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Effects of Waterborne Cadmium Exposure on Its Internal Distribution in *Meretrix meretrix* and Detoxification by Metallothionein and Antioxidant Enzymes

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Cadmium (Cd), one of the most toxic metals found in inshore sediments of China, is a persistent environmental contaminant capable of exerting irreversible toxic effects on aquatic organisms and their associated ecosystems. Although Cd is known to be toxic to marine animals, the underlying mechanism of this toxicity is not clear. In this study, *Meretrix meretrix*, a commercially and ecologically important species of clam, was exposed to different concentrations of cadmium chloride (0, 1.5, 3, 6, and 12 mg L^{-1}) for 5 days, and the levels of Cd accumulation, antioxidant enzyme activity, and expression of metallothionein (MT) in the hepatopancreas, gill, foot, and mantle were evaluated. The results revealed a sharp increase in Cd accumulation in the tissues in response to increased Cd^{2+} concentrations in the water, and significant differences in Cd accumulation were observed among the different tissues. Increased Cd^{2+} level in the tissues also led to a significant increase in malondialdehyde content, caused by increased lipid peroxidation. The activities of superoxide dismutase and catalase also increased, peaking at different Cd^{2+} concentrations, depending on the tissue. Glutathione peroxidase (GPx) activity in the gill and mantle initially increased but then decreased with increasing external Cd^{2+} concentration. In the hepatopancreas and foot, GPx activity was inhibited by Cd^{2+}, even at low concentrations. Furthermore, Cd^{2+} also stimulated the expression of MT in all four tissues. However, the levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) in the gill and mantle, as well as the GSH/GSSG ratios in all four tissues, decreased with increasing external Cd^{2+} concentrations. Taken together, the results suggested that in *M. meretrix*, response to the toxic effect of Cd^{2+} might occur through a combination of mechanisms, which involve both enhanced antioxidant enzyme activities and the ability to bind and sequester Cd^{2+} via cysteine-rich molecules such as GSH and MT, both of which would eventually lead to the reduction of heavy metal-induced oxidative stress.

Keywords: cadmium, *Meretrix meretrix*, oxidative damage, metallothionein, glutathione, antioxidant enzyme

HIGHLIGHTS

- Cd^{2+} accumulation in different tissue of *M. meretrix* is concentration dependent.
- Cd^{2+} induces a significant increase in MDA level in different *M. meretrix* tissues.
- MT response significantly correlates with Cd^{2+} bioaccumulation.
- CAT and SOD activities vary according to external Cd^{2+} concentrations.
- Antioxidant enzymes, GSH and MT, might act cooperatively against oxidative stress.

INTRODUCTION

Toxic metal pollution, which can seriously threaten the biological sustainability of coastal ecosystems, has now become a major problem for the aquatic environment (Liu et al., 2018). Among the toxic metals, cadmium (Cd) is considered to be one of the most dangerous, and it is usually found in marine environments (Liu et al., 2015; Gu et al., 2019). In normal water, the level of dissolved Cd generally ranges from 10 to 500 ng L^{-1}, but levels that exceed 10 μg L^{-1} have also been recorded in some industrialized areas in China (Hu et al., 2011; Meng et al., 2013; Chan and Wang, 2018). Cadmium has a long half-life and can be bioaccumulated in organisms through the food chain. The accumulation of Cd within living aquatic animals would exert a wide range of toxicological effects on the various tissues of the animals and their human consumers (Liu et al., 2015; García-Navarro et al., 2017). Cadmium can induce the body to produce a large number of reactive oxygen species (ROS) (Sandbichler and Höckner, 2016; Jing et al., 2019). The ROS in turn can cause oxidative damage, which is often linked to lipid peroxidation, protein denaturation, and inactivation of enzymes as well as DNA replication errors (Chandurvelan et al., 2015; Xia et al., 2016; Lin et al., 2017). Malondialdehyde (MDA), a final product of the decomposition of lipid peroxidation, has recently been considered as a sensitive biomarker of oxidative damage. It can be used to determine the extent of lipid peroxidation and the accumulation of ROS in an organism (Liu and Wang, 2016; Lin et al., 2017; Haque et al., 2018).

