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Immunological Effect of Cu_2O Nanoparticles on *Biomphalaria alexandrina* Snail the Intermediate Host of *Schistosoma mansoni* in Egypt

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ABSTRACT

Background: Control of the schistosomiasis is dependent on prevention and breaking the life cycle of the trematode by reducing snail populations in the pond. Nanomaterials have unique properties compared with their larger counterparts, due to small size and hence higher specific surface area of the nanoparticles. Aim: The present study aims to evaluate the effect of cuprous oxide nanoparticles (Cu_2O NPs) on enzymatic defense activity of *Biomphalaria alexandrina (B. alexandrina)* snail and its implications to infection rate and cercarial production of exposed snails to *schistosoma mansoni* (*S. mansoni*) miracidia. *Methods:* The activities of catalase (CAT), Glutathione reduced (GSH), Total antioxidant capacity (TAC) and Nitric oxide (NO) were assayed in hemolymph of (*B. alexandrina*) snails exposed to acute (48 h) and chronic (7 day) sub lethal concentration (LC_{25},1.13 PPM) of Cu_2O NPs using colorimetric method. The infection rate and cercarial production, from these snails, was evaluated using light microscope. *Results:* Both TAC and CAT have increased significantly while GSH was has not significantly increased in the hemolymph of snails on acute and chronic exposure than control congeners. On the other hand, NO has significantly decreased in these snails. The infection rate of *B. alexandrina* treated with Cu_2ONPs and then exposed to Egyptian strain of *S. mansoni* miracidia was less than that of control (infected but not treated).This control exhibited a longer life span, duration of cercarial shedding and a higher number of shedding cercariae than snails treated with Cu_2ONPs. *Conclusions:* Exposure to Cu_2O NPs has increased the adaptive defense of snails through Cat, TAC and GSH so that the infection rate and cercarial production have decreased. The non-specific defense through NO has decreased in these snails.

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Introduction

Egypt is one of the many countries that have suffered greatly under the burden of tropical diseases including schistosomiasis (Hotez *et al*., 2012). Human infection with *Schistosoma mansoni* is closely related to the existence of its intermediate snail host of the genus *Biomphalaria*. *Biomphalaria alexandrina* is the only snail host in Egypt (Abou-El-Naga *et al*., 2011). Cu compounds have been used as molluscicide. Nanotechnology is used to modify material at the nano-scale (<100 nm) to create novel properties. Changes in the physicochemical and structural properties of materials caused by the decrease in particle size can lead to new and sometimes unexpected biological effects. Therefore, engineered NPs need to be evaluated in terms of their potential to pose risks to human health and the environment (Handy *et al*., 2008; Nowack, 2009). Nano-CuO was highly toxic when compared to the bulk form of CuO, to other metal oxide nanoparticles as well as to carbon nanoparticles and carbon nanotubes in the human alveolar epithelial cell (Karlsson *et al*., 2008, 2009). Molluscicides mostly have oxidative stress effect on the snails. Oxidative stress occurs in living organisms when the rate of generation of oxygen radicals exceeds the rate of their decomposition (Sies, 1986). Most living organisms depend on ATP generation by oxygen-based metabolism, but one consequence of oxygen dependence is the production of reactive oxygen species (ROS), mainly as byproduct of oxidative metabolism. The mitochondrial electron transport chain and a variety of cellular oxidases are the main sources of ROS generation (Zhang *et al*., 2003).

The present study aims to evaluate the effect of cuprous oxide nanoparticles (Cu_2O NPs) on the non-specific (NO) and specific (CAT, GSH and TAC) immune defense activity of *B. alexandrina* snail and its implications to infection rate and cercarial production of snails exposed to *S. mansoni* miracidia.

Materials and Methods

Chemicals:

Cuprous oxide nanoparticles (Cu_2O NPs):

Cu_2O NPs were prepared with copper sulfate ($CuSO_4$+$5H_2O$, El-Gomhouria Chemical Company, Egypt) as starting material via a simple technique. Exactly 20 mL of NaOH aqueous solution (0.075 mol/L, El-

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Gomhouria Chemical Company, Egypt) was added into 10 mL of $CuSO_4$ aqueous solution (0.5 mol/L) with stirring (pH=10.5). Then, 25 mL of ascorbic acid aqueous solution (0.1 mol/L, Merck Company, Germany) was added drop wise into the above solution with vigorous stirring. After 1 h, a yellow precipitate was obtained (pH = 4–4.5). The particles were separated from the solution by centrifugation at 2000 rpm for 30 min. The product was washed by distilled water and absolute ethanol. The final product was dried in vacuum at 60 ºC for 8 h (Selim *et al.,* 2015).

