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Biochemical monitoring of CAT, GSH and LPO of $CuSO_4$ in the organs of Zebrafish (*Danio rerio*)

Sunaina and B Alam Ansari

Abstract
Heavy metals are natural trace components of the aquatic environment, but their levels have elevated due to domestic, industrial, mining and other activities. These heavy metal toxicant are accumulated in the fish through general body, they damage and weaken the mechanism concerned leading to physiological, pathological and biochemical disorders. High concentration of metals can however cause cellular and histological changes. Copper is an essential element for living organisms. Presence of copper in fresh water in higher concentration is toxic to organisms of experimental fishes (Zebrafish) liver and ovary. Zebrafish were exposed to different concentrations (20% and 80% of 96 hour LC_{50}) of copper. Reduction in CAT and GSH observed whereas LPO was elevated. CAT activity in liver for 7 days 20% is 93% while it decreased at 28 days is 65%. GSH level of 7 days in liver is 90% while much reduction was observed at 28 days is 56%. After 7 days treatment of LPO at 20% increased to 110% and more reduction was observed at 28 days is 136% at 80%. Values are significant at $P<0.05$.

Keywords: Zebrafish, Copper, CAT, GSH, LPO and *Danio rerio*

1. Introduction
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) such as superoxide (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH), mainly as by product 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) [1]. ROS can attach multiple cellular constituents, including proteins, nucleic acids, and lipids. To cope with damaging actions of ROS, organisms have evolved multiple systems of antioxidant defense. So called low-molecular weight anitoxidants include metabolites such as glutathione, ascorbic acid, uric acid, etc. whereas high-molecular weight defences include enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidise (GPx), glutathione reductase (GRx) and glutathione-s-transferase (GST). These enzymatic and nonenzymatic constituents deal directly with radical species and protect against intoxication of any damage to the macro and micro molecules in body (Hermes-Lima and Storey, 1993) [2]. Copper pollution appears in the aquatic environment from natural and anthropogenic sources such as mine washing, agricultural leaching and direct application as algicide and molluscicide (Hinton and Lauren, 1990) [3]. Monitoring biochemical and histo-cytological changes in fish liver is a highly sensitive and accurate way to assess the effects of xenobiotic compounds in laboratory and field studies. Moreover, liver is known to be the primary organ for copper storage in fish. Metals are able to disturb the integrity of the physiological and biochemical mechanisms in fish that are not only an important ecosystem component, but also used as a food source (Toth, 1997) [4], (Wood *et al*., 1999) [5] (Atli *et al.,* 2010) [6].
Oxidative damage after Cu exposure was shown as changes in biomarkers in the liver, kidney, heart and gill of numerous fish species. For instance, zebra fish exposed to Cu led to increase of protein carbonyl content in the gill and liver, along with increases in activity of the ROS defense enzyme catalase (Craig *et al*., 2007) [7]. Cu exposure elevated lipid peroxidation levels in the liver and intestine of Indian flying barb (Esomusdanricus) but results a decline in antioxidant enzyme activity (Vutukuru *et al*., 2006) [8].
In living organisms, oxidative stress can induce by metals exposed to them through the elevation of free radicals and the changes in antioxidant defense mechanisms, including detoxification and scavenging enzymes.

Protein carbonylation and lipid peroxidation are considered consequences of ROS-induced oxidation of protein side chains and lipids, respectively. Generally, a high content of ROS induction can oxidize cell constituents, such as lipids and proteins, causing lipid peroxidation and protein oxidation which are always evaluated by MDA and PC, respectively (Kohen, 2002) [9]. Antioxidant enzymes SOD, CAT, GST, GPx and GR scavenge increased ROS and MDA, and protects organisms from possible oxidative damage by this immediate emergency response mechanism (Rajasekar, G and C. Venkatakrishnaiah, 2016) [10]**.** SOD is the first enzyme to deal with oxyradicals and responsible for catalyzing the dismutation of highly super oxide radical O_2 to O_2 and H_2O_2. CAT is an enzyme located in peroxisomes and facilitates the removal of the H_2O_2, which is metabolized to molecular oxygen and water and protection from the oxidation of unsaturated fatty acids in cell membrane. CAT activities in different organs of carp inhibited with the concentrations of copper exposure and suggest that copper induced oxidative stress responses by suppressing or inactivating the antioxidant activity of CAT (the H_2O_2 scavenger) in fish (Rajasekar, G and C. Venkatakrishnaiah, 2016) [10].

