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TOXIC EFFECTS OF HEAVY METALS POLLUTION ON BIOCHEMICAL ACTIVITIES OF THE ADULT BRINE SHRIMP, *ARTEMIA SALINA*

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ABSTRACT

Toxic metals are widely spread in aquatic ecosystems and aquatic invertebrates are continuously subjected to it. In this study, adult brine shrimp, *Artemia salina* was collected from El Hamra Lake, Wadi El Natrun and acclimated in lake water. They were exposed to sublethal concentrations of $CdCl_2$ (5 mg/l), $FeCl_3$ (30 mg/l) and Cd + Fe (5+30 mg/l) for 7 days. A control group was exposed to lake water only. Antioxidant enzymes activity (Superoxide Dismutase; SOD, Catalase; CAT and Glutathione Peroxidase; GPx), metallothioniens (MTs) and total protein patterns using SDS-PAGE were estimated after exposure to the previous heavy metals for 1, 3 and 7 days. Cd increased the activity of the antioxidants CAT, GPX and SOD significantly (ANOVA/Kruskal Wallis, $P \leq 0.02$) than Fe and Cd + Fe, which increased but insignificantly. Results indicated that concentration of metallothioneins was the significantly higher after Cd exposure (23.9 ± 0.9 ng/100 mg, ANOVA, $P \leq 0.001$) followed by Cd + Fe (14.9 ± 2.4 ng/100 mg) and the lowest was after the Fe exposure (10.8 ± 0.3 ng/100 mg). The total proteins profile of *A. salina* tissue was analyzed after the treatment with Cd and Fe. The total proteins intensity increased in all treatments at the beginning of the experiment then decreased gradually than the control by the end of exposure. In conclusion, Cd exposure alone influenced *A. salina* biological responses than Fe alone or Cd + Fe. *A. salina* is an excellent biomonitor for metals pollution; antioxidant enzymes, metallothioniens and total proteins electrophoresis are good biomarkers to measure *A. salina* biological responses to specific metal exposure.

Keywords: *Artemia*, metals, antioxidants, metallothioneins, total proteins.

INTRODUCTION

Heavy metals enter aquatic environments through different pathways. These can be mainly through natural sources such as weathering of rocks and soil in the catchment and anthropogenic sources like agricultural, urban, domestic, and industrial wastes (Demirak *et al*., 2006 and Cevik *et al*., 2009). During the last century, chemical pollution from human activities has become one of the most important stressors of continental aquatic ecosystems. Aquatic heavy metal pollution usually represents high levels of Hg, Cr, Pb, Cd, Cu, Zn, Ni in water systems (Aoyama *et al*., 1987). Heavy metals are often present in the environment in mixtures, making the assessment of environmental hazards even more difficult due to the antagonistic or synergistic actions that may occur. The investigation of the joint toxic effects of chemicals in a mixture is generally based on comparison of the actual toxic effect of the mixture with the toxic effects of the individual chemicals. In field, the organisms are not safeguarded from the effects of combined toxicants. So, there is a need to quantify and understand the mechanisms of multiple toxicity of metals (Verriopoulos *et al*., 1987; Kungolos *et al*., 1999; Mowat and Bundy, 2002).

Artemia is one of the most valuable test organisms available for ecotoxicity testing and is one of the most sensitive groups to stressors. Its natural tolerance may be faced as an advantage, in comparison to other test organisms. Almost all of tests focused on *Artemia*-based testing which involve cyst-based assays or animals cultured in the laboratory (Nunes *et al*., 2006; Ates *et al*., 2013). Another study, Dhont and Sorgeloos (2002) found that *Artemia franciscana* was chosen as a test organism to investigate the influence of Cadmium (Cd) and Zinc (Zn) exposure on the accumulation of the metals in the tissue. The brine shrimp, *Artemia sinica* possesses a high degree of tolerance, enabling it to cope with various challenging conditions including temperature fluctuations, drought, variable food resources, changes in aeration, and accumulation of heavy metals (Blust *et al*., 1988). After one-year of biomonitoring study, elevated concentrations of metals (Cd, Cu, and Zn) in water of the Arkansas River were paralleled by higher concentrations in benthic macroinvertebrates (Clements, 1994).