In general, aquatic organisms have independently evolved an antioxidant defense mechanism to protect biomolecules from oxidative stress caused by ROS, in which both enzymatic antioxidants and non-enzymatic antioxidants are functionally involved (Lavradas et al., 2014; Xia et al., 2016; Cui et al., 2020). A clear understanding of the link between antioxidants' defense systems and metal bioconcentrations may help to identify markers that can reflect the status of aquatic ecosystems (Haque et al., 2018). Superoxide dismutase (SOD) is considered to be the first line of defense against oxidative stress, since $O_2^{-\cdot}$ is converted to O_2 and H_2O_2, and H_2O_2 is subsequently converted to O_2 and H_2O by catalase (CAT) present in the peroxisomes or by glutathione peroxidase (GPx) present in the mitochondria and cytosol (Chen et al., 2019; Gu et al., 2019). Glutathione is an important sulfhydryl non-protein compound found in the tissue, where it is involved in the protection of cell membrane from lipid peroxidation by scavenging oxygen radicals ($O_2^{-\cdot}$, H_2O_2, and OH). This function plays a key role in the complexation and detoxification of heavy metal ions (Lavradas et al., 2014; Bouzahouane et al., 2018; Chan and Wang, 2018). Glutathione can exist in a thiol-reduced state (GSH) or in an oxidized state (GSSG), which consists of two GSH molecules that are linked together by a disulfide bond (Fraternale et al., 2017). The content of GSH and GSH/GSSG ratio can act as an important indicator of the body's antioxidant capacity (Nair et al., 2015). In the cytosol, hydroxyl (·OH) and superoxide ($O_2^{-\cdot}$) radicals are preferably scavenged by metallothioneins (MTs), which are low-molecular-weight (6–7 kDa) cysteine-rich metal-binding proteins involved in multiple physiological activities, such as metal homeostasis and detoxification (Amiard et al., 2006; Min et al., 2016). Moreover, MT can protect cells from metal toxicity not only by acting as an oxyradical scavenger but also through metal-binding/release dynamics (Gu et al., 2019; Ohta et al., 2019). Metallothioneins can participate directly in antioxidative defenses, and the synthesis of *MT* mRNA is correlated with the extent of metal stress (Zhu et al., 2018; Chen et al., 2019; Gu et al., 2019). In addition, MT can be excreted from the cells and absorbed by the cells via a receptor-mediated mechanism, in which MT remains in an endocytotic compartment while the metal is transported to the cytosol (Amiard et al., 2006; Ohta et al., 2019).

Meretrix meretrix is a clam that is widely found along the coastal areas of China, and it is an economically and ecologically important bivalve mollusk. It has low-metabolism and high filter-feeding activity, and it lives mainly in sandy or muddy substrates in estuarine lower intertidal and shallow subtidal areas, and therefore it is vulnerable to environmental pollutants (Zhang et al., 2011; Xia et al., 2016). *M. meretrix* is known to have a strong capacity to accumulate metals (Zhang et al., 2011; Wang et al., 2013) and is therefore widely used as a biological tool for monitoring toxic metal pollution in the marine environment (Hamad et al., 2011; Meng et al., 2013; Liu et al., 2018). While research has focused on the effect of Cd^{2+} toxicity on mollusks, there is a lack of data with regard to the effect of Cd^{2+} exposure on MT and other antioxidants in the different tissues of the clam. Our objectives were to determine the toxicological and biochemical responses of *M. meretrix* to acute Cd exposure and to investigate the bioaccumulation of Cd in different tissues as a function of exposure concentration. This could allow us to better understand how the antioxidant defense system of these bivalves might respond to Cd^{2+} stress. Furthermore, the relationships between Cd accumulation in the tissues and MT as well as the antioxidants examined were also investigated. Our findings highlight the potential application of Cd toxicity-associated biomarkers in clam in the future selection of biomarkers and bioindicator species for aquatic environmental monitoring.

MATERIALS AND METHODS

Animals and Treatments

Meretrix meretrix clams (shell length, 64.36 ± 2.85 mm; shell width, 31.69 ± 1.42 mm; shell height, 53.57 ± 2.45 mm;

weight, 71.30 ± 8.28 g) were purchased from Linkun aquafarm, Wenzhou, Zhejiang, China. The clams were acclimated in 15‰ artificial seawater without food for 2 days in an auto-temperature-controlled aquarium at 22 ± 1°C and pH 8.0. After acclimation, the clams were randomly assigned to five groups: a control group and four $CdCl_2$-treated groups. Each group consisted of 120 clams, and these were kept in 3 separate aquariums, with 40 clams per aquarium. For Cd^{2+} treatment, the clams were placed in an aquarium containing a different Cd^{2+} concentration (1.5, 3, 6, or 12 mg L^{-1}) for 5 days. The Cd^{2+} solutions were prepared in 15‰ artificial before being added to the aquariums. These concentrations of Cd^{2+} corresponded to 1/10, 1/5, 1/2.5, and 1/1.25 of the previously determined LC_{50} (15.01 mg L^{-1}) (Xia et al., 2016). To maintain the quality of the water and the concentration of Cd^{2+} in the water, the water in the tank was replaced daily with 15‰ fresh artificial seawater containing the same Cd^{2+} concentration and pH as the original water. All other conditions were kept the same as during acclimation. After 5 days of exposure, all clams were collected and the mantle, gill, foot, and hepatopancreas of each animal were quickly removed and immediately frozen in liquid nitrogen before being stored at a −80°C freezer for further analysis.