Copper is one of the key trace minerals required for an effective immune response. The biological functions of copper include electron- transfer catalysis by means of its two accessible oxidation states (Georgopoulos *et al*., 2001) [11].

Regarding to the effect of copper sulphate on the oxidative cascade in liver tissue showed in significant decrease in antioxidant including (GSH) and (GST) in treated groups compared to control group. While (MDA) showed significant increase in treated groups compared to control group. These results were agreed (Engle *et al*., 2000) [12]. These results may be attributed to copper was found to be quite effective in forming toxic oxygen types and starting the process of lipid peroxidation (Weckx and Clijsters 1996 [13]. Copper increases may be due to lipoxygenase activity, catalyzing lipid peroxidation, especially of unsaturated fatty acids, as a result of these reactions, various radicals were formed and these lead to increasing concentration of MDA which is a product of peroxidation, which are an indicator of oxidative stress after heavy metals dosing and the increase level correlates with the increase of metal concentrations (Wu *et al*., 2003) [14]. In present study we used Zebrafish for biomonitoring of environmental contamination. Therefore, examining the change in activity of antioxidant enzymes such as LPO, CAT and GSH should be an effective method ofdenoting oxidative stress and changes in their activity.

2. Materials and Methods

Zebrafish were collected from local ponds, acclimatized for 15 days, stocked and bred under laboratory conditions and used for 7,14, 21 and 28 days of experimental period. The aquaria were continuously aerated through stone diffusers connected to a mechanical air compressor. Water temperature was 25±2ºC and pH was maintained between 6.6 and 8.5. The fish were fed twice daily alternately with egg, goat liver and raw brine shrimp pellets. The experimental fishes were exposed to different concentrations viz, 20% and 80% of 96 hour LC_{50} values i.e. 0.006 and 0.03 mg/l of arsenic calculated earlier (Sunaina and Ansari, 2014) [15]. 20 fishes for each concentration of metal test were used. In the experimental aquaria water was replaced daily with fresh treatment of metal. After the end of the experimental periods (7, 14, 21 and 28 days), required number of treated fish was taken out from experiment and control groups and their tissues were dissected. Six replicates for each concentration of arsenic were arranged.

3. Biochemical Estimation

3.1 Lipid peroxidation (LPO)

LPO levels were estimated with thio-barbituric acid (TBARS) and color reaction for malandialdehyde (MDA) according to procedure by (Placer *et al.*, 1966) [16]. Tissues were homogenized in chilled 0.15 M KCl using a Teflon pestle to obtain 10% w/v homogenate. One ml of homogenate was incubated at 37ºC (± 0.5) for two hours. To each sample, 1 ml of 10% w/v trichloro acetic acid (TCA) (s. d. fine chem. Ltd; Mumbai) was added. After thorough mixing, the reaction mixture was centrifuged at 2000 rpm for 10 minutes 1 ml of supernatant was taken with equal volume of 0.67% w/v TBA (thio-barbituric acid) and kept in boiling water bath for 10 minutes, cooled and diluted with 1 ml of distilled water. The absorbance pink color was observed, which measured at 535 nm against a blank. The concentration of MDA was read from a standard calibration curve plotted using 1, 1, 3, 3` tetra-methoxypropane (Sigma –Aldrich Co., St. Louis, USA) and the results were expressed as µmol of MDA formed 30 min^{-1} mg $protein^{-1}$.

3.2 Reduced glutathione (GSH)

The GSH levels was estimated according to procedures (Paglia *et al.,* 1975) [17], with which it is determined by its reaction with 5,5`- dithiobis-2-nitrobenzoic acid (DTNB) to produce/yield a yellow chromophore that was measured spectrophotometrically at 412 nm. The results were expressed as GSH mg per mg $protein^{-1}$. The protein contents of tissues were assayed using the Lowery *et al*., (1951) [18] method with bovine serum albumin as the standard.

3.3 Catalase (CAT)

The CAT activity was estimated according to procedures by (Sinha, 1972) [19]. This method is based on the fact that in acetic acid dichromate is reduced to chromic acetate when heated in presence of H_2O_2 with the formation of perchromic acid as an unstable intermediate. The chromic acetate is measured colorimetrically at 620 nm. The catalase preparation is allowed to split H_2O_2 at different time intervals by the addition of a dichromatic acetic acid mixture and remaining H_2O_2 is determined colorimetrically. The results were expressed as µmol H_2O_2 utilized min^{-1} mg $protein^{-1}$.

Two way analysis of variance (ANOVA) was applied to test the significance of data. All the data are expressed as means (n=6) ± standard deviation (SD) and differences were considered significant at $P<0.05$.