Of the various toxic heavy metals, cadmium (Cd), lead (Pb), mercury (Hg) and Zinc (Zn) occur frequently in the environment due to their relatively high industrial use. The toxicity of these elements depend on their interactions with essential elements that are necessary for

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the nutrition of live organisms, such as calcium, iron, selenium, copper, chromium and manganese (Pechová *et al*., 1998). Cadmium (Cd) is a non-essential metal that can displace essential metal ions and interfere with the functional characteristics of proteins because of its high affinity for sulphydryl groups (Nieboer and Richards, 1980; Rainbow, 1985). Cadmium belongs to heavy metals widely distributed in the environment. In nature, it is found together with zinc at a ratio of 1:100, or even 1:1000. Relatively high quantities of cadmium are present in phosphate fertilizers which increase the concentration of cadmium in soil and plants. The chemical speciation of cadmium in saline waters is dominated by the formation of complexes with chloride. Only a small fraction of the cadmium exists as free metal ion while most of the cadmium found in chloride complexes. The concentration of the free metal ions increases with decreasing salinity (Turner *et al*., 1981; Nriagu, 1988; Blust et *al*., 1992; Korénekova *et al*., 2002). Iron (Fe) is innocuous to the aquatic organisms but toxic at high concentration (Pandy *et al*., 1988).

Metallothioneins were a potential biomarker for metal stress in invertebrates. Crustacean metallothioneins (MTs) were shown to be markedly similar to vertebrate metallothioneins on molecular weights, UV absorption spectra, isoelectric points and amino acid compositions (Olafson *et al*., 1979). The biological functions for metallothioneins are; firstly; these proteins comprise non-toxic zinc and copper reservoir available for the synthesis of metallo enzymes, allowing the homeostasis of many cellular processes. Secondly, MTs can prevent the binding of non-essential metals (Cd, Hg or Ag) within cells, and so restrict their toxic potential (Zaroogian and Jackim, 2000). Cadmium-binding metallothionein proteins have been isolated from many invertebrates after exposure to Cd. These invertebrates include the crab *Cancer magister* and *Scylla serrata*, adult and nauplii larvae of *Artemia,* the planktonic shrimp *Acetes sibogae* and the chiton *Cryptochiton stelleri* (Olafson *et al*., 1979; Acey *et al*., 1989; Del Ramo *et al*., 1995; Martínez *et al*., 1996).

Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms (Cadenas, 1997). Defence mechanisms against free radicals-induced oxidative stress involve: (i) preventive mechanisms, (ii) repair mechanisms, (iii) physical defences, and (iv) antioxidant defences. Enzymatic antioxidant defences include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants. Under normal conditions, there is a balance between both activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health (Valko *et al*., 2007). The various roles of enzymatic antioxidants (SOD, CAT and GPx) in the protection against oxidative stress have been proved in numerous reviews (Kojo, 2004; Schrauzer, 2006).

This work was designed to study the toxic effect of heavy metals (Cd and Fe single/in combination) on antioxidant enzymes activity, metallothioneins concentration and total protein patterns of adult *Artemia salina* collected from El Hamra Lake, Wadi El Natrun, Egypt.

MATERIALS AND METHODS

Experimental animal

Artemia salina used in the present investigation were collected from El Hamra Lake, Wadi El Natrun, Egypt. Individuals were placed in plastic box contained Lake water. The animals were maintained under laboratory conditions (17± 2ºC) and mild aeration was applied (El-Bermawi *et al*., 2004).

Experimental materials

Cadmium as $CdCl_2$ was prepared in solution at sublethal concentration 5 mg/l (Nováková *et al*., 2007). Iron as $FeCl_3$ was prepared in solution at sublethal concentration 30 mg/l (Gajbhiye and Hirota, 1990).

Experimental design

Adult *Artemia salina* (360 individual) were divided into 4 groups, each group contained three replicates (30 individual/ replicate). The 1st group (control) was kept in filtered Lake water, the 2nd group treated with $CdCl_2$ (5 mg/l), the 3rd group treated with $FeCl_3$ (30 mg/l), and the 4th group treated with mixed solution of $CdCl_2$ (5 mg/l) + $FeCl_3$ (30 mg/l). Animals were fed mixed diet of yeast, wheat flour, soybean meal, squid and *spirulina*. 3 hrs before the semistatic change of the treatments/water. The experimental time intervals were 1, 3 and 7 day (Dvořák *et al*., 2005).

Determination of metallothionein proteins

Metallothioneins was measured using ELISA according to (Viarengo *et al*., 1997) with minor modifications. Breifly, 0.1 g of whole *Artemia salina* tissue was homogenized with 200 µl of Phosphate buffered saline (PBS). Samples were centrifuged at 10,000 rpm for 30 min. The supernatant was collected and diluted with 100 µl of PBS. Chemicals were obtained from Frontier Institute company, LTD, Hokkaido, Japan.