Characterization of Cu_2O nanoparticles:

Nanoparticles prepared were characterized with the help of multiple techniques such as: scanning electron microscopy (SEM, JEOL JSM-5600) to study the surface morphology of nanoparticles (Fig 1A); size of the resulting nanoparticles was analyzed using transmission electron microscope (TEM, EM 208S Philips, Netherlands) connected to a high resolution imaging system. Samples for TEM studies were prepared by placing drops of nanoparticles solutions on carbon-coated TEM copper grids (Fig 1B) and X-ray fluorescence (XRF) that was performed to learn about the main chemical compositions and elemental analysis of the minerals that are present in nanoparticles. XRF measurements were carried out using the JSX-3222 element analyzer (Fig 1C-D).

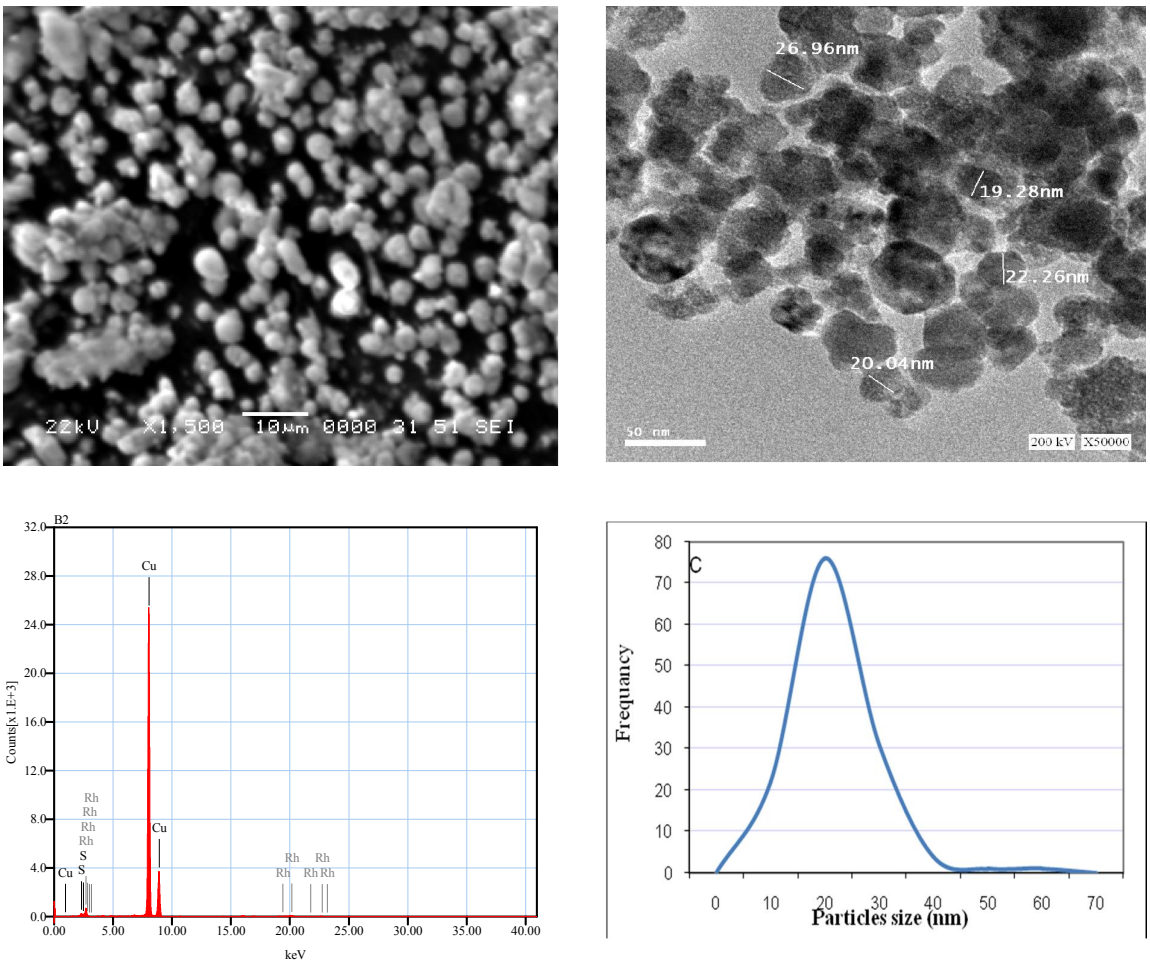

Fig. 1: (A) scanning electron microscopy, (B) transmission electron microscope for cuprous oxide nanoparticles(Cu_2O NPs), (C): XRF of Cu_2O NPs and (D): Particle size distribution of Cu_2O NPs.

Snails:

B. alexandrina snails were obtained from Egyptian laboratory stock at Malacology Department, Theodor Bilharz Institute, Egypt. Snails were maintained, as stock cultures, in a well-prepared snail room, under suitable environmental conditions, in glass aquaria containing dechlorinated tap water in a density of 10 snails / L. The snails were fed on fresh lettuce leaves, supplemented with tetramine (fish food) and chalk, after careful selection on the basis of size and age.

