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Acute Toxicity of Cadmium on the Antioxidant Defense Systems and Lipid Peroxidation in the Juveniles of Genetically Improved Farmed (GIFT) Tilapia *Oreochromis Niloticus*

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Abstract: Heavy metals pose a potential threat to aquatic organisms. In this study, a static-renewal acute toxicity test was conducted to investigate the effects of cadmium on the antioxidant defense systems (both enzymatic and non-enzymatic) and lipid peroxidaton in liver and gill tissues of juvenile GIFT tilapia *Oreochromis niloticus*. After 8 days of exposure to Cd (0, 0.016, 0.08, 0.4 and 2 mg/L), livers accumulated significantly more Cd than gills. Catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferase (GST) activities were stimulated only at the highest concentration tested (2 mg/L). Glutathione peroxidase (GPx) activity was stimulated in the gill while inhibited in the liver, these alternations in gill and liver showed a strong relationship with Cd levels in these tissues. This may indicate either a tissue-specific response of GPx to Cd or, most probably, a hormetic effect of Cd on GPx. Cd increased GSH levels and decreased the ratio GSSG/GSH in fish livers at 2 mg/L. Cd exposure resulted in an elevated level of MDA in the livers of fish at 2 mg/L, indicating that Cd caused lipid peroxidation. Taken together, the results demonstrated that Cd altered the enzymatic and non-enzymatic defensive systems and caused lipid peroxidation in *O. niloticus* at relatively high concentrations (compared to environmentally relevant concentrations). In addition, the results implied that *O. niloticus* could tolerate high level of Cd in sites polluted by Cd.

Key words: Cadmium, antioxidant enzymes, glutathione, lipid peroxidation, *Oreochromis niloticus.*

1. Introduction

Heavy metals, due to their persistence and potential bioaccumulation via trophic transfer, have posed a great threat to aquatic organisms inhabiting contaminated environments [1]. Cadmium is a non-essential metal (i.e., no biological function in animals) [2] that coexists with zinc in zinc ore and is released as a by-product of zinc mining and smelting. Industrially, cadmium is widely used in electroplating, galvanizing, and in batteries. Release of cadmium into aquatic environments includes natural sources (weathering of rocks) and anthropogenic sources such as industrial activities (mining and refining of ores) and agricultural activities (application of phosphate fertilizers) [3].

Reactive oxygen species (ROS), such as hydroxyl radical, hydrogen peroxide and superoxide anion, are produced during normal physiological processes in organisms [4]. Greater production of ROS than an organisms' capacity to detoxify them results in oxidative stress, which could lead to a variety of impacts including DNA damage, peroxidation of unsaturated lipids in the cell membranes, depletion of sulfhydryls, and apoptosis [3]. Recent studies have shown that heavy metals, including cadmium, are pro-oxidants and cause alterations of antioxidant defense systems [1, 3, 5, 6]. However, under normal levels of ROS the antioxidant enzyme defense systems of cells, including both

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enzymatic and non-enzymatic systems, are able to counter the damage caused by ROS by reducing ROS to non-reactive oxygen species. Components of the enzymatic defense system include catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), and glutathione reductase. Non-enzymatic antioxidant defenses are comprised of glutathione, vitamins E and C, uric acid and other small molecular antioxidants.

Previous laboratory studies on the effects of cadmium on antioxidant enzymes used either a single concentration [7, 8] or were under subchronic exposure [9]. Most studies in tilapia have focused on either the enzymatic antioxidant defense systems [7, 10] or the non-enzymatic defense systems, but not both [11]. A few studies have shown that Cd caused lipid peroxidation in fish livers exposed to Cd at mg/L concentrations [9, 11]. The aim of this study was to investigate the acute toxicity of cadmium on both the enzymatic and non-enzymatic antioxidant defense systems, as well as lipid peroxidation in juvenile GIFT tilapia (*Oreochromis niloticus*) exposed to a range of Cd concentrations.

