Role of Vitamins A, C, E and Seleniumin Preventing Heavy Metals Toxicity in Nile tilapia (*Oreochromis niloticus*)

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Received: *11/11/2013*

Abstract: This study was carried out to investigate the potential effects of sublethal concentration (5 mg.L^{-1}) of combined heavy metals (1.25 mg.L^{-1} of each) including cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) on Nile tilapia, *Oreochromis niloticus* after exposure for 1, 3, 5 and 7 days in order to evaluate the protective role of vitamin E and combination of selenium (Se) with vitamins A, C and E against the toxicity of these heavy metals. Exposure of *O. niloticus*to heavy metals only for 1, 3, 5 and 7 days resulted a significant increase in the values of antioxidant enzymes (GSH, CAT, GST, GR and GPx) in liver and gills of *O. niloticus*. While the exposure to the mixture of heavy metals and vitamin E or heavy metals with selenium protected the liver and gill of *O. niloticus*. The activities of antioxidant substances and free radicals (SOD, GSH, GSSG, TAC and MDA) formation were also determined after 1, 3, 5 and 7 days of exposure to heavy metals only or heavy metals with vitamin E or selenium. Antioxidants (both enzymatic and nonenzymatic) can provide protection against deleterious metal-mediated free radical attacks. Vitamin E and combination of selenium (Se) with vitamins A, C and E prevent the majority of metal-mediated (Cd, Cu, Pb, and Zn) damage. Therefore, this study confirms on the powerful protective potential of the antioxidants vitamin E alone and a combination of selenium with vitamins A, C and E against the toxicity of Cd, Cu, Pb and Zn on *O. niloticus*.

Keywords: vitamin E and selenium, oxidative stress, heavy metals, *O. niloticus*.

INTRODUCTION

Aquatic ecosystem is the ultimate recipient of almost all the substances including pesticides and heavy metals which are non-biodegradable in nature. Water pollution imposes a health hazard effect and accumulation of different kinds of pollutants in fish body that reflects negatively on human consuming such fishes. Generally speaking using polluted water sources expose different fish farms to different kinds of pollution in aquatic ecosystem. Water pollution may be checking by the development of biological monitoring based on fish with past responses on the low concentration of direct acting toxicant (El-Shehawi et al., 2007). Heavy metals toxicity is persistent environmental contaminants because they cannot be degraded or destroyed. To a small extent, they enter the body system through food, air and water. They bio-accumulate over a period of time because they cannot be metabolized (Olaifa et al, 2004). Heavy metal toxicity can lead to damaged or reduced mental and central nervous functioning, damage of vital internal organs and lowering of energy levels in the body. Fish constitutes an important aspect of human food due to the high level of quality protein and essential amino acids for the proper growth and functioning of body muscles and tissues. Fish are commonly situated at the top of the food chain and therefore, they can accumulate large amounts of toxicants (Dahunsi*et al.*, 2012). *O. niloticus* are particularly interesting because of their abundance in inland waters, their wide geographical distribution, their economic interest and easy of breeding, which is why they were chosen for this work. This study focuses on the liver and gills, as it is considered for several decades as an important source of biomarkers. Indeed, the vertebrate liver and gills are not only the central body of many vital functions of normal metabolism, but it is also the major site of accumulation, biotransformation and excretion of xenobiotics (Anderson and Luo, 1998).

Antioxidants such as selenium, vitamin E and C act to protect cells against the effects of free radicals, which are potentially damaging by-products of energy metabolism. Free radicals can damage cells and may contribute to the development of many human diseases (Van Gaal et al., 2006). Selenium is an "essential" trace element, which has a variety of functions. It is a key component of several functional selenoproteins required for normal health. Whereas selenium is poisonous to man and animal in large amounts (De Lorgeril and Salen, 2006). Role of selenium as a protective against exposure to metals is a conflict issue. Also vitamin E is an important component in human diet and considered the most effective liposolouble antioxidant found in the biological system. It is composed of various subfamilies of which tocopherols and tocotrienols are the most studied. The structural difference between the two subfamilies is that tocotrienols possess three double bonds in their isoprenoid side chain and this structural difference results in differences in their efficacy and potency as antioxidants (Musalmah et al., 2002). Vitamin E is a fat-soluble vitamin exists in eight naturally occurring forms four tocopherols and four tocotrienols (Traber and Packer, 1995), the most active form is alpha-tocopherol (α- tocopherol) (El-Shebly, 2009).

The objective of this study was to determine whether co-consumption of nutritional supplements as vitamin E and a combination of selenium (Se) with vitamins A, C and E would treat the hazardous effects of exposure fish to heavy metals (Cd , Cu, Pb and Zn).