Toxicity test:

The present experiment was carried out by preparing three replicates of gradual concentrations from each stock solution. Ten snails (8-10mm width) were used in each replicate. The snails were exposed to the tested concentrations (0.16, 0.52, 1.13, 1.53 and 2.29 PPM) for 48 hours, then removed from the experimental concentration, washed with tap water and kept in 1 liter of dechlorinated tap water for next 24 hours for recovery (25±1ºC). Unexposed snails (control) were assayed side by side with the treated groups (WHO, 1965 b). Dead snails were recorded as the average of the three replicates. Death of snails was distinguished by immersion of snails in a small amount of 15–20% sodium hydroxide solution (Nolan *et al*., 1953); if bubbles and blood come out of snail, it is recorded as alive and if not, it is recorded as dead. The effectiveness of these components as a molluscicide has been expressed in terms of LC_{50} and LC_{90} according to the procedure of Litchfield andWiLcoxon (1949).

Miracidia:

Schistosoma mansoni ova used in this study were obtained from Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), where, ova were taken from previously infected mice. The ova were allowed to hatch in small amount of dechlorinated water (24°C) for about 15 minutes under a direct light. Then, the hatched miracidia were used in the experimental tests.

Hemolymph:

Snail hemolymph was collected using the techniques described by Michelson (1966). The hemolymph was obtained via a small hole made in the shell into which capillary tube was inserted then it was drawn into tube by capillary suction.

Enzyme assays:

Total Antioxidant Capacity (TAC): The determination of the antioxidative capacity is performed by the reaction of antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide (H_2O_2). The antioxidants in the sample eliminate a certain amount of the provided hydrogen peroxide. The residual H_2O_2 is determined colorimetrically by an enzymatic reaction which envolves the conversion of 3,5,dichloro –2– hydroxy benzensulphonate to a colored product (Koracevic, 2001).

Glutathione reduced (GSH): The method based on the reduction of 5,5` dithiobis (2 - nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound . The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm (Beutler *et al*., 1963).

Catalase (CAT): The enzyme catalyzes the detoxification of H_2O_2 produced after the dismutation of O^{2-} by SOD. It was assayed according to Lubinksy and Bewley (1979) using 10 mM H_2O_2.The decrease in absorbance was read at 230 nm and the activity of the enzyme was calculated using an extinction coefficient 25 for the enzyme. a-Glycerophosphate dehydrogenase activity was assayed using d-glyceraldehyde 3-phosphate as substrate (10 mM) and NAD (15 mM) in 0.1 M phosphate buffer, pH 7.0 (Lemaire *et al*., 1996). The increase in absorbance was measured at 340 nm.

Nitric Oxide (NO) is synthesized in biological system by the enzyme Nitric Oxide Synthase (NOS). NOS is a remarkably complex enzyme which acts on molecular oxygen, arginine, and NADPH to produce NO, citrulline, and $NADP^+$. NOS O^{2+} $Arginine^+$ NADPH. NO^+ $Citrulline^+$ NADP NO is produced in trace quantities by neurons, endothelial cells, platelets, and neutrophils in response to homeostatic stimuli. This NO is scavenged rapidly ($t_{1/2}$ = 4 seconds) and acts in a paracrine fashion to transducer cellular signals. NO is also produced by other cells (macrophages, fibroblasts, hepatocytes) in micromolar concentrations in response to inflammatory or mitogenic stimuli. The final products of NO in vivo are nitrite (NO^{2-}) and nitrate (NO^{3-}). The relative proportion of NO^{2-} and NO^{3-} produced from NO is variable. The exogenous source of NO_3 ingested in the diet should be considred and cannot be ignored (none-NO origine). Thus, one of the indexes of NO production is the NO_2. The Biodiagnostic Nitrite Assay Kit provides an accurate and convenient method for measurement of endogenous nitrite concentration as indicatore of nitric oxide production in biological fluides. It depend on the addition of Griess Reagents which convert nitrite into a deep purple azo compound, photometric measurement of the absorbance due to this azo chromophore accurately determines NO^{2-} concentration (Montgomery and Dymock 1961).

Infection and cercarial production:
Snail Exposed to Miracidia:

Three replicates, each of 30 lab-bred *B. alexandrina* snails'offspring (4-6 mm in diameter) were exposed to newly hatched miracidia. The number of miracidia used was 10 miracidia / snail. The snails were exposed to miracidia individually in 15x17 mm glass vials with 1.0 ml dechlorinated aerated tap water, under fluorescent light from 20 watt tubes, 30 cm far and temperature 25°C±2°C. On the next day, the exposed snails were transferred to and maintained in standard aquaria previously described.