2. Materials and Methods

All glassware and containers were acid-washed with 10% nitric acid and rinsed with deionized water prior to use. All chemicals were purchased from Sigma (Sigma China, Hangzhou, China) unless mentioned otherwise and purity > 99% (except for $CdCl_2$ which had 98% purity).

2.1 Fish and Cd Exposure

GIFT tilapia was developed by crossing eight strains of Nile tilapia (four wild African strains and four domestic Asian strains) [12]. It was first introduced to China in 1994 [13]. It is cultured in more than one hundred countries and regions and is estimated to account for approximately 70% of the total production of tilapia (approximately 1 million tons in 2006) in China [14]. Approximately 200 juvenile *O. niloticus* were obtained from Guangxi Tilapia Seed Center (location) and acclimated to well water (pH 7.71; hardness 131.4 mg/L; SO_4^{2-} 2.80 mg/L; Na^+ 0.99 mg/L; Cl^- 4.16 mg/L) for seven days prior to Cd exposure. A static-renewal system was used to conduct the exposure and all animals were of similar mass and length in all treatments (n = 15, mean weight = 18.02 ± 2.26 g, mean length = 8.06 ± 0.39 cm). Animals were exposed to Cd (in the form of $CdCl_2$) at five concentrations (0, 0.016, 0.08, 0.4 and 2 mg/L) in plastic aquaria with moderate aeration at 20 ± 2 ^oC. The two low Cd concentrations (0.016 and 0.08 mg/L) represented Cd levels in water from polluted sites (U. S. Environmental Protection Agency 2001). The two high concentrations (0.4 and 2 mg/L) were based on previous studies which showed these concentrations altered the activities of the antioxidant enzymes in *O. niloticus* [7, 9, 15]. Each treatment had three replicates with five fish per replicate. Light: dark cycle was maintained at 12 h:12 h and exposure solutions were renewed every other day by siphoning out the old solution and adding the freshly prepared solutions with the designated Cd levels. Exposures lasted for 8 days and food was withheld throughout. Cd levels in solution were monitored by taking water samples 2 hours after renewal and measured by inductively coupled plasma mass spectroscopy (ICP-MS) (X-7, Thermo Scientific,Waltham, MA, USA). Measured Cd levels were in good agreement with the nominal Cd concentrations (87 ± 5%). No mortality was observed during the exposures.

2.2 Antioxidant Enzymes, Glutathione, and Lipid Peroxidation Determination

The following procedures were performed on ice or at 4 ^oC. After 8 days of exposure all fish were rinsed well with PBS buffer to clean the external surface and blotted dry (n = 12). Fish were processed immediately to obtain the liver and gill tissues and any individuals with apparent external bruises due to collision were excluded from analysis. Half of the liver and the gill

from each fish were used for the determination of Cd in these tissues; the other half of each tissue type was used for biochemical analyses. All tissues were rinsed with ice-cold 50 mM phosphate buffer (PBS, pH 7.4, with 1mM EDTA solution), blotted dry, and weighed. The liver and gill tissues were then separately homogenized in 50 mM PBS buffer at ratio of 10: 1 (volume : weight) using a glass homogenizer. The homogenates were then centrifuged at 10,000 g for 15 min and the resulting supernatant was aliquoted and frozen at-20 ^{o}C until analysis.

All enzyme activities (CAT, SOD, GPx and GST), oxidized glutathione (GSSG), reduced glutathione and lipid peroxidation were assayed using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, P. R. China) in a 96-well plate format. These assay kits have been successfully applied in different organisms including fish [15-17]. All procedures for each assay were followed according to the manufacturer's instructions. All the biochemical endpoints were normalized to total protein, which was determined by the method of Bradford [18] using a bovine serum albumin standard. One unit (U) of SOD activity was defined as the amount of enzyme required for 1 mg proteins in 1 mL of a reaction mixture SOD inhibition rates to 50% as monitored at 550 nm. One unit of CAT activity was defined as 1 mg proteins consumed 1 μmol H_2O_2 at 405 nm for 1 min. One unit of GPx and GST activity represented a decrease in GSH concentration of 1 μmol/mg protein per minute after subtraction of non-enzymatic increase in absorbance at 37 ^{o}C. Lipid peroxidation was estimated by measuring the formation of thiobarbituric acid reactive substances (TBARS) and quantified as malondialdehyde (MDA) equivalent.

