humoral factors, or internal defense system, mainly circulating hemocytes and plasma factors [23-25]. Furthermore, the lack of tissue reaction towards the parasite in untreated snails was noticed. This may be attributed to the compatibility of B. alexandrina snails to S. mansoni. These results were in agreement with [26] who found that *B. alexandrina* stocks of SBSC-TBRI have more susceptibility and higher cercarial production than the other snail groups that were collected from different Egyptian governorates. This was also, in accordance with [27] who noticed that there were neither dead parasites nor cellular reactions around the living ones. It seems that there is a tolerance in the snail tissue to the presence, growth and multiplication of the larval stages of the parasite.

Conflict of interest: The author alone is responsible for the content and writing of this article.

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