The experimental snails were randomly divided into two groups. The snails of the first group were exposed for 48 hrs to Cu_2O NPs Infection was carried out by the method described by Watson & Abdel- Azim (1949).The second group was used as control. The experimental snails were kept individually in 500 ml jars and maintained under constant temperature of 25 ± 2°C. A photoperiod of 12 h per day was applied; each snail was supplied daily with one bunch of lettuce (about 15 cm^2). Observations were recorded for both control and infected snails.

Examination of exposed snails for cercarial shedding:

Starting from the day 21 post miracidial exposure, the snails were examined individually and repeatedly for cercarial shedding in multi dishes under artificial light for two hours (stimulant period) and 2 ml of dechlorinated tape water/snail. After initial shedding was observed, snails were screened individually twice weekly till the death of snails (Chernin and Dunavan, 1962). The snail's infection rate was calculated at the end of experiment by dividing number of shedding on the number of exposed snails and the survival rate was calculated by dividing the number of snails at first shedding on the total number of exposed snails (Yousif *et al.,* 1996). At the first day of detecting cercariae, positive snails were separated individually in a plastic cups. The produced cercariae/snail were transferred to a small Petri dish by a Pasteur pipette, fixed in Bouin's solution and counted under a stereomicroscope. This examination was repeated weekly. The period between miracidial exposure and the first shedding of cercariae for each snail was considered as the incubation period (prepatent period). The time elapsed from first cercarial shedding till stopping of cercariae production was reported as duration of cercarial shedding.

Statistical analysis:

Data were analyzed applying the Chi Square and T-test, and ratio test to achieve these statistical Tests, Minitab software (MINITAB® Release 14.1) was used.

Results

The current work was carried out to evaluate some immune defense antioxidant enzymes in hemocytes of adult *B. alexandrina* snails that had been acutely and chronically exposed to cuprous oxide nanoparticles (Cu_2O NPs), with its implications to infection rate and cercarial production of snails exposed to *S. mansoni* miracidia.

Toxicity test:

This experiment was planned to elucidate the molluscicidal properties of Cu_2O NPs against adult *B. alexandrina* snails after 48 hours (hrs) of exposure followed by another 24 hrs for recovery.

Data presented in Table (1) show values of LC_{90}, LC_{50} and LC_{25} against *B. alexandrina* snails treated with tested Cu_2O NPs (2.29, 1.53 and 1.13ppm respectively).

Table 1: Molluscicidal activity of cuprous oxide nanoparticles (Cu_2O NPs) against adult *Biomphalaria alexandrina* snails (48 hours exposure).

Lethal concentration	LC_0Ppm	LC_{10}Ppm	LC_{25}Ppm	LC_{50}Ppm	LC_{90}Ppm	Slope
Cu_2O NPs	0.16	0.52	1.13	1.53	2.29	1.54

Antioxidant:

The chronic exposure of *B. alexandrina* snail to LC_{25} causing dose of Cu_2O NPs showed that, catalase (CAT) and total antioxidant capacity (TAC) has a high significantly increased (22.12± 1.20 and 7.31± 0.39) than control (2.32± 0.20 and 1.29± 0.17 respectively, Table 2 and Fig.2). Glutathione reduced (GSH) has also elevated (10.25± 0.30) than control (9.32± 0.09) but this increase is not significant. While, there was a high significant decrease to Nitric oxide assay (NO, 95.72± 3.80) than control (134.19± 4.00). On the other hand *B. alexandrina snail* that had acutely treated with LC_{25} of Cu_2O NPs showed that CAT significantly increased (4.15± 0.50) and TAC high significantly increased (5.74±0.16) than control (2.32± 0.20 and 1.29± 0.17 respectively, Table 2 and Fig.2)).GSH has also increased but not significantly (9.88± 0.63) than control (9.32± 0.09). Finally, NO high significantly decreased (64.85± 3.30) than control (134.19± 4.00).

Infection rate and cercarial production:

Effect of LC_{25} (1.13 PPM) of Cu_2O NPs on infection rate and cercarial production from *B. alexandrina* exposed to *S. mansoni* miracidia has been studied with particular reference to the control snails (untreated) on the cercarial production. Cercarial production is one of the most important factors constituting the transmission dynamics of schistosomiasis.

Table 2: Effect of acute and chronic exposure to sub-lethal concentration (LC_{25}) of cuprous oxide nanoparticles (Cu_2O NPs) on some antioxidants (immune defense) parameters of *Biomphalaria alexandrina* snail.