2.3 Determination of Cd Levels in Tissues

Fresh liver and gill tissues were digestion in 2 mL of 70% ultrapure nitric acid for 96 hours at 60 ^{o}C in Teflon tubes. Cd levels in the digestate were determined after proper dilutions using ICP-MS. Quality control and assurance included blanks (acid and method) and standard reference material (Pig liver from the National Research Center for Certified Reference Material, P. R. China, standard reference number GBW08551). Cd in the Certified Reference Material was recovered at 98 ± 5%.

2.4 Statistical Analysis

Data are expressed as mean ± standard error unless otherwise stated. All statistical analyses were performed using SAS software (version 8.0, SAS Institute, Cary, North Carolina, USA). All data met assumptions of normal distribution and equality of variances. Analysis of variance (ANOVA) was then used to determine the difference between the control and treatment groups. Tukey's least significant difference (LSD) was applied for multiple comparison when needed. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1 Cadmium Accumulation in the Gill and Liver Tissues

After 8 day exposures Cd levels in the gill and the liver increased with the ambient Cd exposure concentration (Table 1). At each concentration tested (including controls), the liver accumulated 1-fold to 5-fold more Cd than the gill ($P < 0.05$ for all pair-wise comparisons). At the highest tested concentration (2 mg/L), the liver had a Cd level of 25.15 ± 8.90 ug/g wet wt, which was approximately 5 times more Cd than the gill (Table 1).

3.2 Responses of Oxidized (GSSG) and Reduced Glutathione (GSH) to Cd

Liver GSSG levels were 29 ± 7% and 40 ± 16% ($P < 0.05$) higher than those in the livers of animals from 0.08 and 2 mg/L treatment groups respectively but remained relatively stable in the livers of the fish from other treatment groups (Fig. 1, Panel A). Increased GSSG levels in gills were only observed in animals from the 0.08 mg/L treatment. GSH levels in the gill

Table 1 Measured Cd in the gill and liver (ug·g^{-1} wet wt) of tilapia exposed to different concentrations of Cd for 8 days. Data are expressed as mean ± standard deviation.

Treatment	Cd in gill	Cd in liver
0 (control)	0.005 ± 0.0008 (ND)	0.057 ± 0.009
0.016 mg·L^{-1}	0.23 ± 0.09^{a}	0.57 ± 0.31^{a}
0.08 mg·L^{-1}	0.97 ± 0.46^{b}	4.24 ± 1.35^{b}
0.4 mg·L^{-1}	1.56 ± 0.52^{c}	10.57 ±4.00^{c}
2 mg·L^{-1}	3.68 ± 1.05^{d}	25.15 ± 8.90^{d}

Means with different letters indicated significant differences within the tissue. ND: near detection limit.