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MATERIALS AND METHODS

Experimental setup:

Analytical graded Cadmium Sulfate ($3CdSO_4.8H_2O$), Lead nitrate ($Pb(NO_3)2$), Cupric Sulfate pentahydrated ($CuSO_4.5H_2O$), Zink Sulfate heptahydrate ($ZnSO_4.7H_2O$) were supplied by (Sigma Aldrich USA). The antioxidant vitamin E (di-alpha-tocopherylacetat) is supplied by European Egyptian Company (Egypt). Combined selenium (Se) with vitamin A, vitamin C and vitamin E (Se-ACE) as antioxidants are supplied by Wassen international Ltd., the Mole Business Park, Leatherhead, KT22 7BA, UK (Great Britain).

Specimens collection:

In the present work, specimens of apparently healthy Nile tilapia, *O. niloticus*(100 ± 8 g) were obtained from Jeddah fish market, Prior to the experiment, fish were acclimatized in Zoology lab, Faculty of Science, King Abdualaziz University North Campus, for two weeks in 120 L glass aquaria (100 × 30 × 40 cm) under laboratory conditions; glass aquaria (30 fish per each aquarium) containing tap water (temperature 26 ± 2 °C; pH 7.4 ± 0.18; dissolved oxygen (DO) 6.6 ± 0.78 mg/1; photoperiod 12:12 Light: Dark). Fish were fed on a commercial pellet diet containing 25% crude protein (3% of body weight per day) twice a day.

Experimental setup:

Sublethal concentration (5 mg/L) of combined heavy metal solution (Cd + Cu + Pb + Zn) containing sublethal concentration (1.25 mg/L) of each metal ion was applied as toxicants. The heavy metal concentrations were selected on the basis of studies of Vinodhini and Narayanan (2009), Abdel-Tawwab et al. (2013). Vitamin E was applied as 400 mg/Kg diet. The combined selenium-vitamins (Se-ACE) were applied as a dose of Se (75 μg/Kg diet), vitamin A (300 μg/Kg diet), vitamin C (60 mg/Kg diet) and vitamin E (10 mg/Kg diet).

Experimental design:

After two week of acclimatization, fishes were randomly classified into 4 groups: control and three treated groups. Each group consists of forty five fishes divided into three replicates (15 fish/tank). The first group is the control, the second group, heavy metals only (HM). The third group, heavy metals and separate vitamin E (HM+E) treated group and the fourth group, heavy metals and a combination of selenium (Se) with vitamins A, C and E (HM+Se-ACE). Exposure duration was 1, 3, 5 and 7 days and the water and heavy metals were completely replenished each day.

Ethical statement:

All experiments were carried out in accordance with the Saudi Arabian laws and University guidelines for the care of experimental animals. All procedures of the current experiment have been approved by the Committee of the Faculty of Science, North Jeddah, King Abdulaziz University, Jeddah, Saudi Arabia.

Biochemical assay:

Ten Fishes from each group were slaughtered at 1, 3, 5 and 7 days of treatment, livers and gills were removed and rinsed with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots. One gram from each organ was homogenized in 10 ml cold 20 mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210 mMmannitol, and 70 mM sucrose per gram tissue. Homogenates were centrifuged at 10.000 x g for 15 minutes at 4 °C and the supernatant was removed and stored at -80 °C until further biochemical analysis .

In the liver and gills homogenate, reduced glutathione (GSH), Oxidized glutathione (GSSG) and total glutathione were determined using a kit supplied by Cayman (Cat. No. 703002, Cayman, USA) according to the manufacturer's instructions (Ellman, 1959). Total antioxidant capacity according to (Janaszewska and Bartosz 2002). Catalase (CAT) activity was determined using a kit (Cat. No. NWK-CAT01) purchased from Northwest Life Science Specialties (NWLSSTM), Vancouver, Canada, according to the manufacturer's instructions (Aebi, 1984). Superoxide dismutase (SOD) activity was using Cayman SOD diagnostic kit (Cat. No. 706002, Cayman, USA) according to the manufacturer's instructions.Glutathion-S-Transferase (GST) was determined using a kit supplied by Bio-diagnostic (CAT. No. GT 25 18, Giza, Egypt) according to the manufacturer's instructions (Habig and Pabst 1974). Glutathione reductase (GR) activity in liver homogenate was determined according to the method described by Beutler (1969) using a kit supplied by Northwest Life Science Specialties (NWLSSTM), Vancouver, Canada, (Cat. No. NWK-GR01). Glutathione peroxidase (GPx) was determined using a kit (Cat. No. NWK-GPX01) purchased from Northwest Life Science Specialties (NWLSSTM) according to the manufacturer's instructions (Lawrence and Burk, 1976). Malondialdehyde (MDA) was analyzed by measuring the production of thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust (1978) using TBARS assay kit (Cat. No. 10009055, Cayman, USA). Nitric oxide was determined using a kit supplied by Bio-diagnostic (CAT. No. NO 25 32, Giza, Egypt) according to the manufacturer's instructions (Montgomery et al., 2002). Protein carbonyls was determined according to Loro et al (2012).