Antioxidant parameter	Acute exposure (LC_{25}1.13 PPM)	Chronic exposure (LC_{25}1.13 PPM)	Control
Catalase assay, CAT (mU/ L)	4.15± 0.50^{a*,b**}	22.12± 1.20^{a**}	2.32± 0.20
Glutathione reduced, GSH (mg/dl)	9.88± 0.63	10.25± 0.30	9.32± 0.09
Total antioxidant capacity, TAC (mM/L)	5.74±0.16^{a**b*}	7.31± 0.39^{a**}	1.29± 0.17
Nitric oxide assay, NO (mM/L)	64.85± 3.30^{a**b**}	95.72± 3.80^{a**}	134.19± 4.00

^{a} P< 0.05, ^{a}** P< 0.01 (acute and chronic compared to control), ^{b}* P< 0.05, ^{b}** P< 0.01 (acute compared to chronic)and Data expressed as Mean± Standard Error*

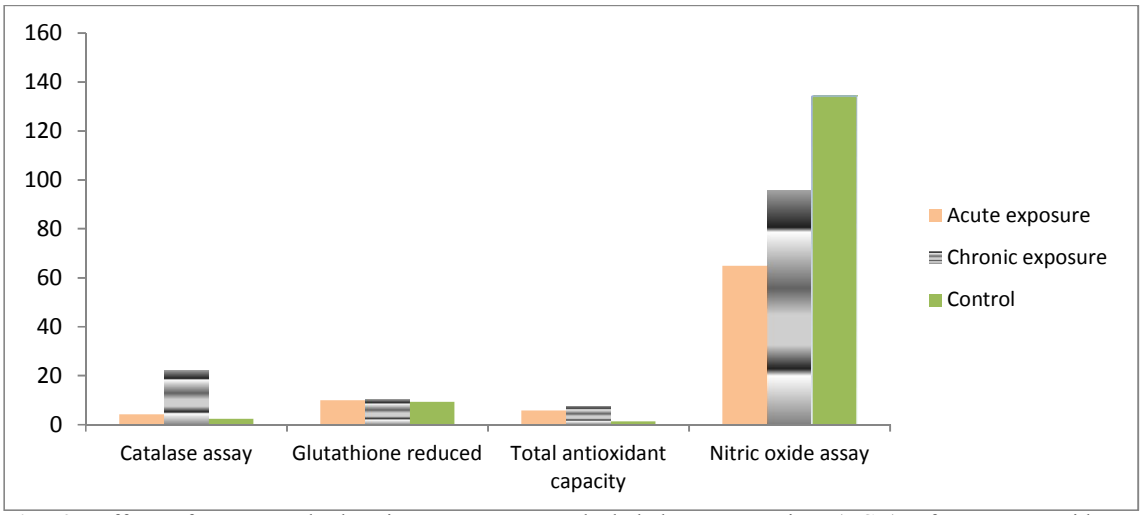

Fig. 2: Effect of acute and chronic exposure to sub lethal concentration (LC_{25}) of cuprous oxide nanoparticles (Cu_2O NPs) on some antioxidants (immune defense) parameters of *Biomphalaria alexandrina* snail.

Survival rate at first shedding and Infection rate:

The survival rate (Table 3) of two snail groups exposed to *S. mansoni* miracidia (SBSC-TBRI strain), at first cercarial shedding, showed that treated snails exhibited a non-significant decrease (60%) comparing to control (80%, P = 0.091). The decreasing in the infection rate (Table 3) also was not significant between snails treated with Cu_2O NPs (75%) and control group (87.5%, P = 0.327).

Table 3: Effect of sub-lethal concentration LC_{25} of cuprous oxide nanoparticles (Cu_2O NPs) on infection rate and cercarial production from *Biomphalaria alexandrina* infected with *Schistosoma mansoni.*

B. alexandrina Snails exposed to *S. mansoni* miracidia	Cu_2O NPs	Control	Pvalue
Infection rate	75%	87.5%	0.327
Survival rate at 1st shedding	60%	80%	0.091
Mean life span of snail at 1st shedding	14.11±2.8	25.14± 2.1	0.006**
Mean duration of shedding	11.25±2.7	20.07±1.9	0.021*
Duration of cercarial shedding	29 day	33 day	0.611
Mean no of cercariae/ snail	676.00±275	2324.00±364	0.001***
Cercarial production range	25-837	56-2390	
Mean no of cercariae/ stimulant	869.14± 176	3751.50± 868	0.014*
Mean no of cercariae/ snail/stimulant	264.52± 58	516.65± 84	0.001***

** P< 0.05, ** P< 0.01, ***P< 0.001 and Data expressed as Mean± Standard Error*

Life span of snailsand duration of cercarial shedding:

The mean life span of snail at 1st shedding of cercariae (Table 3) of the *B. alexandrina* snail treated with Cu_2O NPs (14.11±2.8) was high significantly decreased than control group (25.14± 2.1, P<0.01). The Mean duration of shedding from the *B. alexandrina* snail treated with Cu_2O NPs (11.25±2.7) was significantly decreased than control group (20.07±1.9, P<0.05).

Duration of cercarial shedding:

In the current study, there was no significantly decrease regarding the duration of cercarial shedding (Table 3) between *B. alexandrina* snail treated with Cu_2O NPs (29 day) and control (33 day).