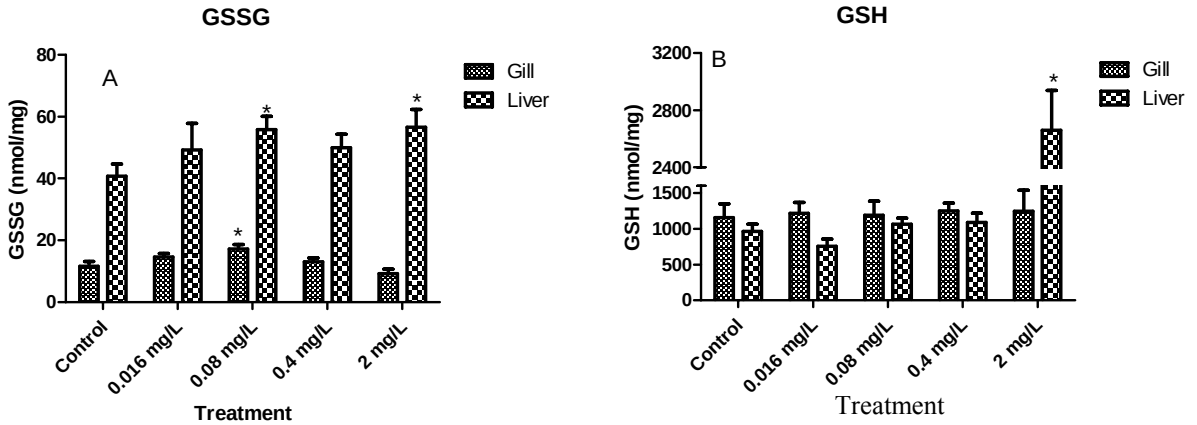

Fig. 1 Oxidized glutathione (GSSG) and reduced glutathione (GSH) in the gills and the livers of tilapia exposed to different levels of tilapia. * indicates significant difference at $P < 0.05$.

did not show significant alterations in fish from any of the treatment groups compared to those from controls. Enhanced GSH levels were only observed in the livers of fish from the 2 mg/L treatment, which had a 176% increase compared to those in the livers of control fish (Fig. 1, Panel B). The ratio of GSSG/GSH was approximately 0.04 ± 0.0011 in the control fish and remained relatively constant in fish from Cd treatment groups except 2.0 mg·L^{-1}, where the ratio dropped to 0.02 ± 0.0008 due to the marked increase in GSH levels in these fish (Fig. 1, Panel B).

3.3 Responses of Antioxidant Enzymes to Cd

All the antioxidant enzymes responded to Cd challenge but with different patterns. CAT and GST activities in liver and gill tissues of the exposed animals increased significantly at 2 mg·L^{-1} but remained relatively unchanged at the other exposure concentrations as compared to controls (Fig. 2, Panels A and D). CAT activities in the gill and liver of the 2 mg·L^{-1} treatment animals were enhanced at approximately 18 ± 4% ($P < 0.05$) and 47 ± 10% ($P < 0.05$), respectively. GST activities in the gill and liver were 27 ± 4% ($P < 0.05$) and 71 ± 18% ($P < 0.05$) higher in the 2 mg·L^{-1} treatment animals than those in controls. SOD activities were not altered in the gill, however, activity was significantly stimulated ~71 ± 14% more in the livers of the 2 mg·L^{-1} treatment animals than those in controls (Fig. 2, Panel B). Interestingly, GPx in the liver and gill responded differently to Cd exposure. The gills of animals exposed to 0.016, 0.08 and 0.4 mg/L had significantly higher GPx activities than controls but had similar GPx activities to the 2 mg/L treated animals. Conversely, the livers of animals exposed to the full range of Cd concentrations had significantly lower GPx activities than those in control animals (Fig. 2, Panel C). Furthermore, the stimulation of GPx in the gills (excluding data from the 2 mg/L treatment due to their non-significance from the control values, see Fig. 2, Pane C) and the inhibition of GPx in the livers was concentration dependent (Fig. 3).