Determination of Cadmium Content in Different Tissues of the Clam

Cadmium concentrations in the various tissues (mantle, gill, foot, and hepatopancreas) of the clams were measured according to Lin et al. (2017) and expressed as mg kg^{-1} wet weight tissue.

MT mRNA Expression Analysis

Total RNA was extracted from 20–40 mg of gill, mantle, foot, or hepatopancreas using a UNIQ-10 column TRIzol Total RNA Extraction Kit (Shanghai Shenggong, China) according to the manufacturer's protocol. Briefly, the tissue sample was ground in TRIzol Reagent and then extracted with chloroform followed by centrifugation. After centrifugation, the upper aqueous layer was withdrawn and the RNA in it was precipitated with ethanol. The precipitated RNA was washed with PRE solution to remove the impurities and then resuspended in DPEC-distilled water and stored at −80°C for subsequent experiments. The RNA quality was evaluated by electrophoresis in 1.5% agarose gel (28S/18S RNA), and RNA concentration, as well as purity, was determined by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, United States). cDNA was synthesized using a commercial cDNA synthesis kit (PrimeScript™ RT Reagent, TaKaRa Bio. Inc., Dalian) according to the manufacturer's instructions. Briefly, for reverse transcription, the reaction mixture contained 1 μg total RNA, 1 μL oligo dT Primer, 4 μL of 5 × PrimeScript Buffer (for Real Time), 1 μL PrimeScript RT enzyme mix, and RNase-free water in a final volume of 20 μL. The mixture was incubated at 37°C for 15 min and then heated at 85°C for 5 s to inactivate the reverse transcriptase. The cDNA product was stored at −80°C until use. The following gene-specific primers were used in the amplification: MT RT1f (forward) CGAGGACTGTTCATCAACCACTG, MT RT1r (reverse) GCAAACAACTTTACACCCTGGAC, β-actin1f (forward) TTGTCTGGTGGTTCAACTATG, and β-actin1r (reverse) TCCACATCTGCTGGAAGGTG (Wang et al., 2010). The expression of *MT* mRNA in different tissues was determined by quantitative real-time PCR (qRT-PCR) using a Roche LC480 fluorescent quantitative PCR (Switzerland) and AccuPower 2 × GreenStar™ qPCR Master Mix Kit (Bioneer Co., Shanghai, China) according to the manufacturer's instructions. The qRT-PCR reaction mixture consisted of 2 μL of cDNA template, 10 μL of 2 × Green Master Mix, 0.4 μM of each forward and reverse primers, and 0.4 μL of 50 × ROX dye and DEPC-distilled water in a final volume of 20 μL. The PCR conditions were as follows: 5 min at 95°C, and 40 cycles of 15 s at 95°C, 15 s at 58°C, and 20 s at 72°C. The relative expression level of *MT* mRNA was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Assays of SOD, CAT, and GPx Activities and MDA, GSH, and GSSG Contents

Frozen samples of different tissues were macerated on ice in 9 vols of 0.9% physiological saline using a SCIENTZ DY89-II type motor-driven homogenizer and then centrifuged at 1200 × *g* and 4°C for 15 min. Each supernatant was collected, and the activities of SOD, GPx, and CAT and the levels of MDA, GSH, and GSSG were then determined using a commercial kit according to the manufacturer's instructions (Nanjing Jiancheng Biological Engineering Institute). Catalase activity was assayed spectrophotometrically by monitoring the decrease in absorbance at 240 nm due to H_2O_2 consumption (Saint-Denis et al., 1998). Superoxide dismutase activity was determined from the extent of inhibition of cytochrome C reduction at 550 nm (Beauchamp and Fridovich, 1971). Glutathione peroxidase activity was determined by detecting the rate of NADPH oxidation at 412 nm (Rotruck et al., 1973). All enzyme activities were defined as units of activity per mg of protein (U mg^{-1} protein). Thiobarbituric acid-reactive substance (TBARS) was expressed as MDA equivalents, and the absorbance of samples was read at 532 nm. Malondialdehyde level was expressed in nmol mg^{-1} protein (Ohkawa et al., 1979). Total glutathione (GSH plus GSSG) content was detected by a colorimetric method (5,5'-dithio-bis-2-nitrobenzoic acid, DTNB). The absorbance of the sample was measured at 405 nm using a microplate reader (Epoch, BioTek Co., United States). Oxidized glutathione was determined by the same method in the presence of 2-vinylpyridine, and GSH content was calculated from the difference between total glutathione and GSSG (Baker et al., 1990). Reduced glutathione and GSSG levels were expressed in mg g^{-1} protein. Soluble protein concentration was assayed by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Statistical Analysis