The Phosphate buffer, standard (Metallothioneins), samples and antibody solution (PBS containing MTs antibody) were kept at 10˚C. Then the strips were washed with washing buffer, 96 well plates were turned upside-down and taped out on a paper towel until the remaining buffer has been removed. 50 µl of assay sample and standard were added into each well and the antibody solution was added to the same wells. The contents in the

wells were stirred then the plate was covered and incubated at 4˚C for 1 hr. After incubation, the solution was discarded from the well then washed 3 times with washing solution (350 µl). 100 µl of the 2nd antibody solution was added in each well and incubated for 1 hr at room temperature then the contents in the wells were stirred. 100µl of the substrate and 50 µl of stop solution were added to each well and incubated for 10 min at room temperature. The optical density (OD) was measured at 450 nm using the spectrophotometer.

Total Proteins analysis

The protein analysis was assayed by SDS- PAGE according to Laemmli (1970) method. 0.02 g of the tissue was homogenized with 200 µl of Tris buffer saline (50 mM tris- HCl, pH 7.5, containing 75 mM NaCl), 1:10 (w/v) ratio. The homogenate was centrifuged at 10,000 rpm for 10 min at 4˚C. The pellet was discarded and the supernatant was aliquoted (Bradford, 1976). Total tissue proteins of *Artemia salina* were separated on 12% resolving gel with 4% stacking gel using. The protein bands were visualized by staining the gel with Coomassie Brilliant Blue (CBB) stain (De-Moreno *et al*., 1985). The gel was photographed and analyzed using gel docu advanced program.

Antioxidant enzymes assays

0.1 g of *Artemia salina* tissue was homogenized in 0.2 ml cold assay buffer, centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was collected for the assay.

Superoxide dismutase (SOD)

SOD activity in *Artemia salina* tissue was measured according to Rest and Spitznagel (1977) using Superoxide Dismutase Assay kit (CAB-574601). One unit of SOD is defined as the amount of the enzyme in 20 µl of sample solution that inhibits the reduction reaction of WST-1 with superoxide anion by 50%. The absorbance (A) was read at 450 nm using the following equation:

$$\text{SOD activity (Inhibition rate \%)} = \frac{[(\text{A blank 1} - \text{A blank 3}) - (\text{A sample} - \text{A blank 2})]}{(\text{A blank 1} - \text{A blank 3})} \times 100$$

Where: **blank 1** is the coloring without inhibitor, **blank 2** is the sample blank and **blank 3** is the reagent blank.

Catalase (CAT)

CAT activity in *Artemia salina* tissue was determined according to Aebi (1983) using Oxiselect ™ Catalase Activity assay kit (STA-341). One unit of catalase was expressed by the amount of catalase decomposes 1 µmol of H_2O_2 per min at pH 4.5 at 25°C. Samples were stored at (-30)°C. Optical density (OD) was measured at 570 nm using the following equation:

$$\text{Catalase Activity} = \frac{\text{B}}{30 \times \text{V}} \times \text{Sample dilution factor}$$

Where: **B** is the amount of decomposed H_2O_2, **V** is the pretreated sample volume added into the reaction well (in ml) and **30** is the reaction time (30 min).

Glutathione Peroxidase (GPx)

GPx activity in *Artemia salina* tissue was measured according to Prins and Loose (1969) using Total Glutathione Peroxidase Assay kit (ZMC-0805002). One unit GPx is defined as the amount of enzyme that will cause the oxidation of 1 µmol of NADPH to NADP per minute at 25 °C. Samples were kept at (-30)°C for storage. OD was measured at 340 nm at T1 to read A1, OD was measured at 340 nm again at T2 after incubating the reaction at 25 °C for 5 min to read A2 according to the following equation:

$$\text{GPx Activity} = \frac{(\text{B - B0})}{(\text{T2 - T1}) \times \text{V}} \times \text{Sample dilution}$$

Where: **B** is the amount of NADPH (in nmols) that was decreased between T1 and T2, **B0** is the background change (without Cumene Hydroperoxide) between T1 and T2, **T1** is the time of first reading (A1 in min), **T2** is the time of second reading (A2 in min) and **V** is the pretreated sample volume added into the reaction well (in ml).

Statistical analysis

All data were analysed using Statgraphics (v5.1 software). Data were expressed as mean ± SD. The statistical analysis was carried by One-way ANOVA was applied to set the difference between the control and treated groups of the experiment, setting the probability level to $P \leq 0.05$, where ANOVA could not be applied, Kruskal Wallis test was used.