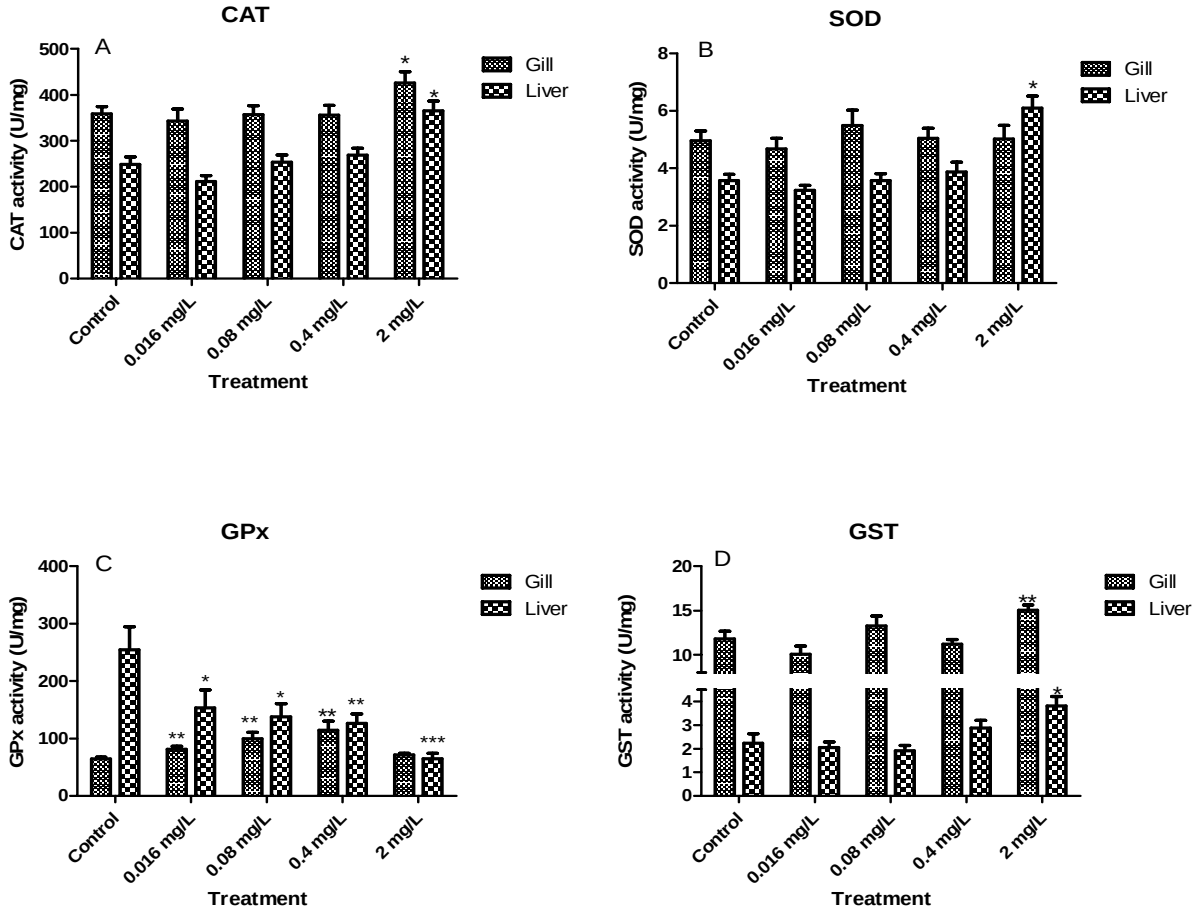

Fig. 2 Antioxidant enzyme activities in the gills and the livers of tilapia exposed to different concentrations of Cd. For all the figures, *, **, and * indicate significance at $P < 0.05$, 0.01, and 0.001 respectively from control.**

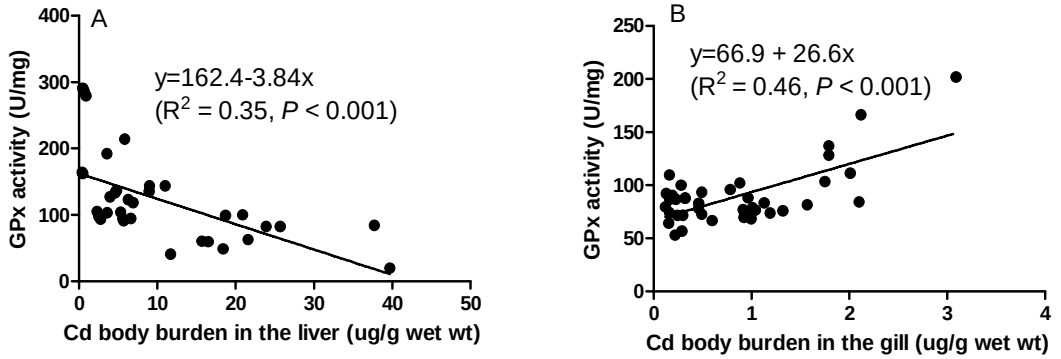

Fig. 3 The relationship between measured Cd levels in the gills or the livers and glutathione peroxidase activities. Data for GPx in the gill from treatment 2 mg/L were not included since this treatment did not increase the GPx activities significantly compared to control (see Fig. 1).