Number of cercariae per snail and cercarial production Range:

According to the data presented in this work (Table 3), there was a very high significant decrease between the mean numbers of cercariae per snails treated with Cu_2O NPs (676.00±275 with Cercarial production Range 25-837) compared to control group (2324.00±364 with Cercarial production Range 56-2390, $P<0.001$).

Number of cercariae per stimulant:

Results presented in Table (3), showed that the mean number of cercariae per stimulant from *B. alexandrina* snail treated withLC_{25} doses of Cu_2O NPs was significantly decrease compared to control (869.14± 176 and 3751.50± 868, respectively, $P<0.05$).

Number of cercariae per snails per stimulant:

According to the current results, there was a very high significant decrease between the mean numbers of cercariae per snails per stimulant treated with Cu_2O NPs (264.52± 58) compared to control group (516.65± 84, $P<0.001$).

Discussion

Immunity of snails is determined mainly by the activity of circulating hemocytes and plasma factors (Barbosa *et al*., 2006 a, b). Haemocytes are mobile defence cells producing NO molecules (Bernice Wright *et al*., 2006) that eliminate pathogens in a nonspecific immune response (Moncada *et al*., 1991). Recently antioxidant enzymes have been used as biomarkers of pollutants that generate oxidative stress in aquatic animals (Ait Alla *et al*., 2006; Zapata-Vivenes and Nusetti, 2007; Siwela *et al*., 2010). The up-regulation of antioxidants' activity in the snails from the polluted sites could be an adaptive mechanism to prevent the accumulation of toxic reactive oxygen intermediates (Torres *et al*., 2002; Regoli *et al*., 2006). In the current investigation CAT, GSH, TAC and NO have been measured to determine the generated oxidative stress (both in-specific and adaptive) in *B. alexandrina* snails treated with Cu_2O NPs.

Oxidative stress occurs in living organisms when the rate of generation of oxygen radicals exceeds the rate of their decomposition (Jiaoqin Liu *et al*., 2016). Free radicals also are formed by homolytic bond fission, which can be induced by electron transfer to the molecule (reductive fission). It can also generate hydroxyl radical (OH^-), a free radical of paramount toxicological fission from hydrogen peroxide (Klaassen, 2001). The Fenton reaction, which is catalyzed by transition metal ions, typically Cu (I) or Cr (V), Fe (II) is a major toxicity mechanism for HOOH and its precursor O^{2-} as well as transition metals may be one of the major reasons for producing ROS (Klaassen, 2001 and Segner and Braunbeck, 1998). Fenton reaction will not allow the conversion of hydrogen peroxide into water molecule and instead of it produces ROS.

superoxide dismutase (SOD) and CAT are two key antioxidant enzymes responsible for elimination of cellular reactive oxygen species (ROS) induced by toxicants, in which SOD firstly disproportionates the highly reactive and potentially toxic superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) (Reddy and Sreenivasula, 1997; Kumar *et al*., 2003). Then H_2O_2 was converted to molecular oxygen and water by CAT catalyzing.

The tendency of CAT activity change was similar to that of SOD and some researchers believed that CAT activity showed a positive relationship with SOD activity (Porte *et al*., 1991; Wu *et al*., 2011; Richardson *et al*., 2008). The present study had focused on CAT due to the disproportionation reaction of O_2 is not the only source of H_2O_2 that could also be generated by amino acids or cytochrome P450 oxidation activated (Livingstone *et al*.,1992).

CAT is an important component of intracellular and antioxidant defences of organisms (Jamil, 2001). It reduces the H_2O_2 into water and oxygen to prevent oxidative stress and for maintaining cell homeostasis. Many studies have found varying responses of catalase to increased metal concentrations, with some organisms exhibiting increased activity, others exhibiting depressed activity, and still others showing no catalase response at all (Regoli *et al*., 1998). CAT is regarded as an enzyme presenting a clear and early response to contamination (Radwan *et al*., 2010b). Under oxidative stress, CAT activity often increases due to up- regulation by ROS (Hermes-Lima, 2004).

In the present study, it is observed that catalase activity was significantly increased in hemolymph of *B. alexandrina* snails that was acutely treated with Cu_2ONPs. CAT high significantly increased in acute compared to chronic treatments and both were higher than control. The obtained results are in agreement with the findings of Almeida *et al*. (2004), that found the catalase activity was increased in mussels after exposure to lead, also this result was in agreement with the findings of Radwan *et al*. (2010a, 2010b) and Regoli *et al*. (2006) the CAT activity was significantly higher in the snails from the polluted sites as compared to the value obtained for the same species from the reference site by 4-fold. However these antioxidant enzymes are sensitive to damage by ROS (Kono and Fridovich, 1982; Goldstone *et al*., 2006). Therefore, measured activities of these enzymes are a result of two processes, their synthesis and inactivation. Studies in aquatic invertebrates exposed to a variety of

organic and metal contaminants have shown that antioxidant enzyme responses are transient and variable for different species and chemicals (Livingstone, 2001; Orbea *et al*., 2002; Barata *et al*., 2005; Cochon *et al*., 2007).