RESULTS

Effect of heavy metals (Cd and Fe) exposure on the activity of antioxidant enzymes of *Artemia salina*

Exposure to Cd (5 mg/l) caused insignificant increase in the activity of Superoxide dismutase (SOD) on the 1st and 3rd days (ANOVA/ Kruskal Wallis, $P > 0.05$), and significant increase at day 7 (Kruskal Wallis, $P = 0.02$) when compared to the control. It recorded 89.7 ± 5.6 u/100 mg on the 1st day, 96.4 ± 4.3 u/100 mg on the 3rd day and 101.1 ± 3.6 u/100 mg on the 7th day, while the control value was 72.2 ± 0.3, 73.7 ± 1.3 and 73.8 ± 1.4 u/100 mg. Catalase (CAT) showed significant increase in its activity on the 1st and 3rd days (Kruskal Wallis, $P \leq 0.01$), and insignificant increase on the 7th day (Kruskal Wallis, $P > 0.05$) when compared to the control. It was 23.6 ± 4.3 u/100 mg on the 1st day, 27.4 ± 1.9 u/100 mg on the 3rd day and 29.28 ± 1.1 u/100 mg on the 7th day when compared to the control value, 18.6 ± 0.5, 19.1 ± 0.4 and 19.2 ± 0.6 u/100 mg. Glutathione Peroxidase (GPx) showed significant increase in its activity during all time points of exposure. On the 1st day, it recorded 106.6

± 5.8 u/100 mg; on the 3rd day was 110.7 ± 5.8 u/100 mg and on the 7th day was 114.3 ± 0.4 u/100 mg (ANOVA / Kruskal Wallis, $P \leq 0.004$) when compared to the control value, 80.5 ± 0.8, 80.9 ± 0.8 and 81.2 ± 1.1 u/100 mg (Fig. 1).

Data showed in Fig. (1), indicated that exposure to Fe (30 mg/l) caused insignificant increase in the activity of SOD during all time points of exposure (ANOVA/ Kruskal Wallis, $P > 0.05$) when compared to the control. It was 85.6 ± 5.7 u/100 mg on the 1st day, 92.2 ± 0.6 u/100 mg