3.4 Lipid Peroxidation

There were no significant differences in MDA levels in the gills of fish from any treatment groups (Fig. 4). MDA levels were augmented 34 ± 8% ($P < 0.05$) higher in the livers of fish from 2 mg·L^{-1} treatment group compared to MDA in the livers of control fish, but were non-significant the other treatment groups.

4. Discussion

A gross visualization of the data may suggest that the

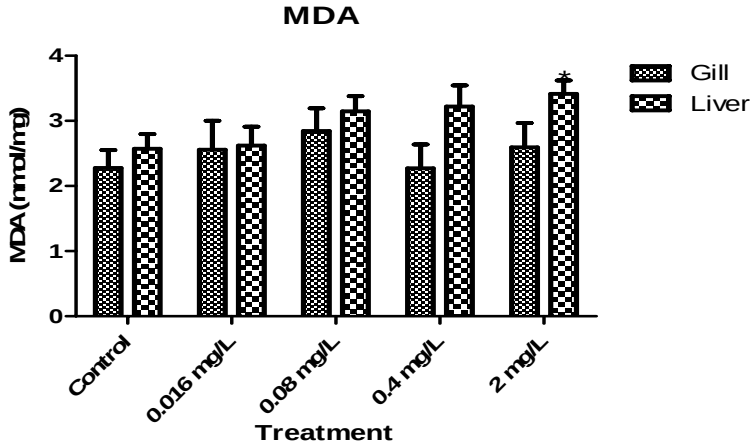

Fig. 4 Malondialdehyde (MDA) levels in the gills and livers of tilapia exposed to different concentrations of Cd. * indicates significant difference at $P < 0.05$.

gills had higher CAT, SOD and GST activities than the livers. This was mainly due to the lower total protein levels in the gill homogenate (with similar mass to liver tissues) compared to the liver tissues. However, increased or similar CAT and SOD activities in fish gills compared to those of the liver have been reported [19].

In this study, both the livers and gills accumulated Cd as a function of increasing exposure concentrations. The livers of juvenile GIFT *O. niloticus* accumulated more Cd than the gills at all test concentrations. This is consistent with a recent study showing that accumulation of Cd in the liver and the gill of tilapia depends on the ambient Cd concentrations [11]. The results indicate that liver could accumulate Cd from the dissolved route, which has been shown in other laboratory and field studies [11, 19].

Glutathione is one of the small thiols which play a role in ameliorating metal toxicity by chelating metals with the –SH group and participating in cellular redox reactions [6, 20]. GSH functions as an antioxidant by scavenging free radicals, resulting in the oxidation of GSH to GSSG. GSH is also a substrate of two enzymes, GPx and GST, and essential to decomposing hydroperoxides of the two enzymes. GSH has been considered as the first line of defense of excessive ROS, along with some other non-enzymatic, small molecular weight antoxidants, such as vitamins C and E, uric acid, and carotenoids [21]. No obvious changes in GSH evels in the gill occurred at any concentration tested or in the livers from animals exposed to concentrations below 0.4 mg/L. This implies that the constitutive GSH levels were sufficient for chelating the accumulated Cd in the tissues. The increased levels of GSSG in the livers of fish from the 0.08 and 2 mg/L suggested that some reduced glutathione was oxidized to become GSSG. The ratio of GSSG/GSH was 0.04 in the control fish, which was close to value of 0.05 in another study using the same species [15]. The ratio of GSSG/GSH remained relatively constant in fish exposed to concentrations other than 2 mg/L. This was opposite to the increase in this ratio with exposure duration in tilapia exposed to 3 mg/L (but the increased initiated after 20 days of exposure) as seen in other study [15]. In fish from the 2 mg/L treatment, the ratio decreased due to the increase of GSH levels in those fish. In accordance with the present study, an increase of GSH by Cd stress was documented previously in tilapia [11], rainbow trout [22], and mice [23]. In addition, the elevated GSH in tilapia may be related to the tolerance of tilapia to Cd stress. Studies have shown that tilapia can tolerate high levels of waterborne Cd [24, 25]. The high tolerance to Cd may require high basal and induced GSH to ameliorate Cd toxicity, since elevated levels of GSH correlate with environmental stress tolerance [26].