Statistical analysis of the data was performed with the SPSS Statistical Package (Version 16.0, Chicago, IL, United States),

TABLE 1 | Accumulation of Cd^{2+} in different tissues of *M. meretrix* exposed to various Cd^{2+} concentrations in water.

Cd^{2+} concentration (mg L^{-1})	Hepatopancreas (mg kg^{-1})	Foot (mg kg^{-1})	Mantle (mg kg^{-1})	Gill (mg kg^{-1})
0	0.36 ± 0.02^{Ce}	0.52 ± 0.01^{Be}	0.39 ± 0.01^{Cd}	0.83 ± 0.01^{Ae}
1.5	4.24 ± 0.11^{Dd}	6.38 ± 0.07^{Cd}	10.87 ± 0.13^{Bc}	23.35 ± 0.29^{Ad}
3	7.45 ± 0.14^{Dc}	10.62 ± 0.13^{Cc}	12.65 ± 0.17^{Bc}	40.24 ± 1.21^{Ac}
6	19.26 ± 0.17^{Db}	22.11 ± 0.55^{Cb}	44.89 ± 0.66^{Bb}	73.58 ± 1.25^{Ab}
12	38.63 ± 0.54^{Ca}	37.65 ± 0.78^{Ca}	72.19 ± 1.45^{Ba}	133.17 ± 2.88^{Aa}

Different uppercase letters indicate significant differences among different tissues under the same concentration, whereas different lowercase letters indicate significant differences among different Cd^{2+} concentrations for the same tissue at the $P < 0.05$ level. Data are the means ± standard errors (SEs) ($n = 6$).

and the regression analysis between GSH and MDA or GPx was performed using Statistica 6.0. Tukey's multiple comparison test with one-way analysis of variance (ANOVA) was used to evaluate the differences among the treatment and control groups, and differences were considered statistically significant at the $P < 0.05$ level. The least significant difference test (LSD) was used to perform multiple comparisons among different Cd-treated groups.

RESULTS

Cd^{2+} Accumulation in Different Tissues of *Meretrix meretrix*

Meretrix meretrix individuals that were exposed to Cd^{2+} all had significantly ($P < 0.05$) higher Cd^{2+} levels in their tissues than their non-exposed counterparts (controls) (**Table 1**). In the control group, there were significant differences among the different tissues except for the mantle and hepatopancreas. The gill was found to contain the highest Cd^{2+} concentration, followed by the foot, mantle, and hepatopancreas. As for all Cd^{2+}-treated groups, the order was gill > mantle > foot > hepatopancreas, with significant differences among the different tissues from the same group, except for the hepatopancreas and foot in the group exposed to 12 mg L^{-1} Cd^{2+}. The highest increase in tissue Cd^{2+} concentration between the control and Cd^{2+}-exposed groups was found in the mantle (18,510%) and the lowest increase in the foot (7240%). Thus, the accumulation of Cd^{2+} in *M. meretrix* appeared to be tissue-specific.

Effects of Cd^{2+} on MDA Content

Meretrix meretrix individuals exposed to Cd^{2+} exhibited significant ($P < 0.05$) increase in MDA concentration compared with the non-exposed individuals, with the maximum concentration found in the group exposed to 12 mg L^{-1} Cd^{2+} (**Figure 1**). For the gill and mantle, significant differences were observed among groups exposed to different Cd^{2+} concentrations. The order of MDA content was gill > foot > mantle > hepatopancreas for the control group and gill > mantle > foot > hepatopancreas for the Cd^{2+}-exposed groups. Thus, the levels of MDA in the various tissues of *M. meretrix* were consistent with the levels of Cd^{2+} in these tissues.