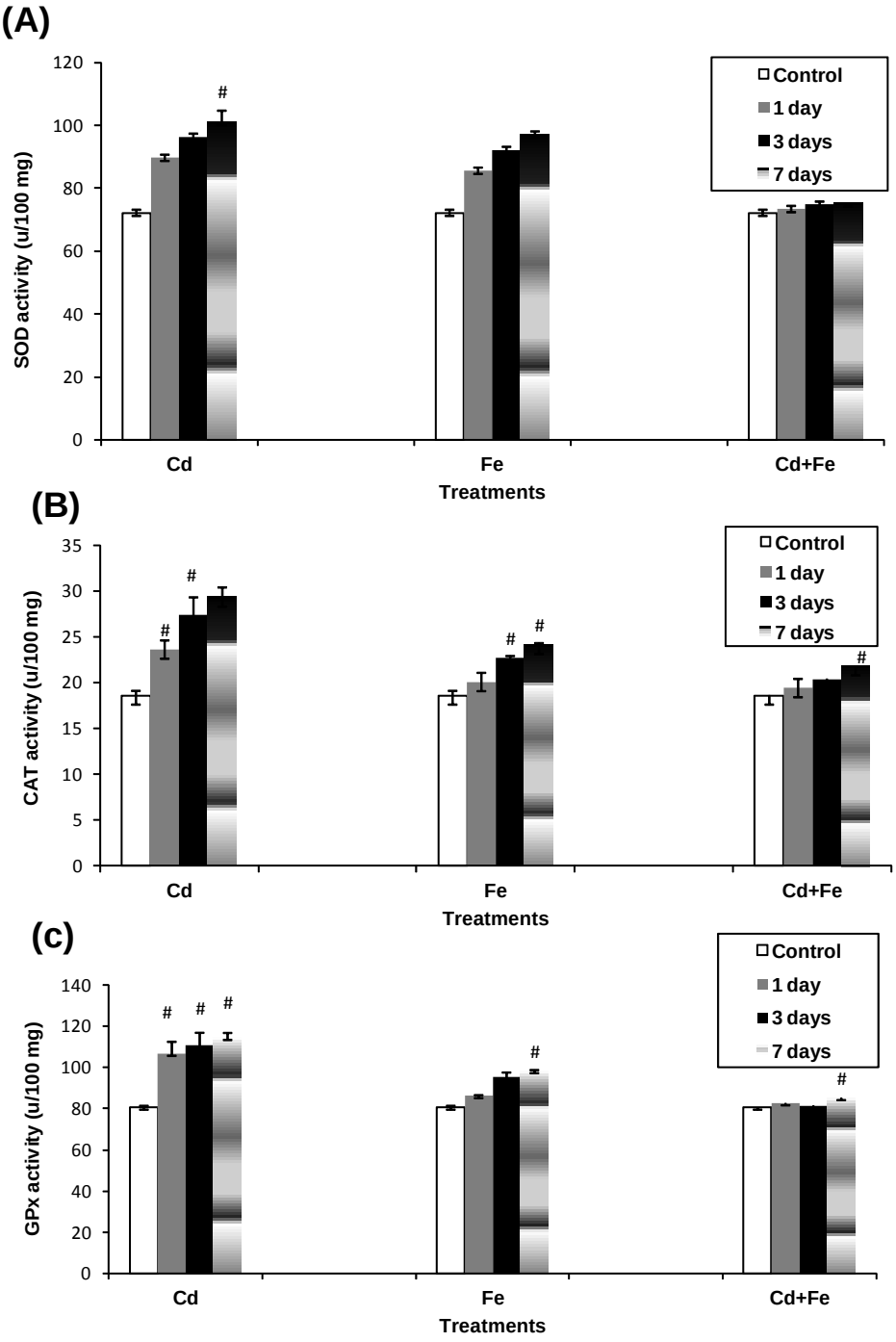

Fig. 1. Effect of Cd, Fe and Cd + Fe exposure on the activity of antioxidant enzymes **(A)** SOD, **(B)** CAT and **(C)** GPx of *Artemia salina* for 7 days, # indicates significant difference when $P < 0.05$ (ANOVA/ Kruskal Wallis).

on the 3rd day and 96.9 ±1u/100 mg on the 7th day when compared to the control value 72.2 ± 0.3, 73.7 ± 1.3 and 73.8 ± 1.4 u/100 mg. CAT showed insignificant increase in activity on the 1st day (Kruskal Wallis, $P > 0.05$), significant increase on the 3rd and the 7th days (ANOVA / Kruskal Wallis, $P \leq 0.01$) when compared to the control. It was 20.06 ± 0.5 u/100 mg on the 1st day, 22.7 ± 0.6 u/100 mg on the 3rd day and 24.1 ± 0.7 u/100 mg on the 7th day when compared to the control value 18 ± 0.5, 19.1 ± 0.4 and 19.2 ± 0.6 u/100 mg. GPx showed insignificant increase in its activity on the 1st and the 3rd days (ANOVA/ Kruskal Wallis $P > 0.05$) and significant increase on the 7th day (Kruskal Wallis, $P = 0.0005$) when compared to the control. It was 86.1 ± 6.04 u/100 mg on the 1st day, 95.5 ± 0.6 u/100 mg on the 3rd day and 98.2 ± 2 u/100 mg on the 7th day when compared to the control value 80.5 ± 0.8, 80.9 ± 0.8 and 81.2 ± 1.1 u/100 mg.

As showed in figure 1, exposure to Cd+Fe caused insignficant increase in the activity of SOD during all time points of exposure (ANOVA/ Kruskal Wallis, $P > 0.05$) when compared to the control. It recorded 73.4 ± 1.1 u/100 mg on the 1st day, 74.8 ± 0.5 u/100 mg on the 3rd day and 75.2 ± 1.2 u/100 mg on the 7th day when compared to the control value 72.2 ± 0.3, 73.7 ± 1.3 and 73.8 ± 1.4 u/100 mg. CAT showed insignficant increase of activity on the 1st and the 3rd days (Kruskal Wallis, $P > 0.05$) and significant increase on the 7th day (ANOVA, $P = 0.0001$) when compared to the control. It was 19.4 ± 0.4 u/100 mg on the 1st day, 20.4 ± 0.2 u/100 mg on the 3rd