Changes in the level of antioxidants have been proposed as biomarker of a contaminant-mediated pro-oxidant challenge in a variety of invertebrates (Regoli *et al*., 2002)In the present study total antioxidant capacity (TAC) was high significantly increased in the snail treated with Cu_2O NPs than control. These data was accordant with Torres *et al*. (2002) finding who stated that the increase in antioxidant defenses enzymes would be due to enhanced oxygen free radicals production, which could stimulate antioxidant activities to cope with increased oxidative stress and protect the cells from damage. Contrarlly, Liesivuori and Savolainen (1991) stated that excess of ROS accumulation leads to a decrease in antioxidant defenses or causes oxidative damage in organisms. From this point of view a sub lethal (LC_{25}) of Cu_2O NPs which was used in the current work increased ROS and up regulated TAC that enhanced the adaptive immuneresponses in the snail to protect it from oxidative damage.

The antioxidant responses were different between the organisms. The main differences were found in the content of t-GSH. GSH is the most abundant cellular thiol, being found in the millimolar range in most cells. GSH, in addition to being a necessary cofactor for Glutathione peroxidase (GPx) it plays a central role in maintaining cellular redox status and protecting cells from oxidative injury (Doyotte *et al*., 1997; Dickinson and Forman, 2002). GSH is one of the most important factors protecting from oxidative attacks by active oxygen species, because GSH acts as a reducing agent and free- radical trapper and is known to be a cofactor substrate and/or GSH-related enzymes (Verma *et al*., 2007).

Interaction of toxic metals with GSH metabolism is an essential part of the toxic response of many metals (Hultberg *et al*., 2001). When GSH is depleted by any metal, GSH synthesizing systems start making more GSH from cysteine via the γ-glutamyl cycle. Glutathione is a tripeptide non enzymatic antioxidant with a single cysteine residue and constitutes an important pathway of the antioxidant and detoxification defense. Chemical compounds, such as trace metals, are bio-transformed to a conjugate of GSH. GSH is usually not effectively supplied;

In the current results there was no significant increasing in GSH level from hemolymphe of *B. alexandrina* snails that had acutely or chronically treated with Cu_2ONPs than control. These findings are in agreement with Dafre *et al*, (2004) that indicate no significant changes of total GSH levels in pigmented snails exposed to azinphos-methyl, while in the case of L. *variegatus*, exposure to the pesticide triggered significant increases in total GSH in all exposed groups. In contrast, other authors have reported decreases in GSH content after acute exposure of fish to azinphos-methyl (Ferrari *et al*., 2007). Total GSH depletion was also observed in marine bivalves exposed to the organophosphate insecticide fenitrothion (Pena-Llopis *et al*., 2002), also Radwan *et al*. (2010a) and Farid *et al*. (2009) observed that the decrease in GSH concentrations in the digestive gland of *E. vermiculata* snails might be attributed to the intensification of turnover between reduced and oxidized glutathione under the conditions, which cause increased consumption of this peptide for the synthesis of heavy metal- binding proteins, like metallothioneins. In addition, Chandran *et al*. (2005) reported that the level of GSH was decreased in the digestive gland in Zn-treated *A. fulica* snails. Also, Sudama *et al* (2013) stated that lead binds with glutathione and decreases GSH level. However, if GSH depletion continues because of chronic metal exposure (Quig, 1998; Hultberg *et al*., 2001) several enzymes in antioxidant defense systems may protect this imbalance.

NO is an important molecule in innate immune responses. In mollusks NO is produced by haemocytes (Bernice Wright *et al*., 2006). NO is a highly reactive molecule produced by mammalian, invertebrate, and plant cells. NO is synthesized by the oxidation of L-arginine to L-citrulline, which is catalyzed by the enzyme NO synthase (NOS, Rodeberg *et al*., 1995). NOS is a conserved enzyme with a great degree of sequence similarity between invertebrates and vertebrates (Matsuo *et al*., 2008). In mollusks, NOS-like activity has been identified in *Mytilus galloprovincialis*, L. *stagnalis*, and *B. glabrata* defense cells (Hahn *et al*. 2001; Novas *et al*. 2004; Wright *et al*., 2006). The present investigation showed a high significant decreasing in the activity of the NO after acute and chronic treated with Cu_2ONPs in hemolymph of *B. alexandrina* snails compared with their corresponding control. The direct toxicity of NO is enhanced by reacting with superoxide radical-forming peroxynitrite, which is capable of oxidizing cellular structures and causing lipid peroxidation (Weinstein *et al*., 2000).