Some heavy metals including Cd have been shown

to act as prooxidants by different mechanisms [3, 27]. Various endogenous antioxidants protect against the propagation of oxygen radical reactions in organisms exposed to heavy metals. CAT and SOD universally occur and co-exist in most invertebrates and vertebrates. These two enzymes work collaboratively on the detoxification of superoxide anion [1]. SOD catalyzes the destruction of the superoxide anion by dismutation and H_2O_2 formation. CAT and GPx are responsible for eliminating the intracellular H_2O_2 formed from the previous step [28]. Our results showed that juvenile tilapia exhibited elevated CAT and SOD activities upon exposure to 2 mg·L^{-1} for 8 days. This increase was in agreement with findings from other studies using tilapia [7, 8] and using rainbow trout [22]. A more than 140% increase in CAT activity was observed in the liver of juvenile *O. niloticus* exposed to 1.5 mg/L of Cd for 96 hrs [7]. A 62% increase in CAT activity and 60% increase in SOD activity were reported in the liver of similar-sized juvenile tilapia *O. mossambica* exposed to 5 mg/L [8]. The increase in CAT and SOD activities in the fish livers may be associated with increased oxidative stress by Cd [29], as evidenced by the significant increase in lipid peroxidation in fish from this treatment (Fig. 4). However, in contrast to other studies [7, 9], which reported increased CAT activity in juvenile *O. mossambica* exposed to 0.1, 0.5 and 1 mg/L of Cd for 96 hrs and an increase in SOD activity in the liver of *O. niloticus* exposed to 0.35, 0.75 and 1.5 mg/L for 60 days, CAT and SOD activities in our study remained relatively unaltered by exposure to concentrations lower than 2 mg/L. These discrepancies may be accounted for by the differences in species used, strain of fish, concentrations used, size of the fish and exposure duration [15]. For example, an increase in CAT activity was observed following 0.5, 1 and 3 days of exposure to Cd in a crab (*Charybdis japonica*), but CAT levels stabilized after 6 days of exposure and remained relatively constant with longer exposure duration to the same concentrations [30]. It was found that catfish exposed to Cd concentrations lower than 0.4 mg L^{-1} did not show alternations in CAT and SOD activities for 7 days but had a significant increase in SOD activity but not CAT after 21 days of exposure [31].

GST is a group of multifunctional proteins, which play a critical role in detoxification of electrophilic chemicals and the hepatic removal of potentially harmful hydrophobic compounds [32]. GST activity was stimulated in both the liver and the gill tissues in fish from 2 mg/L but remained unchanged in fish from other treatment groups. In another study, it was found that GST activity was increased in the livers of *O. niloticus* exposed to 3 mg/L of Cd for 5, 10, and 20 days but leveled off after 30 days [15].

GPx detoxifies ROS by reducing lipid hydroperoxides to stable alcohols and by removing hydrogen peroxide formed in the cytosol [33]. Unlike previous studies [5, 7, 9, 15], this study highlighted the different responses of GPx to Cd in the gill and liver tissues. GPx activity was stimulated in the gill of fish exposed to 0.016, 0.08 and 0.4 mg/L of Cd but not in fish exposed to 2 mg/L Cd. On the other hand, Cd significantly inhibited GPx activities in the fish livers of all the Cd treatment groups. Either stimulation or inhibition of GPx activities by Cd have been measured in fish [15, 34]. These apparently different responses to Cd in the gills and in the livers in our study suggested GPx was likely to show a tissue-specific response to Cd exposure. Tissue specific alternations of CAT and SOD have been documented in *O. niloticus* exposed to diazinon [35]. However, after scrutinization of Cd burdens in these two tissues, it seemed more likely that Cd caused a hormetic response of GPx in *O. niloticus* (characterized by a low dose of stimulation and a high dose of inhibition). The highest Cd tissue burden to stimulate GPx activity in the gill was 1.57 ug/g wet wt, which was slightly higher than the lowest Cd body burden level of 0.57 ug/g wet wt to inhibit the GPx activity in the liver. Since fish gill has direct contact with waterborne Cd, it is possible that part of the Cd in the gill from the acute test might be adsorbed to the cell