4. Results and Discussion

4.1 Changes in liver

The effect of copper on the catalase activity in the liver of zebrafish is shown in the table 1. Copper was found to be toxic as it causes much reduction in the CAT activity. Two concentrations of copper *i.e.* 20% (15.63 mg/L) and 80% (62.53 mg/L) of 96–h LC_{50} causes much decrease at 7 days of treatment period 93% (148.79±0.37 µM H_2O_2 utilized/min/mg protein) and 79% (132.79±0.42 µM H_2O_2 utilized/min/mg protein) and 28 days treatment period 65% (102.05±0.26 µM H_2O_2 utilized/min/mg protein) and 57% (89.49±0.34 µM H_2O_2 utilized/min/mg protein) Minimum change in CAT activity was observed after 14 days of treatment period at both

concentrations which was 86% (137.60±0.08 µM H_2O_2 utilized/min/mg protein) and 74% (121.60±0.28 µM H_2O_2 utilized/min/mg protein) (Table 1 and Fig. 1) whereas at 21 days of treatment period at all concentrations the maximum change in CAT activity were observed which was 77% (123.19±0.24 µM H_2O_2 utilized/min/mg protein) and 68% (108.79±0.32 µM H_2O_2 utilized/min/mg protein).

Effect of Copper on GSH was significantly reduced at 7 days 20% and 80% of values, 90% (5.40±0.53 µM H_2O_2 utilized/min/mg protein) 85% (5.10±0.56 µM of GSH utilized/min/mg protein). The GSH level was decreased to 83 % (5.00±0.42±0.63 µM of GSH utilized/min/mg protein) as compared to 14 days of 20%. It was recorded that there was a gradual decrease in GSH level as the time and concentration increases but at 80% the reduction was more significant after 21 days of exposure periods. The reduction of GSH level was maximum at 28 days of treatment of 80% of the 96-h LC_{50} and it was found to be only 56% (3.28±0.62 µM of GSH utilized/min/mg protein) (Table 2 and Fig. 2). Analysis of variance confirmed that the inhibition was concentration dependent ($P<0.05$).

The effect of copper on lipid peroxidation (LPO) in the liver of zebrafish showed a significant ($P<0.05$) changes at different concentrations and exposure periods. After 7 days of treatment at 20% and 80% concentrations the LPO was increased to 105% (10.23±0.06 µM of LPO utilized/min/mg protein) and 110% (10.98±0.43 µM of LPO utilized/min/mg protein) as compared to control (100). After 14 days exposure to 20% of 96–h LC_{50} of copper LPO was increased to 111% (11.03±0.09 µM of LPO utilized/min/mg protein). At 20% for 21 days the level was increased to 118% (11.82±0.23 µM of LPO utilized/min/mg protein) which further enhanced to 123% (12.32±0.29 µM of LPO utilized/min/mg protein) at 80%. A significant change was recorded at each concentration after 28 days of exposure period. At 20% the level was 129% (12.96±0.29 µM of LPO utilized/min/mg protein) and highly increased 136% (14.22±0.31 µM of LPO utilized/min/mg protein) at 80% of 96-h LC_{50}. With the increase in concentration there was significant ($P<0.05$) increase in the LPO showing the concentration dependent enhancement (Table 3, Fig. 3).

4.2 Changes in ovary

After copper treatment the CAT activity was gradually decrease to 87% (136.59±0.20112 µM of H_2O_2 utilized/min/mg protein), 83% (20±0.63µM of H_2O_2 utilized/min/mg protein), 71% (124.17±0.20 µM of H_2O_2 utilized/min/mg protein) and 62% (100.00±0.60 µM of H_2O_2utilized/min/mg protein) after 7, 14, 21 and 28 days of exposure. While maximum reduction was observed at 80% concentration and it was found to be 73% (100.00±0.60 µM of H_2O_2 utilized/min/mg protein), 69% (107.10±0.46 µM of H_2O_2 utilized/min/mg protein), 63% (99.55±0.59 µM of H_2O_2 utilized/min/mg protein) and 51% (81.09±0.50 µM of H_2O_2 utilized/min/mg protein) respectively for all exposure periods as compared to control (100%). Analysis of variance showed that the inhibition was concentration dependent ($P<0.05$) *i.e.,* increased in concentration enhanced the inhibition of CAT activity (Table 1 and ANOVA table of table 4, Fig. 4).