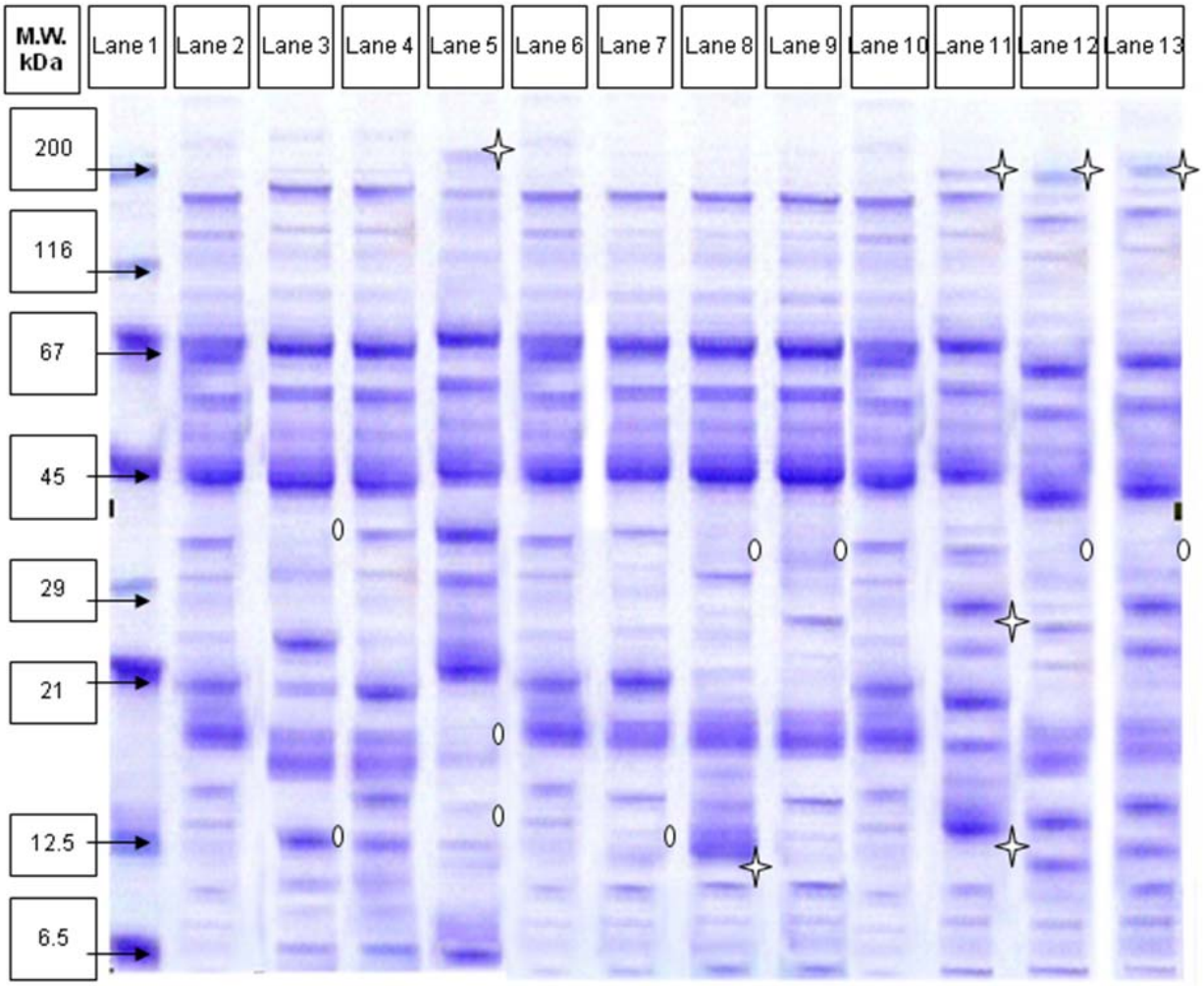

Fig. 2. SDS-PAGE profile of total proteins of *Artemia salina* stained with CBB treated with Cd and Fe for 7 days of exposure. Lane 1, marker, Lane 2, 3, 4 and 5, control and treated with Cd, Fe and Cd + Fe for 1 day, respectively; Lane 6, 7, 8 and 9, control and treated with Cd, Fe and Cd + Fe for 3 day, respectively; Lane 10, 11, 12 and 13, control and treated with Cd, Fe and Cd + Fe for 7 day, respectively and represent appearance and disappearance of bands, respectively. ✦ and O represent appearance and disappearance of bands, respectively.

Table 1. Effect of heavy metals (Cd + Fe) on metallothioneins concentration of *Artemia salina* for 7 days of exposure

Treatments / Time points	Control	Cd	Fe	Cd + Fe
1 day	3.1 ± 0.5	11.1 ± 0.8 #	6.3 ± 0.6	9.6 ± 0.3
3 days	3.2 ± 0.6	18.8 ± 0.5 #	9.4 ± 0.1 #	12.4 ± 2.7
7 days	3.4 ± 0.7	23.9 ± 0.9 #	10.8 ± 0.3 #	14.9 ± 2.4 #

Note; n = 3, data were expressed as means ± SD, ng/100 mg, # indicates significant difference when $P < 0.05$ (ANOVA/ Kruskal Wallis).