Statistical analysis:

The data was statistically analyzed by SPSS version 20 (SPSS, Richmond, USA) was used as described by Dytham 1999). Statistical packages (IBM 1 New Orchard Road Armonk, New York 10504-1722 United States), and presented as a mean ± SD, n = 10. Statistical differences between groups performed using One-way analysis of variance (ANOVA). Duncan's test was used for testing the inter-grouping homogeneity (Duncan 1995). Statistical significance was set $P \leq 0.05$ and $P \leq 0.01$.

RESULTS

Oxidative stress markers and antioxidant enzymes activities:

It was observed that heavy metals induces SOD and CAT activities in liver and gills tissues (Table 1 and 2) when compared with control, vitamin E alone or with Se significantly decreases the activities of SOD at 3, 5 and 7 days in both tissues and at 1, 3, 5 and 7 days for CAT in liver tissue, but at 5 and 7 days for gills tissue when compared with heavy metals groups. GR, GPx and GST activities were decreased after 3, 5 and 7 days of heavy metals exposure in both tissues in comparison to control. Vitamin E alone or with Se succeeded to induce the activities these enzymes after 3, 5 and 7 days of treatment when compared to heavy metals exposed group.

Oxidative stress were manifested through monitoring of GSH, GSSG, Total glutathione and TAC levels in both liver and gills tissues (Table 3 and 4). In concerning to GSH, Total glutathione and TAC concentrations were decreased significantly in both tissues upon heavy metals toxicity and at all periods of measurements comparing to the control fishes. Vitamin E alone not succeeded to increase both GSH and total glutathione in both tissues but did that to TAC when compared to heavy metals treated group. With Se it succeeded to increase the levels of GSH, Total glutathione and TAC in gills tissue and that of GSH and TCA but not total glutathione in liver tissue. GSSG levels were increased significantly in either heavy metals or heavy metals with vitamin E groups when compared with control. Vitamin E with selenium (Se) with vitamins A, C and E have the ability to decrease the level of GSSG in both tissues to become as the control levels.

Table (1): Levels of SOD, CAT, GR, GPx and GST (mean ± SD) in liver of *O. niloticus* exposed to heavy metals, heavy metals plus vitamin E and heavy metals plus selenium (Se) with vitamins A and C.

Parameter	Time (days)	Control group	HM group	HM+E group	HM+Se-ACE group
SOD (µmol/ mg wt.w / min)	1	8.2 ± 2.0^{d}	20.3 ± 1.1^{a}	16.9 ± 2.9^{b}	14.7 ± 1.5^{c}
	3	8.1 ± 1.8^{d}	24.0 ± 2.0^{a}	16.1 ± 2.8^{b}	13.9 ± 2.7^{c}
	5	8.3 ± 2.0^{d}	30.0 ± 3.0^{a}	15.3 ± 2.1^{b}	12.7 ± 1.2^{b}
	7	8.1 ± 2.1^{c}	32.0 ± 1.9^{a}	13.7 ± 2.5^{b}	14.7 ± 1.5^{b}
CAT (µmol/ H_2O_2 decomposed/ mg P/ min)	1	510.0 ± 10.0^{b}	500.0 ± 4.5^{b}	720 ± 10^{a}	750 ± 20^{a}
	3	513.0 ± 12.0^{b}	497.0 ± 15.0^{b}	720 ± 10^{a}	753 ± 25^{a}
	5	512.0 ± 10.0^{c}	800.0 ± 30.0^{a}	717 ± 15^{b}	760 ± 20^{a}
	7	500.0 ± 15.0^{c}	850.0 ± 20.0^{a}	730 ± 10^{b}	728 ± 11^{b}
GR (U/ mg P)	1	4.4 ± 1.0^{b}	5.0 ± 0.5^{a}	5.3 ± 0.3^{a}	4.4 ± 0.4^{b}
	3	4.4 ± 1.0^{c}	4.8 ± 0.3^{c}	5.6 ± 0.6^{a}	6.0 ± 0.4^{a}
	5	4.3 ± 1.1^{b}	2.8 ± 0.3^{c}	5.9 ± 0.3^{a}	6.2 ± 0.2^{a}
	7	4.3 ± 1.0^{b}	2.0 ± 0.3^{c}	6.0 ± 0.4^{a}	6.5 ± 0.5^{a}
GPx (µ mol/ mg P/ min)	1	124.0 ± 10.0^{b}	120.0 ± 5.0^{b}	130.0 ± 10.0^{a}	135.0 ± 5.0^{a}
	3	134.0 ± 12.0^{a}	68.0 ± 8.0^{c}	88.0 ± 8.0^{b}	90.0 ± 10^{b}
	5	130.0 ± 13.0^{a}	48.0 ± 7.6^{c}	90.0 ± 9.6^{b}	93.0 ± 3.0^{b}
	7	131.0 ± 11.0^{a}	46.0 ± 6.0^{b}	120.0 ± 10.0^{a}	122.0 ± 7.5^{a}
GST (nmol/ mg P/ min)	1	365.0 ± 5.0^{a}	360.0 ± 9.6^{b}	370.0 ± 9.6^{a}	375.0 ± 5.0^{a}
	3	360.0 ± 9.9^{a}	355.0 ± 6.0^{b}	370.0 ± 9.2^{a}	370.0 ± 5.0^{a}
	5	361.0 ± 1.1^{a}	299.0 ± 7.6^{b}	300.0 ± 8.9^{b}	308.0 ± 7.6^{b}
	7	365.0 ± 7.8^{a}	260.0 ± 10.0^{b}	250.0 ± 4.7^{b}	260.0 ± 7.0^{b}