The effect of copper on GSH in ovary shows a significant reduction. The GSH level in the control was considered as 100%. At the 20% concentration there was a significant decrease in GSH level at 7, 14, 21 and 28 days exposure periods and it was found 83% (4.15±0.89 µM of GSH utilized/min/mg protein), 78% (3.88±0.88 µM of GSH utilized/min/mg protein), 65% (3.22±0.88 µM of GSH utilized/min/mg protein) and 59% (2.30±0.55 µM of GSH utilized/min/mg protein) respectively as a compared to control whereas at 80% also decrease in GSH level was observed all exposure periods 74% (3.70±0.72 µM of GSH utilized/min/mg protein), 67% (3.33±0.75 µM of GSH O_2 utilized/min/mg protein), 61% (3.17±0.76 µM of GSH utilized/min/mg protein) and 52% (2.83±0.79 µM of GSH utilized/min/mg protein), maximum reduction was observed after 28 days of treatment period. Overall reduction in the GSH level after copper treatment statistically significant ($P < 0.05$) (Table 5, Fig. 5).

LPO level was increased in copper treated fish ovary. After both exposure period and at each concentration (Table 6, Fig.6), there was a gradual increase in LPO level were observed after 14 to 28 days of treatment, 109% (7.36±0.43 µM of LPO utilized/min/mg protein) and 121% (8.44±0.42 µM of LPO utilized/min/mg protein) of 20%. At 80% treatment there was an increase of 132% (6.75±0.56 µM of LPO utilized/min/mg protein) in the LPO level after 28 days of treatment as compared to control (100). With increase in concentration there was significant ($P < 0.05$) increase in LPO level showing the concentration-dependent enhancement.

Despite copper's important biological functions, copper can be harmful to fish. Chronic waterborne exposure to slightly higher copper concentrations than environmental ones could have negative effects on the health status of common carp. Such effects include behavioural changes, the impairment of haematological and biochemical indices, an imbalance in antioxidant defence, and also pathological lesions in tissues (Sevcikova *et al*., 2016) [20].

Copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*). Hazards of pollutants accumulated in sediments since low concentrations of heavy metals could result in non-negligible deleterious effects on early developmental stages of various fish species nanocopper is acutely toxic to zebra fish, with a 48-h LC_{50} concentration of 1.5 mg/L. Histological and biochemical analysis revealed that the gill is the primary target organ for nanocopper. To further investigate the effects of nanocopper on the gill, zebrafish were exposed to 100 µg/L of nanocopper or to the concentration of soluble copper matching that present due to dissolution of the particles. Under these conditions, nanocopper produced different morphological effects and global gene expression patterns in the gill than soluble copper, clearly demonstrating the effects of nanocopper on gill are not mediated solely by dissolution (Griffitt *et al.* 2007) [21].

High intracellular copper has been described as toxic to the cells via generation of reactive oxygen species (ROS) (Pourahmad and O`Brien, 2000) [22]. A major cytotoxic role for ROS includes the activation of the apoptosis and some studies clearly implicate a role for ROS in copper-induced apoptosis *in vitro* (Ma *et al*., 1998) [23], (Pourahmad and O`Brien, 2000) [22].

(Zhai *et al*., 2000) [24]. Most sensitive organs to stress induced by waterborne copper are the central nervous system and the liver, even though the most affected in terms of cell death are the gills and pronephrons. Cell death is likely to be elicited by the induction of ROS, providing possible immediate cause for death of exposed larvae of zebrafish. They studied that copper is toxic for zebrafish larvae because the metal is incorporated and accumulates in diverse tissues (Allende *et al*. 2011) [25].

In present study the zebrafish was exposed to $CuSO_4$ for a

period of 7, 14, 21 and 28 days at suitable concentrations and recorded a significant reduction in CAT (Catalase) and GSH (glutathione reduced) but in the LPO we observed significant induction in liver and ovary of zebrafish. Maximum reduction was recorded in GSH and CAT at the higher concentration 20% of 96- h LC_{50} as compared to the lower concentration of 80% of 96- h and maximum induction of LPO was recorded at the 80% of 96- h as compared to the higher concentration of 20% 96–h. Same trend with the liver values of (Farombi *et al*. 2007) [26], MDA levels were significantly elevated (177%) in the liver of fish (*Clarias gariepinus*) obtained from the Ogun River compared with those of control fish from the Agodi fish farm. GSH is the main intracellular thiol antioxidant and has a key role in the detoxification process of pollutants, not only as a substrate of antioxidant enzymes, but also as a direct reducing agent and a nucleophile able to block the toxicity of heavy metals and organic chemicals with thiol affinity by covalent binding. It has an important role in scavenging of cellular ROS (such as H_2O_2, O_2 •— and •OH). A variety of environmental pollutants are known to change the GSH level in aquatic organisms, including heavy metals. Due to the high sensitivity of the aquatic life towards dissolving toxicant, the fish has been utilized as the biological measurement (Biomarker) to indicate the existence of toxicant exposure and/or the impact towards the evaluation of molecular, cellular to physiological level (Sabullah *et al.,* 2015) [27].