Table 2. Effect of heavy metals (Cd + Fe) on the intensity and profile of total proteins of *Artemia salina* for 7 days of exposure

Treatments / Time points	Control		Cd		Fe		Cd + Fe	
	Intensity	Bands No.	Intensity	Bands No.	Intensity	Bands No.	Intensity	Bands No.
1 day	16810,6784	17	272892,8800	14	38180,9223	20	27998,1197	14
3 days	26257,0989	16	4403,0011	16	46432,7554	15	36274,1365	14
7 days	49380,2113	16	24491,4061	18	34671,7158	15	15459,3467	16

day and 21.8 ± 0.2 u/100 mg on the 7th day when compared to the control value 18.6 ± 0.5, 19.1 ± 0.4 and 19.2 ± 0.6 u/100 mg. GPx showed insignificant increase in its activity on the 1st day, insignificant decrease on the 3rd day (ANOVA/ Kruskal Wallis $P > 0.05$) and significant increase on the 7th day (ANOVA, $P = 0.03$) when compared to the control. It was 82.6 ± 2.4u/100 mg on the 1st day, 81.4 ± 1.8 u/100 mg on the 3rd day and 85.1 ± 0.5 u/100 mg on the 7th day when compared to the control value 80.5 ± 0.8, 80.9 ± 0.8 and 81.2 ± 1.1 u/100 mg.

Effect of heavy metals (Cd and Fe) on metallothioneins concentration of *Artemia salina*

Treatment with Cd significantly increased the concentration of metallothioneins on the 1st, 3rd and 7th day (ANOVA, $P \leq 0.001$) when compared to the control. It was 11.1 ± 0.8 ng/100 mg on the 1st day, 18.8 ± 0.5 ng/100 mg on the 3rd day and 23.9 ± 0.9 ng/100 mg on the 7th day while the control values were 3.1 ± 0.5, 3.2 ± 0.6 and 3.4 ± 0.7 ng/100 mg at 1, 3 and 7 days, respectively. Fe caused insignificant increase in the concentration of metallothioneins at 1st day (Kruskal Wallis, $P > 0.05$), significant increase on the 3rd and 7th days (ANOVA/Kruskal Wallis, $P \leq 0.002$) when compared to the control. It was 6.3 ± 0.6 ng/100mg on the 1st day, 9.4 ± 0.1 ng/100 mg on the 3rd day and 10.8 ± 0.3 ng/100 mg on the 7th day compared to control values 3.1 ± 0.5, 3.2 ± 0.6 and 3.4 ± 0.7 ng/100 mg, respectively. Cd + Fe recorded insignificant increase in the concentration of metallothioneins on the 1st and 3rd days (ANOVA/ Kruskal Wallis, $P > 0.05$), significant increase on the 7th day (ANOVA, $P = 0008$) when compared to the control. It was 9.6 ± 0.3 ng/100 mg on the 1st day, 12.4 ± 2.7 ng/100 mg on the 3rd day and 14.9 ± 2.4 ng/100 mg on the 7th day when compared to the control values 3.1 ± 0.5, 3.2 ± 0.6 and 3.4 ± 0.7 ng/100 mg, respectively (Table 1).

Effect of heavy metals (Cd and Fe) on the intensity and profile of total proteins of *Artemia salina*

On the 1st day of treatment, Cd (5 mg/l) exposure caused disappearance of the bands with molecular weight 32.08 and 14.47 kDa, increased protein intensity (272892,8) and decreased band numbers to 14 bands. Fe (30 mg/l) increased in the number of protein fractions to 20 with intensity (38180,9) with appearance of low molecular weight bands (6, 7 and 8 kDa). Cd + Fe showed decreased in the number of protein fractions to 14 with increased intensity to 27998, 1 when compared to the control (17 and 16810,6), respectively. The protein bands 16.69 and 13.41disappeared under the effect of treatment with Cd + Fe (Fig. 2 and Table 2).

On the 3rd day of treatment, Cd caused decrease in the protein intensity (4403,0) and no change in the number of protein fractions as the control (16). The band with molecular weight 12.35 kDa disappeared under the effect of treatment with Cd. Fe treatment caused increase in the protein intensity (46432,7) and decrease in the number of bands to 15. The protein band 32.16 kDa disappeared under the effect of treatment with Fe. Cd + Fe showed decrease in the number of protein fractions to 15 with increased intensity to 36274 when compared to the control (16 and 26257,1), respectively. The protein band 34.02 kDa disappeared under the effect of treatment with Cd + Fe (Fig. 2 and Table 2).

On the 7th day of treatment, Cd caused increase in the number of protein fractions to 18 but decreased intensity to 24491,4. The bands with molecular low weight as 7.2 and 6.2 kDa appeared under the effect of treatment with Cd. Fe treatment decreased both protein bands and intensity (15 and 34671,7), with appearance of 180.79 kDa and disappearance of 33.48 kDa bands. Cd + Fe caused decreased in the protein intensity 15459,3 with the same number of fractions 16, when compared to the control (16 and 49380,2), respectively. The protein band 181.28 kDa appeared and the band 34.23 kDa disappeared under the effect of treatment with Cd + Fe (Fig. 2 and Table 2).