SOD; superoxidedismutase, CAT; catalaseenzyme, GR; Glutathionereducatase; GPx; glutathioneperoxidase; GST; glutathionetransferase,
Means of treatments with the same superscript letter within each interval in the same row are not significantly different at P≤ 0.05.

Lipid peroxidation propagation in fish liver and gills tissue (Table 3 and 4) were increased by 383.9% and 428.5% respectively upon heavy metals exposure when compared with control groups at all the periods of measurement (1, 3, 5 and 7 days), up on administration of vitamin E alone or with Se the lipid peroxidation levels were decreased significantly when compared with heavy metals toxicity till nearly become as control levels in both tissues at 5 and 7 days. In concerning to protein oxidation (estimated by Nitric oxide and Protein carbonyls) both are significantly increased in both tissues upon heavy metals exposure by but vitamin E either alone or with Se have the ability to overcome on protein peroxide and decrease it significantly as compared with heavy metals till become as control at 7 days.

Table (2): Levels of SOD, CAT, GR, GPx and GST (mean ± SD) in gills of *O. niloticus* exposed to heavy metals, heavy metals plus vitamin E and heavy metals plus selenium (Se) with vitamins A and C.

Parameter	Time (days)	Control group	HM group	HM+E group	HM+Se-ACE group
SOD (µmol/ mg wt.w / min)	1	6.3 ± 0.30^{d}	18.1± 2.0^{a}	14.9 ± 2.0^{b}	11.7 ± 1.5^{c}
	3	6.2 ± 0.31^{d}	21.0 ± 0.3^{a}	14.7 ± 2.5^{b}	11.8 ± 1.8^{c}
	5	6.4 ± 0.4^{d}	25.0 ± 0.5^{a}	12.7 ± 1.5^{b}	10.7 ± 1.5^{c}
	7	6.3 ± 0.3^{d}	20.0 ± 0.3^{a}	13.0 ± 2.0^{b}	9.7 ± 1.1^{c}
CAT (µmol/ H_2O_2 decomposed/ mg P/ min)	1	480.0 ± 10.0^{b}	458.0 ± 7.6^{b}	648.0 ± 7.6^{a}	660.0 ± 10.0^{a}
	3	485.0 ± 15.0^{c}	425.0 ± 9.9^{d}	650.0 ± 4.8^{a}	620.0 ± 10.0^{b}
	5	489.0 ± 11.0^{c}	665.0 ± 6.8^{a}	620.0 ± 4.8^{b}	650.0 ± 12.0^{a}
	7	482.0 ± 13.0^{c}	690.0 ± 9.5^{a}	610.0 ± 10.0^{b}	602.0 ± 9.6^{b}
GR (U/ mg P)	1	4.03 ± 0.35^{b}	3.8 ± 0.3^{c}	4.6 ± 0.6^{a}	4.8 ± 0.3^{a}
	3	4.02 ± 0.3^{b}	3.6 ± 0.4^{b}	4.7 ± 0.5^{a}	4.8 ± 0.3^{a}
	5	4.06 ± 0.2^{b}	2.7 ± 0.4^{c}	4.8 ± 0.5^{a}	4.8 ± 0.3^{a}
	7	4.03 ± 0.4^{b}	2.2 ± 0.2^{c}	5.4 ± 0.7^{a}	5.2 ± 0.6^{a}
GPx (µ mol/ mg P/ min)	1	120.0 ± 10.0^{a}	115.0 ± 4.9^{b}	125.0 ± 5.0^{a}	130.0 ± 5.0^{a}
	3	125.0 ± 15.0^{a}	75.0 ± 4.7^{c}	85.0 ± 6.9^{b}	88.0 ± 7.4^{b}
	5	122.0 ± 13.0^{a}	60.0 ± 5.8^{c}	90.0 ± 12.0^{b}	91.0 ± 5.8^{b}
	7	120.0 ± 12.0^{a}	55.0 ± 5.9^{c}	120.0 ± 5.0^{a}	116.0 ± 15.0^{b}
GST (nmol/ mg P/ min)	1	320.0 ± 4.9^{a}	315 ± 4.9^{b}	325.0 ± 4.5^{a}	330.0 ± 10.1^{a}
	3	325.0 ± 12.0^{a}	300.0 ± 10.0^{b}	320.0 ± 5.5^{a}	326.0 ± 3.9^{a}
	5	330.0 ± 7.5^{a}	217.0 ± 15.0^{c}	250.0 ± 10.0^{b}	250.0 ± 4.9^{b}
	7	320.0 ± 4.9^{a}	200.0 ± 4.7^{c}	228.0 ± 7.6^{c}	250.0 ± 6.8^{b}