The highest copper concentration in the kidney may be due to the fact that fish kidney contains a cystine rich copper binding protein which is thought to have either a detoxifying or storage function (Luckey and Venugopal, 1977) [28]. The high concentration of copper in the liver compared to gills and heart can be ascribed to the binding of Cu to metallothionein in the liver, which serves as a detoxification mechanism. Copper though essential in the diet can be harmful when large intake occurs. The harmful toxicity is largely attributed to its cupric (Cu^{2+}) forms which, is the specie commonly found (Olafia *et al*., 2004) [29]. Our results indicate a significant elevation of lipid peroxidation in all the organs. The apparent increase in lipid peroxidation may be attributed to the accumulation of the heavy metals in the organs as our data indicate significant concentration of heavy metals in the various organs. Metal catalyzed formation of ROS capable of damaging tissues such as DNA, proteins and lipids is well documented (McCord, 1996) [30], (Pandey *et al.*, 2003) [31]. Furthermore, the activities of SOD, GST and the redox sensitive thiol compound GSH were elevated in all the organs except the gills. The significant increase in these organs may be a response to oxidative stress caused by the presence of heavy metals. The accumulation of heavy metals might have led to the production of superoxide anions which led to the induction of SOD to convert the superoxide radical to H_2O_2. SOD catalytically scavenges superoxide radical which appears to be an important agent of toxicity of oxygen and this provides a defense against this aspect of oxygen toxicity (Kadar *et al*. 2005) [32].

Copper sulfate is highly toxic to fish. Even at recommended rates of application, this material may be poisonous to trout and other fish, especially in soft or acid waters. Fish eggs are more resistant than young fish fry to the toxic effects of copper. When copper dissolves in the pond, it breaks down into copper ions that are presumed to be toxic to algae, parasites and fish. In ponds that have high pH and alkalinity, copper ions quickly react with ions stay in water for such a long period that it is difficult to kill algae and parasites without killing the fish. Copper sulfate is toxic to aquatic invertebrates, such as crab, shrimp, and oysters (Trivedi *et al*., 2012) [33].

Fish are often used as sentinel organisms for ecotoxicological studies because they play number of roles in the trophic web, accumulate toxic substances and respond to low concentration of mutagens therefore, the use of fish biomarkers as indices of the effects of pollution are of increasing importance and can permit early detection of aquatic environmental problems (Van Der Oost, 2003) [34].

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 ($OH^{\cdot}$), a free radical of paramount toxicological fission from hydrogen peroxide (Klaassen, 2001) [35]. 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 $O2^{\cdot}$ as well as transition metals may be one of the major reasons for producing ROS (Klaassen, 2001) [35]. Fenton reaction will not allow the conversion of hydrogen peroxide in to water molecule and instead of it produces ROS. Cu is known to induce the formation of reactive form of oxygen which can produce enzymatic deactivation, lipid peroxidation and DNA damage. Cu has a great capacity to alter membrane structural lipids and could provoke membranous disruption (Segner and Braunbeck, 1998) [36]. Moreover, the elevated level of lipid peroxidation in the liver of *C. auratus* in response to the exposure to Copper sulphate suggests that there is increased production of ROS (Trivedi *et al*., 2012) [33]. The liver is also noted as site of multiple oxidative reactions and maximal free radical generation (Atli *et al.,* 2006) [37]. Reduction in the activity of different oxidative enzymes like CAT, GPx, GR, GST, and SOD may reduce the secondary antioxidative defence mechanism in the hepatic cells of goldfish (Yuanyuan Sun *et al*, 2006) [38].

However, organisms are equipped with interdependent cascades of enzymes to alleviate oxidative stress and repair damaged macromolecules, produced during normal metabolism or due to exposure to xenobiotics. In this cascade, SOD and CAT are the major enzymes in eliminating ROS formed during bioactivation of xenobiotics in the hepatic tissues (Sk and Bhattacharya 2006) [39] and the induction of SOD/CAT system provides a first line of defense against ROS. SOD help to dismutase superoxide radical O2- to hydrogen peroxide (H_2O_2).