DISCUSSION

Effect of heavy metals Cd and Fe exposure on the activity of antioxidant enzymes of *Artemia salina*

The present results indicated that sublethal concentrations of Cd and Fe single or in combination caused increase of antioxidant enzymes (SOD, CAT and GPx) activity in the treated tissue of *Artemia salina*. This supported by Cadenas (1997), who stated that antioxidant enzymes considered one of defence mechanisms after exposure to free radicals from a variety of sources. Cd significantly induced stimulation in SOD, CAT and GPx activities after 0.5 day at concentrations 0.025 and 0.05 mg/l in the gills and hepatopancreas of the crab *Charybdis japonica* (Pan and zhang, 2006). Ercal *et al*. (2001) stated that redox-active metals such as Fe, Cu and Cr or redox-inactive metals such as Pb, Cd and Hg, may cause an increase in production of reactive oxygen species (ROS) such as hydroxyl radical (HO), superoxide radical (O_2^-) or hydrogen peroxide (H_2O_2). Enhanced generation of ROS can result in a condition known as "oxidative stress". Cells under oxidative stress display various dysfunctions due to lesions caused by ROS to lipids, proteins and DNA. Kojo (2004) and Schrauzer (2006) stated that the enzymatic antioxidant defences include SOD, CAT and GPx are considered one of the defence mechanisms against oxidative stress and may play an important role in reducing some hazards of heavy metals.

Effect of heavy metals Cd and Fe exposure on metallothioneins concentration and the total proteins profile of *Artemia salina*

The present results indicated that Cd exposure highly increased concentration of metallothioneins of *A. salina* followed by Cd + Fe and finally the lowest recorded after Fe exposure. This supported by the findings that invertebrates exposed to elevated metal concentrations respond to this stress by metal-induced synthesis of metal-binding proteins, metallothioneins (Roesijadi *et al*., 1982; Dallinger, 1994). In addition, laboratory experiments showed significant rise in metallothioneins concentrations after exposure to heavy metals (Bodar *et al*., 1988 on *Daphnia magna*; Martínez *et al*., 1996 on *Echinogammarus echinosetosus* and Barka *et al*., 2001 on copepod, *Tigriopus brevicornis***)**. Increase in the concentration of metallothioneins may relate to its biological role, where these proteins can prevent the binding of non-essential metals (Cd, Hg or Ag) within cells, and so restrict their toxic potentials (Viarengo and Nott, 1993; Roesijadi, 1996). In the present work, decreased metallothioneins concentration under the effect of Cd + Fe exposure is supported by Nováková *et al*. (2007) who found that higher concentration of Cd and Zn caused synergistic effects on *A. franciscana*. They explained that the low concentration of zinc and cadmium bound to metallothioneins and after blocking all binding sites on this protein, the metals pass to the blood and tissues as free ions and cause toxicity. However, if the time for metallothioneins synthesis is insufficient due to parallel administration of zinc and cadmium, the influence of zinc on the prevention of cadmium toxicity can`t be observed.

The analysis of SDS- PAGE profile of proteins of treated *Artemia* revealed changes in total protein intensity and fractions number. Variations in protein content reflect the enzymatic changes in *Artemia* tissue. The level of enzymes changes in response to infection or stressors (Cheng *et al*., 1978). Tolba *et al*. (1997) stated that the reduction in the total protein content caused by heavy metals might suggest disturbance in functions of the internal organs as a sequence of structural damage that may lead to inhibition of protein synthesis. An occasional appearance of protein bands indicated that *Artemia* produces additional proteins in response to the heavy metals. Many invertebrates exposed to elevated metal concentrations respond to this stress by metal-induced synthesis of metallothioneins (Rainbow, 1985). In the present study, some low molecular weight proteins appeared as a response to metals exposure, especially Cd. In a recent study, Seebaugh and Wallace (2004) recorded expression of metal-binding proteins (MTs) in response to the exposure to Cd and Zn in *Artemia franciscana*. Ubiquitin (Ub)/proteasome-dependent proteolytic systems that break down proteins to reduce muscle mass of the claw in land crab, *Gecarcinus lateralis*, and american lobster, *Homarus americanus* increased to facilitate withdrawal of the appendage at ecdysis (Koenders *et al*., 2002). Because ubiquitin is a low molecular weight protein (8.5 kDa) appeared under stress, like inflammation, which is characteristic to Cd exposure, *Artemia* expressed low protein intensity under treatment.

CONCLUSION

It is concluded that, *A. salina* is an excellent biomonitor for heavy metals toxicity. Antioxidant enzymes, metallothioniens and total proteins electrophoresis are good biomarkers to measure *A. salina* specific biological responses to specific metal exposure.

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