SOD; superoxidedismutase, CAT; catalaseenzyme, GR; Glutathionereducatase; GPx; glutathioneperoxidase; GST; glutathionetransferase, Means of treatments with the same superscript letter within each interval in the same row are not significantly different at $P \leq 0.05$.

Table (3): Levels of GSH, GSSG, Total glutathione, TAC, MDA, Nitric oxide and Protein carbonyls (mean ± SD) in liver of *O. niloticus* exposed to heavy metals, heavy metals plus vitamin E and heavy metals plus selenium (Se) with vitamins A and C.

Parameter	Time (days)	Control group	HM group	HM+E group	HM+Se-ACE group
GSH ($\mu molg^{-1}$ wt. w)	1	158.0 ± 8.0^a	95.0 ± 5^c	100.0 ± 10.0^c	123.0 ± 10.0^b
	3	165.0 ± 10.0^a	78.0 ± 8^c	84.0 ± 4.0^c	96.0 ± 6.0^b
	5	155.0 ± 9.0^a	62.0 ± 2.0^c	66.0 ± 6.0^c	93.0 ± 3.0^b
	7	158.0 ± 8.0^a	60.0 ± 5.0^c	61.0 ± 6.0^c	94.0 ± 4.6^b
GSSG ($\mu molg^{-1}$ wt. w)	1	16.1 ± 2.6^d	29.0 ± 3.2^a	26.0 ± 1.0^b	20.7 ± 2^c
	3	16.3 ± 2.3^d	33.0 ± 1.5^a	27.0 ± 2.6^b	20.0 ± 2.6^c
	5	16.5 ± 2.6^d	39.0 ± 3.2^a	36.0 ± 2.0^b	20.7 ± 2^c
	7	16.3 ± 1.8^c	42.0 ± 2.6^a	35.0 ± 2.5^b	18.3 ± 1.5^c
Total glutathione ($\mu molg^{-1}$ wt. w)	1	174.0 ± 10.0^a	125.0 ± 7.6^c	126.0 ± 11^c	143.7 ± 12.0^b
	3	173.0 ± 13^a	111.0 ± 9.5^b	111.0 ± 6.2^b	116.0 ± 8.5^b
	5	176.0 ± 12^a	101.0 ± 4.9^c	105.0 ± 9.5^c	113.7 ± 5.0^b
	7	174.0 ± 11.0^a	$102.0 \pm 7.b^d$	96.7 ± 5.5^c	112.3 ± 6.0^b
TAC (μMg^{-1} wt. w)	1	5.5 ± 0.5^a	4.4 ± 0.4^b	4.9 ± 0.4^b	5.2 ± 0.2^a
	3	5.5 ± 0.6^a	4.2 ± 0.8^b	5.2 ± 0.4^a	5.3 ± 0.3^a
	5	5.6 ± 0.7^a	2.9 ± 0.4^b	5.4 ± 0.4^a	5.9 ± 0.5^a
	7	5.5 ± 0.5^a	4.3 ± 0.3^b	5.9 ± 0.5^a	5.7 ± 0.6^a
MDA ($nmolg^{-1}$wt.w)	1	1.2 ± 0.1^d	4.1 ± 0.5^a	3.2 ± 0.2^b	2.5 ± 0.2^c
	3	1.2 ± 0.12^c	4.6 ± 0.6^a	1.9 ± 0.3^b	2.0 ± 0.4^b
	5	1.3 ± 0.13^c	5.9 ± 0.4^a	1.7 ± 0.2^b	1.5 ± 0.3^b
	7	1.2 ± 0.1^b	5.1 ± 0.4^a	1.1 ± 0.2^b	1.1 ± 0.2^b
Nitric oxide ($\mu molg^{-1}$wt.w)	1	0.43 ± 0.10^c	2.2 ± 0.3^a	1.2 ± 0.2^b	1.1 ± 0.4^b
	3	0.43 ± 0.11^d	2.1 ± 0.4^a	1.1 ± 0.3^b	0.8 ± 0.2^c
	5	0.44 ± 0.1^d	2.5 ± 0.3^a	0.9 ± 0.2^b	0.6 ± 0.2^c
	7	0.44 ± 0.1^c	2.8 ± 0.2^a	0.6 ± 0.2^b	0.4 ± 0.1^c
Proteincarbonyls ($\mu molg^{-1}$wt.w)	1	1.8 ± 0.2^c	2.9 ± 0.3^b	8.1 ± 0.4^a	2.6 ± 0.3^b
	3	1.9 ± 0.2^c	2.6 ± 0.6^b	8.2 ± 0.2^a	2.1 ± 0.4^b
	5	1.8 ± 0.3^c	3.1 ± 0.3^b	8.3 ± 0.4^a	1.8 ± 0.3^c
	7	1.8 ± 0.2^d	2.1 ± 0.2^b	8.2 ± 0.2^a	1.5 ± 0.5^c