The same pattern of elevation in LPO and reduction in GSH and CAT in Zebrafish liver and ovary, exposed to arsenic tri oxide reported, (Ansari *et al*., 2016) [40]. In the present study we observed, LPO level has been significantly increased in liver and ovary of zebrafish *Danio rerio* at 7, 14, 21 and 28 days exposure period. The findings revealed that heavy metal, copper create harmful effects by generating reactive oxygen species that damage the cells by disturbing the fluidity balance.

5. Conclusion

The above mention findings suggested that oxidative stress induced by arsenic is an important issue in aquatic ecosystems. The present observations on the induction of oxidative stress and antioxidant system would make it clear that arsenic has a high degree of impact on antioxidant system in liver and ovary of zebrafish particularly during the exposures to arsenic would normally affect and alter the fish health.

Table 1: Effect of Copper on CAT activity (µM CAT utilized/min/mg protein) in the liver of zebrafish, *Danio rerio*.†

Concentration(mg/l)*	Treatment period (Days)			
	7	14	21	28
0.00 (Control)	159.99±0.26 (100%)	160.00±0.09 (100%)	159.99±0.12 (100%)	157.00±0.15 (100%)
15.63	148.79±0.37 (93%)	137.60±0.08 (86%)	123.19±0.24 (77%)	102.05±0.26 (65%)
62.53	132.79±0.42 (79%)	121.60±0.28 (74%)	108.79±0.32 (68%)	89.49±0.34 (57%)

†Values represent mean (n=6) ± standard deviation (SD); data were significant at P< 0.05 (Two-way ANOVA). Numbers in parentheses indicate the percent activity of CAT rounded to the nearest values in comparison to control value taken as 100%.
*The exposure concentrations used were 20% (15.63 mg/L) and 80% (62.53 mg/L) of the 96-h LC_{50} value.

Summary of computation for ANOVA of the table 1.

Source of variation	Degree of Freedom	Sum of squares	Variance	F-values	P<
Variation due to operation	3	1602.97	534.323	4.85325	0.05
Variation due to concentration	2	4429.92	2214.96	20.1185	0.05
Total interaction	6	660.575	110.096		
Total	11	6693.47			

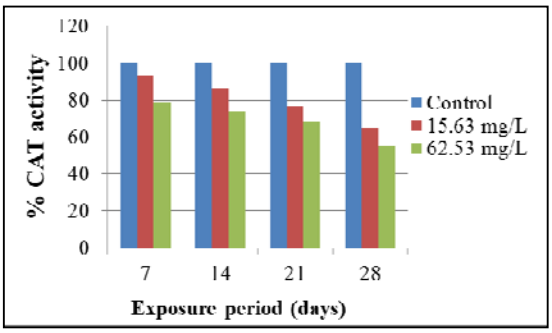

Fig 1: Effect of copper on CAT activity in the liver of zebrafish.

Table 2: Effect of Copper on GSH activity (µM GSH utilized/min/mg protein) in the liver of zebrafish, *Danio rerio*.†

Concentration(mg/L)*	Treatment period (Days)			
	7	14	21	28
Control (0.00)	6.00±0.39 (100%)	6.03±0.43 (100%)	5.93±0.46 (100%)	5.86±0.45 (100%)
15.63	5.40±0.53 (90%)	5.00±0.42 (83%)	4.44±0.49 (75%)	3.63±0.51 (63%)
62.53	5.10±0.56 (85%)	4.70±0.59 (78%)	4.03±0.61 (68%)	3.28±0.62 (56%)

†Values represent mean (n=6) ± standard deviation (SD); data were significant at P< 0.05 (Two-way ANOVA). Numbers in parentheses indicate the percent activity of GSH rounded to the nearest values in comparison to control value taken as 100%.
*The exposure concentrations used were 20% (15.63 mg/L) and 80% (62.53 mg/L) of the 96-h LC_{50} value.

Summary of computation for ANOVA of the table 2.

Source of variation	Degree of Freedom	Sum of squares	Variance	F-values	P<
Variation due to operation	3	2.67527	0.89176	5.25292	0.05
Variation due to concentration	2	6.29135	3.14568	18.5297	0.05
Total interaction	6	1.01858	0.16976		
Total	11	9.9852			

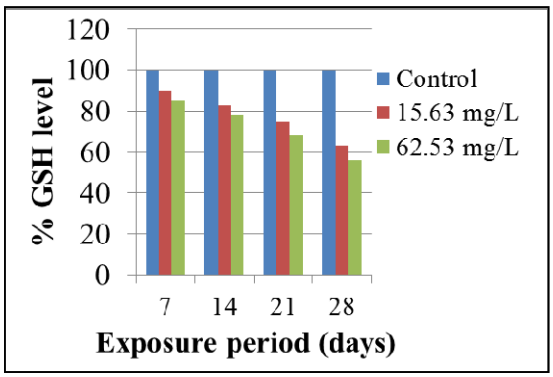

Fig 2: Effect of Copper on GSH level in the liver of zebrafish.