surface or might be associated with mucus. Therefore the real amount of Cd acting on the target sites inside the cells might less than the measured value. On the other hand, Cd accumulated in the liver was probably inside the cells. In addition, the subcellular fractionation of Cd which affects the bioavailable Cd may not be the same in the gill as in the liver, as demonstrated in rainbow trout [36]. More research is needed to test these hypotheses using more sensitive methods such as ^{109}Cd. The inhibition of GPx was probably the direct toxic effect of Cd on GPx. Cd has high affinity to bind –SH groups and therefore deactivates the enzyme activity, as reported in other studies [23, 29]. Nonetheless, a similar hormetic effect of Cd on GPx was observed in a marine fish (*Salaria basilisca*) which had an increased activity of GPx after 14 days exposure but a decreased activity after 28 days of exposure to Cd [37]. In addition, a hormetic effect by Cd on CAT and SOD has also been demonstrated in the earthworm *Eisenia fetida* [38]. Furthermore, inhibited GSH-GPx activity may give rise to increased formation of free radicals, which can cause subsequent oxidation of fatty acids [39]. Indeed, increased lipid peroxidation was observed in the livers of 2 mg/L Cd treated fish. The results support the claim that Cd induces ROS production by indirect mechanisms (i.e., inhibiting the activities of antioxidant enzymes) [3].

Lipid peroxidation has been considered as the first step of cellular membrane damage by heavy metals [1, 32, 40]. After 8 days of exposure, only livers from fish exposed to 2 mg/L Cd exhibited elevated MDA levels. Cd did not increase lipid peroxidation in the livers of fish from other treatments and in the gills of any Cd treated fish. The increase in lipid peroxidation was also observed in *O. niloticus* exposed to 3 mg/L [5] and other fish species challenged with Cd [31, 39]. The increase in lipid peroxidation implied that excess ROS had been produced in the liver upon exposure to Cd. The lack of change in the MDA levels in the gills and livers of fish exposed to other treatment groups indicated that the antioxidant defense systems including the enzymatic and non-enzymatic systems were capable of detoxifying the ROS generated from Cd exposure. The increase in lipid peroxidation in the livers of fish exposed to 2 mg/L was accompanied by increased expression of antioxidant enzymes and GSH. Increased lipid peroxidation with increased antioxidant enzyme activities was reported in a copepod *Tigriopus japonicus* exposed to 100 μg/L of Cd [41] and *Daphnia magna* exposed to endosulfan [42].

5. Conclusions

Taken together, antioxidant enzymes CAT, SOD and GST activities increased in the livers of fish exposed to 2 mg/L of Cd, while gills of fish exposed to this concentration had elevated CAT and GST activities. Cd probably exhibited a hormetic effect on GPx activity in *O. niloticus*. The livers from the 2 mg/L treatment group also had a marked increase in GSH level. The relative unresponsiveness of the antioxidant defense systems in *O. niloticus* to concentrations lower than 2 mg/L implied that antioxidant enzymes CAT and SOD, together with GSH, might be sufficient to protect the fish from Cd generated ROS toxicity. However, we could not rule out the roles of metallothioneins (MTs) in protecting the fish from the Cd-exerted toxicities. MTs are a family of small molecular weight, cysteine-rich, metal binding proteins which play important roles in binding and regulating heavy metals and as scavengers for free radicals, and are inducible in response to heavy metal exposure and oxidative stress [43]. In addition, The findings imply that *O. niloticus* could tolerate Cd concentrations above 1 or 2 magnitudes higher than those found in polluted sites. The stimulation of GSH and antioxidant enzymes may suggest that this species could survive in heavy metal polluted sites in the natural aquatic environment, which was shown from field work [44]. The findings suggested that *O. niloticus* may not be a sensitive species for biomonitoring Cd pollution in natural aquatic systems.

Acknowledgments

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