GSH; reduced glutathione, GSSG; oxidized glutathione, TAC; total antioxidant capacity, MDA; malondialdehyd,
Means of treatments with the same superscript letter within each interval in the same row are not significantly different at $P \leq 0.05$.

Table (4): Levels of GSh, GSSG, Total glutathione, TAC, MDA, Nitric oxide and Protein carbonyls (mean ± SD) in gill of *O. niloticus* exposed to heavy metals, heavy metals plus vitamin E and heavy metals plus selenium (Se) with vitamins A and C.

Parameter	Time (days)	Control group	HM group	HM+E group	HM+Se-ACE group
GSH (μmolg^{-1} wt. w)	1	138.3 ± 8.0^{a}	94.0 ± 3.0^{c}	94.0 ± 4.0^{c}	117.0 ± 9.0^{b}
	3	135.0 ± 10.0^{a}	76.0 ± 6.0^{b}	77.0 ± 7.0^{b}	77.0 ± 4.0^{b}
	5	140.0 ± 15.0^{a}	60.0 ± 5.0^{c}	64.0 ± 4.0^{c}	99.0 ± 9.0^{b}
	7	138.0 ± 8.0^{a}	58.0 ± 7.0^{c}	61 ± 6.0^{c}	100.0 ± 7.0^{b}
GSSG (μmolg^{-1} wt. w)	1	14.3 ± 1.5^{d}	24.4 ± 1.1^{a}	21.9 ± 2.6^{b}	17.0 ± 2.6^{c}
	3	14.1 ± 1.3^{d}	31.3 ± 1.5^{a}	23.9 ± 2.1^{c}	25.0 ± 2.7^{b}
	5	13.9 ± 1.1^{d}	35.3 ± 3.2^{a}	31.3 ± 3.2^{b}	17.0 ± 2.6^{c}
	7	14.3 ± 1.5^{c}	37.1 ± 2.6^{a}	33.7 ± 2.1^{b}	14.7 ± 2.0^{c}
Total glutathione (μmolg^{-1} wt. w)	1	153.0 ± 9.5^{a}	117.4 ± 4^{c}	116.0 ± 6.2^{c}	134.0 ± 12.5^{b}
	3	150.0 ± 13^{a}	104.0 ± 2.6^{b}	101.0 ± 8.1^{b}	102.0 ± 6.6^{b}
	5	155.0 ± 16^{a}	95.3 ± 8^{c}	95.3 ± 7^{c}	116.0 ± 11.5^{b}
	7	152.0 ± 10^{a}	94.7 ± 9.6^{c}	96.3 ± 5.9^{c}	115.0 ± 6.6^{b}
TAC (μMg^{-1} wt. w)	1	6.0 ± 0.5^{a}	4.0 ± 0.4^{c}	5.0 ± 0.4^{b}	5.3 ± 0.3^{b}
	3	6.03 ± 0.6^{a}	3.2 ± 0.2^{c}	5.3 ± 0.3^{b}	5.0 ± 0.5^{b}
	5	6.1 ± 0.7^{b}	2.8 ± 0.3^{d}	6.6 ± 0.6^{a}	5.7 ± 0.6^{c}
	7	6.0 ± 0.5^{a}	4.1 ± 0.6^{c}	5.7 ± 0.6^{b}	6.1 ± 0.15^{a}
MDA (nmolg^{-1}wt.w)	1	0.9 ± 0.1^{a}	3.8 ± 0.8^{b}	2.8 ± 0.3^{c}	2.3 ± 0.3^{c}
	3	1.0 ± 0.2^{c}	4.0 ± 0.5^{a}	2.1 ± 0.3^{b}	2.0 ± 0.4^{b}
	5	0.95 ± 0.1^{c}	4.3 ± 0.3^{a}	1.6 ± 0.3^{b}	1.4 ± 0.4^{b}
	7	1.0 ± 0.2^{b}	4.4 ± 0.4^{a}	1.2 ± 0.2^{b}	1.0 ± 0.3^{b}
Nitric oxide (μmolg^{-1}wt.w)	1	0.36 ± 0.05^{d}	1.6 ± 0.2^{b}	2.8 ± 0.2^{a}	1.0 ± 0.2^{c}
	3	0.37 ± 0.04^{d}	1.8 ± 0.3^{a}	0.9 ± 0.1^{b}	0.6 ± 0.1^{c}
	5	0.35 ± 0.03^{d}	2 ± 0.6^{a}	0.9 ± 0.2^{b}	0.5 ± 0.13^{c}
	7	0.36 ± 0.05^{d}	2.3 ± 0.3^{a}	0.6 ± 0.1^{b}	0.4 ± 0.15^{c}
Proteincarbonyls (μmolg^{-1}wt.w)	1	1.6 ± 0.2^{c}	4.0 ± 0.5^{a}	2.0 ± 0.2^{b}	2.0 ± 0.2^{b}
	3	1.7 ± 0.25^{c}	7.3 ± 0.3^{a}	2.8 ± 0.3^{b}	2.8 ± 0.3^{b}
	5	1.5 ± 0.3^{c}	6.0 ± 0.4^{a}	2.5 ± 0.4^{b}	2.5 ± 0.4^{b}
	7	1.6 ± 0.2^{b}	6.3 ± 0.3^{a}	1.7 ± 0.2^{b}	1.7 ± 0.2^{b}