Table 3: Effect of Copper n LPO activity (µM LPO utilized/min/mg protein) in the liver of zebrafish, *Danio rerio*.†

Concentration(mg/L)*	Treatment period (Days)			
	7	14	21	28
Control (0.00)	9.99±0.04 (100%)	10.12±0.03 (100%)	10.02±0.02 (100%)	10.45±0.04 (100%)
15.63	10.23±0.06 (105%)	11.03±0.09 (111%)	11.82±0.23 (118%)	12.96±0.29 (129%)
62.53	10.98±0.43 (110%)	12.05±0.32 (119%)	12.32±0.29 (123%)	14.22±0.31 (136%)

†Values represent mean (n=6) ± standard deviation (SD); data were significant at P< 0.05 (Two-way ANOVA). Numbers in parentheses indicate the percent activity of LPO rounded to the nearest values in comparison to control value taken as 100%.
*The exposure concentrations used were 20% (15.63 mg/L) and 80% (62.53 mg/L) of the 96-h LC_{50} value.

Summary of computation for ANOVA of the table 3.

Source of variation	Degree of Freedom	Sum of squares	Variance	F-values	P<
Variation due to operation	3	7.22449	2.40816	5.93708	0.05
Variation due to concentration	2	10.2577	5.12886	12.6447	0.05
Total interaction	6	2.43368	0.40561		
Total	11	19.9159			

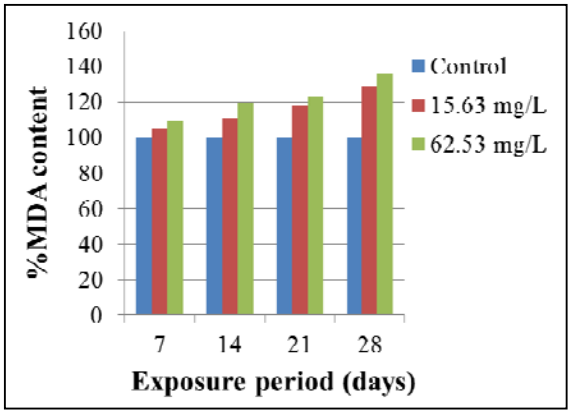

Fig 3: Effect of Copper on LPO level in the liver of zebrafish.

Table 4: Effect of Copper on activity of CAT (µM CAT utilized/min/mg protein) in the ovary of zebrafish, *Danio rerio*.†

Concentration(mg/L)*	Treatment period (Days)			
	7	14	21	28
0.00 (Control)	157.00±0.23 (100%)	155.22±0.45 (100%)	158.03±0.11 (100%)	159.00±0.21 (100%)
15.63	136.59±0.20 (87%)	124.17±0.20 (83%)	112.20±0.63 (71%)	100.00±0.60 (62%)
62.53	100.00±0.60 (73%)	107.10±0.46 (69%)	99.55±0.59 (63%)	81.09±0.50 (51%)

†Values represent mean (n=6) ± standard deviation (SD); data were significant at P< 0.05 (Two-way ANOVA). Numbers in parentheses indicate the percent activity of CAT rounded to the nearest values in comparison to control value taken as 100%.
*The exposure concentrations used were 20% (15.63 mg/L) and 80% (62.53 mg/L) of the 96-h LC_{50} value.

Summary of computation for ANOVA of the table 4.

Source of variation	Degree of Freedom	Sum of squares	Variance	F-values	P<
Variation due to operation	3	1398.07	466.024	4.79931	0.05
Variation due to concentration	2	6434.69	3217.35	33.1336	0.05
Total interaction	6	582.614	97.1023		
Total	11	8415.38			

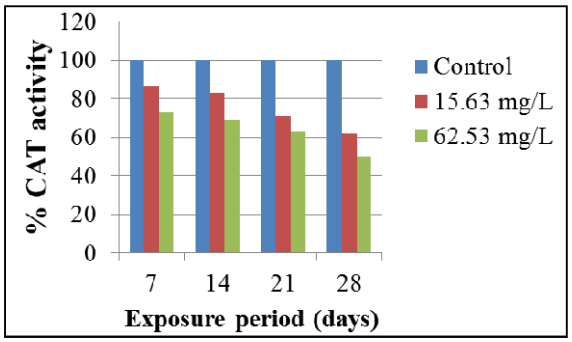

Fig 4: Effect of Copper on CAT level in the ovary of zebrafish.