NO is a molecular messenger with numerous functions, including regulation of vascular tone, cellular signaling in the brain, and the elimination of pathogens in a nonspecific immune response (Moncada *et al*., 1991). NOS is a key enzyme to catalyze L-arginine and molecular oxygen to generate of NO that plays an important role in sensory and motor systems (Funakoshi *et al*., 1999), learning and memory (Susswein *et al*., 2004), neurogenesis (Estrada and Murillo-Carretero, 2005), and autonomic nervous activities (Guo and Longhurst, 2003), as it was stated that the organophosphorus pesticides negatively affect the neurotransmittors in the nervous system of treated organisms (Van Cong *et al*., 2009), This finding agree with the decreasing in the NO value in snails treated with Cu_2ONPs in the current work which plays an important role in resistance to disease and immune regulation in the immune system of aquatic animals.

So in the present study, the disturbances in activities of CAT, GSH, TAC and NO in hemolymphe of snails treated with the tested agents could be explained on the hypothesis of cells rupturing and injuries of different snails' organs, which had suppressive effect on innate immunity and nervous systems, and activate the adaptive immunity systemof these snails, that most has implications on infection rate and cercarial production in these snails.

The rate of infection of *B. alexandrina* snails with their compatible parasites (*S. mansoni*) reached 90%. Frandsen, (1979) found infection rates of *B. alexandrina* snails with *S. mansoni* from Egypt to be about 50-90% and Yousif *et al*., (1996) exposed *B. alexandrina* to *S. mansoni* and recorded 100% infection rates. On the other hand, Reda *et al*., (1991) found that the infection rate of *B. alexandrina* increased up to 88.2%. Although the difference was not significant, infection rate in the current work decreased up to 75% in acute treatments with Cu_2ONPs compared to control which reached 87.5%. This finding is in accordance with Hariston (1973)who reported that low percentage of natural infection is the rule and it depends upon a complex interaction of different factors. Low infection rate may reflect a small degree of biotic pollution but may also indicate a small proportion of susceptible snails in a population exposed to heavy pollution (Paraense and Correa, 1963).

The present results showed that survival rates of snails at 1st shedding post 48 h of exposure to the tested compounds were significantly less than their corresponding control groups. Similar conclusion was recorded by Massoud *et al*. (1973) on *B. truncatus* snails exposed to Bayluscide and infected with S. *haematobium*. This was also; recorded for *B. alexandrina* snails post their subjection to the fungicide Topas (Esmaeil, 2009) and the pesticides Match and Vertimec (Youssef, 2010) after 3 weeks of snail's exposure to *S. mansoni* miracidia, However in contrast, Hira and Webbe (1972) found that *B. glabrata* snails treated with triphenyl lead acetate after 20 days of exposure to *S. mansoni* miracidia, had a similar mortality rate at 1st shedding as control group.

Abd Allah *et al*. (1997) reported that infection also decreased survival and growth of snail hosts and infected individuals exposed to heavy metals displayed the greatest mortality. A significant interaction between heavy metal exposure and infection was apparent. Cercarial shedding by infected snails was significantly reduced in the presence of heavy metals and by 6 weeks shedding had ceased at the highest metal concentrations.

This study revealed a marked reduction of infection rates and cercarial production of snails treated with the tested Cu_2ONPs in comparison with control group. This was supported by the present records on disturbances in activities of the antioxidant defense enzymes, in the presence of the tested compound.The harmful stress of NPs has raised the snails' death rates during the prepatent period, reduced their survival and infection rates and shortened duration of cercarial shedding and cercarial production from infected snails.

The present suppressive effect on cercarial production from infected treated snails could be due to the increased antioxidants (adaptive immune response) hence their subsequent developmental stages could be deteriorated leading to decrement in cercarial output and shortening the duration of cercarial shedding from these snails.

These findingsare supported by Mahmoud (2006) who worked on the insecticides Regent and Mimic, Esmaeil (2009) on the fungicide Topas and Youssef (2010) on the pesticides Match and Vertimec against infection of *B. alexandrina* snails with *S. mansoni.* Similar observations on infection of *B. truncatus* with *S. haematobium* post their exposure to the pesticides Chlorpyrifos and profenofos were recorded (Hasheesh and Mohamed, 2011). In addition, reduction in schistosomes cercarial output from infected-treated snails may be resulted from their active defense system. As, it was stated by Ataev and Coustau (1999) and Barbosa *et al*. (2006 a, b) that the success or failure of *B. glabrata* infection with trematodes depends on snails' humoral factors, mainly circulating hemocytes and plasma factors.

From the foregoing data, it is concluded that low concentrations of Cu_2ONPsdisturb the compatibility of *B. alexandrina* snails to *S. mansoni* through alterations of snails' Immunity, nervous and metabolic processes. Although the innate (nonspecific) immunity (as NO) decreased that make the snail easy to be infected by the miracidia and survive less, the adaptive (specific) immunity (as CAT, TAC and GSH) increased, that attack the trematodes'larvae inside the snail preventing it to produce cercariae. Therefore, introduction of such nanoparticles to snails' habitats could decrease/or prevent schistosomiasis transmission.

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