GSH; reduced glutathione, GSSG; oxidized glutathione, TAC; total antioxidant capacity, MDA; malondialdehyd,
Means of treatments with the same superscript letter within each interval in the same roware not significantly different at $P \leq 0.05$.

DISCUSSION

There are relation between reduced glutathione (GSH) and numerous effects that produced by heavy metals toxicity in fish. The activities GST, CAT, SOD and of GR were increased in fish after 1, 3, 5 and 7 days of exposure to heavy metals. GSH is the most important non protein thiol in all living cells; it has a vital role in protection of intracellular against toxin such as Cu and Zn through the action of GR, GST and GPx (Anderson and Luo, 1998; Mosleh et al., 2005a and b; Mosleh et al., 2006). GSH concentration and GST activity evaluation has been reported in the context of their involvement in the metabolism of phase II detoxification. However, these molecules are also involved with the catalase activity and glutathione reductase (GRD) as cellular antioxidants. While the glutathione S- transferases (GST) are a group of mainly cytosolic enzymes, some membrane-bound forms have been described. The activity of SH group of glutathione with reactive molecules resulting from the activity of phase I enzymes are important when catalyzed by glutathione S- transferases. Glutathione conjugation with these reactive molecules neutralizes their electrophilic site and makes them more soluble. While superoxide dismutase may be best known antioxidant enzymes (Lackner, 1998; Cossu et al., 1997). In addition, a high superoxide dimutase activity level is induced following exposure of fish to the mixture of heavy metals only. High concentrations of pollutants, such as heavy metals will instead induce a decrease in the activity explained by the oxidation of superoxide dismutase by reactive oxygen species. The glutathione peroxidases are the most important peroxidase for detoxification of hydroperoxides. Indeed, they catalyze the reduction of organic hydroperoxides (ROOH) and lipid. They thus prevent the spread of chain reactions leading to lipid peroxidation (Cossu et al., 1997). In addition, they are involved in catalyzing the oxidation of GSH to GSSG. The glutathione S- transferases (GST) are cytosolic enzymes, they contribute to the elimination of certain products of catabolism or cell decontamination when glycosylated. The glutathione S-transferases catalyze the conjugation reactions of reduced glutathione GSH with electrophilic xenobiotics, resulting in an increase in solubility and facilitating their removal from the cell (Paris-Palacios et al., 2010; Mofeed and Mosleh 2013). Recent studies have shown that many substrates GST result of oxidative stress such as peroxides. In addition, certain GST isoenzymes have glutathione peroxidase activities and are able to reduce lipid hydroperoxides to the corresponding alcohol(Tappel et al., 1978) described an activity Se-independent glutathione peroxidase which appear due to glutathione S- transferases. And gradually the difference of action existed between the glutathione S -transferase and glutathione peroxidases are gradually reduced. It is likely that these two enzymes correspond to two different types of activities belonging to one same molecule. While catalase is a tetramerichemoprotein (240,000 daltons) comprising a Fe atom per subunit. This enzyme has three localized in different cellular compartments isoforms: CAT1 and CAT2 are located in peroxisomes and cytoplasm while CAT3 is mitochondrial. However the majority of cellular catalase activity was due to peroxisomal forms of the enzymes and peroxisome proliferation is also well correlated with increases in the activity (Scandalios et al., 2000). This enzyme catalyzes the reduction of hydrogen peroxide (H_2O_2) to water and molecular oxygen. While GPx, catalyzes the reduction of H_2O_2 to tow molecules of water, in this reaction GSH is the source of hydrogen and converted to the oxidized form