Table 5: Effect of Copper on activity of GSH (µM GSH utilized/min/mg protein) in the ovary of zebrafish, *Danio rerio*.†

Concentration(mg/L)*	Treatment period (Days)			
	7	14	21	28
0.00 (Control)	5.00±0.93 (100%)	4.98±0.92 (100%)	5.21±0.97 (100%)	5.46±0.96 (100%)
15.63	4.15±0.89 (83%)	3.88±0.88 (78%)	3.38±0.87 (65%)	3.22±0.88 (59%)
62.53	3.70±0.72 (74%)	3.33±0.75 (67%)	3.17±0.76 (61%)	2.83±0.79 (52%)

†Values represent mean (n=6) ± standard deviation (SD); data were significant at P< 0.05 (Two-way ANOVA). Numbers in parentheses indicate the percent activity of GSH rounded to the nearest values in comparison to control value taken as 100%.
*The exposure concentrations used were 20% (15.63 mg/L) and 80% (62.53 mg/L) of the 96-h LC_{50} value.

Summary of computation for ANOVA of the table 5.

Source of variation	Degree of Freedom	Sum of squares	Variance	F-values	P<
Variation due to operation	3	0.83033	0.27678	5.63097	0.05
Variation due to concentration	2	6.30002	3.15001	64.0861	0.05
Total interaction	6	0.29492	0.04915		
Total	11	7.42527			

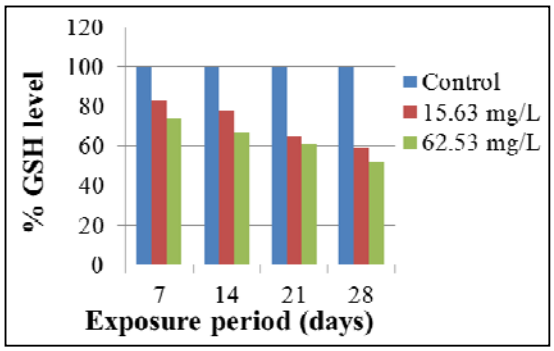

Fig 5: Effect of Copper on GSH level in the ovary of zebrafish.

Table 6: Effect of Copper on activity of LPO (µM LPO utilized/min/mg protein) in the ovary of zebrafish, *Danio rerio*.†

Concentration(mg/L)*	Treatment period (Days)			
	7	14	21	28
Control (0.00)	7.05±0.36 (100%)	7.02±0.32 (100%)	7.03±0.69 (100%)	8.03±0.49 (100%)
15.63	7.31±0.39 (103%)	7.36±0.43 (109%)	7.69±0.58 (115%)	8.44±0.42 (121%)
62.53	4.22±0.29 (109%)	5.05±0.49 (115%)	5.49±0.68 (120%)	6.75±0.56 (132%)

†Values represent mean (n=6) ± standard deviation (SD); data were significant at P< 0.05 (Two-way ANOVA). Numbers in parentheses indicate the percent activity of LPO rounded to the nearest values in comparison to control value taken as 100%.
*The exposure concentrations used were 20% (15.63 mg/L) and 80% (62.53 mg/L) of the 96-h LC_{50} values.

Summary of computation for ANOVA of the table 6.

Source of variation	Degree of Freedom	Sum of squares	Variance	F-values	P<
Variation due to operation	3	4.07847	1.35949	9.87662	0.05
Variation due to concentration	2	12.2631	6.13156	44.5455	0.05
Total interaction	6	0.82588	0.13765		
Total	11	17.1675			

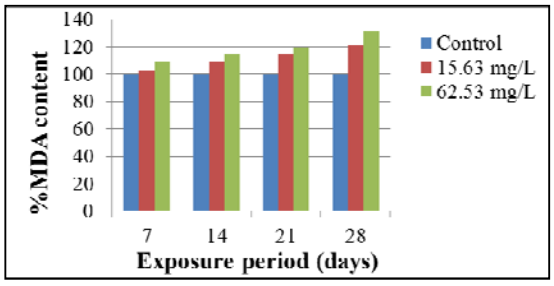

Fig 6: Effect of Copper on LPO level in the ovary of zebrafish.

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