glutathione disulphide (GSSG) so GPx activity is strictly linked to GSH concentrations (Paris-Palacios et al., 2010; Mofeed and Mosleh, 2013). The reduction in GPx activity when fish exposed to heavy metal only is an indicator for decreasing its capacity to break H_2O_2 and lipid peroxides, at the same time this reduction may be resulted from over production of H_2O_2 or a direct action of heavy metals on the enzyme synthesis. The levels of protein carbonylation in this study were significantly higher in fish treated with heavy metals only indicating that the fish suffering from oxidative stress when exposed to heavy metals. Because metals are known to induce directly the protein carbonyls formation via metal catalyzed oxidation reactions (Stadtman and Oliver, 1991). The increased MDA rates in fish in the present study indicates the disturbance in membranes phospholipids of the exposed *O. niloticus* due to metal exposure. The MDA concentration was increased up to 233% in fish exposed to heavy metals after 24 h only, when compared to control group several studies reported the increase of MDA concentrations in aquatics exposed to xenobiotics (Üner et al., 2006), pollutants (Ji et al., 2010) and heavy metals. Antioxidants such as selenium and some vitamins have antioxidant activities to protect the cells against the damaging effects of the free radicals through preventing its production and antioxidant defense; the most powerful antioxidant vitamins are vitamins A, C and E (Keleştemur, 2012). Treatment with heavy metals and vitamin E and a combination of selenium (Se) with vitamins A, C significantly reduced the changes caused by heavy metals exposure in all examinedparameters. Moreover, the results indicate that alterations caused by heavy metals are connected with free radicals generation and used antioxidants effectively to protect against heavy metals intoxication. Moreover, vitamin E pretreatment partially attenuates heavy metals-induced oxidative stress by altering antioxidant enzymes. Ognjanovic et al. (2003) demonstrated the effectiveness of vitamin E in reducing oxidative stress in heavy metals-treated animals and suggested that reductions in increased lipid peroxidation due to heavy metals toxicity may be an important factor in the action of vitamin E. Vitamin E has a greater effect than selenium in reducing lipid peroxidation in various brain regions (Whanger, 2001). Many studies reported the protection action of the antioxidants like vitamin C and Vitamin E against cadmium toxicity in animals (Ognjanovic et al., 2003). But Cosic et al. (2007), reported the increase of DNA damage in presence of antioxidants as glutathione and vitamin C in in-vitro studies. Vitamin E is the most important fat soluble antioxidants, it plays a key role in

protection of lipoproteins, membranes phospholipids, and stored lipids from oxidation (Mekkawy et al., 2013); also, it plays important role in healthy of red blood corpuscle, capillaries, cardiac muscle and immunity (Halver, 2002). While vitamin C plays important roles as anticarcinogenic (Draz et al., 2009), anticlastogenic and antimutagenic.Teleosts lack L-gulonolactone oxidase, so it can't be synthesize vitamin C, which must be supplied in the diet. Vitamin C is necessary for collagen synthesis, iran metabolism and it has anti-stress properties, it consider the most important water soluble antioxidant, it characterized by nucleophilic properties, intercept reactive electrophilic metabolites and preventing their attack on nucleophilic sites on DNA and hence blocking adduct formation (Ognjanovic, et al., 2003), therefore, it prevent the free radical and ROS harmful action.

CONCLUSION

Therefore, this study confirms on the powerful protective potential of the antioxidants vitamin E alone and a combination of and a combination of selenium (Se) with vitamins A, C and E against the toxicity of Cd, Cu, Pb and Zn on *O. niloticusniloticus*.

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