Effect of Cd^{2+} on *MT* mRNA Expression Level

In general, the expression of *MT* mRNA in the various tissues of Cd^{2+}-treated *M. meretrix* increased significantly compared with the control ($P < 0.05$) (**Figure 2**), but variations in the levels of increase among the tissues were obvious for the different groups. For example, at the lowest Cd^{2+} concentration, maximal *MT* mRNA level occurred in the gill and declined to the level of the control group at the highest Cd^{2+} concentration. However, at the two intermediate concentrations, the mantle exhibited the highest level of *MT* mRNA, whereas, at the highest Cd^{2+} concentration, the hepatopancreas exhibited the highest level of *MT* mRNA. The result appeared to suggest that lower concentrations of Cd^{2+} in these tissues could stimulate the expression of MT to enhance the binding of Cd^{2+} in the cell, but the stimulation seemed to be less profound at higher concentrations of Cd^{2+}.

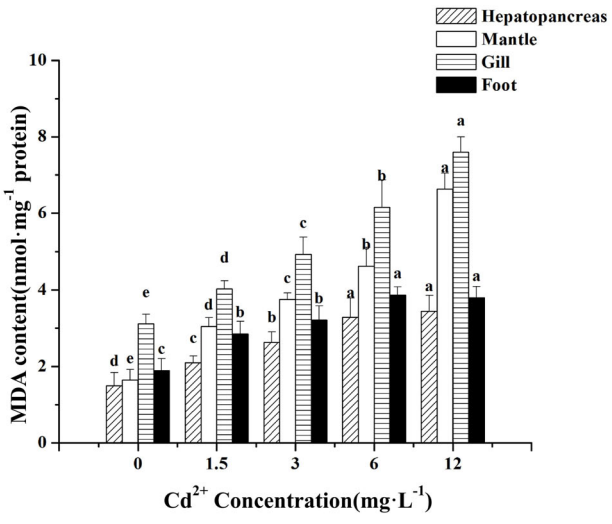

FIGURE 1 | Effect of Cd^{2+} on the levels of MDA in different tissues of *M. meretrix* following Cd^{2+} exposure. Hepatopancreas (twill pillars), mantle (white pillars), gill (horizontal stripes pillars), and foot (black pillars). Data are the means ± standard errors (SEs) ($n = 6$). Different letters above the pillars indicate significant ($P < 0.05$) differences among the different Cd^{2+}-treated groups (0, 1.5, 3, 6, or 12 mg L^{-1}) for the same tissue as revealed by Tukey's *post hoc* multiple comparison tests.

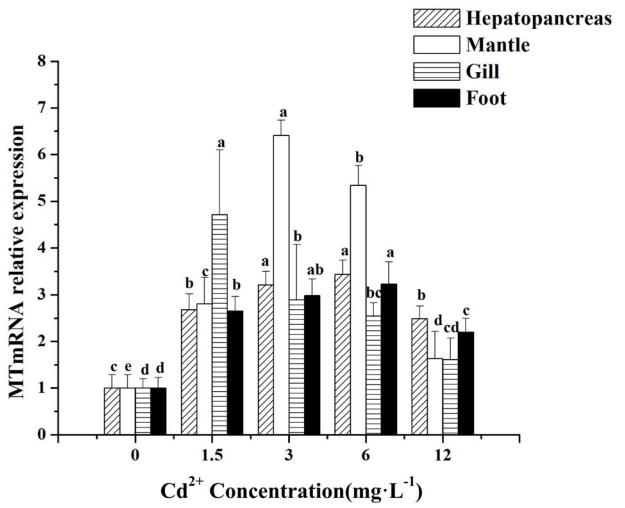

FIGURE 2 | Effect of Cd^{2+} on *MT* expression in different tissues of *M. meretrix* following Cd^{2+} exposure. Hepatopancreas (twill pillars), mantle (white pillars), gill (horizontal stripes pillars), and foot (black pillars). Data are the means ± standard errors (SEs) (n = 6). Different letters above the pillars indicate significant ($P < 0.05$) differences among the different Cd^{2+}-treated groups (0, 1.5, 3, 6, or 12 mg L^{-1}) for the same tissue as revealed by Tukey's *post hoc* multiple comparison tests.

Effects of Cd^{2+} on CAT, SOD, and GPx Activities

Among the four tissues tested, the hepatopancreas was found to have the highest level of CAT activity, followed by the gill, mantle, and foot, both for the control and Cd^{2+}-exposed groups (**Figure 3A**). Significant increases in CAT activity were observed in the hepatopancreas and foot of the groups exposed to 1.5 and 3 mg L^{-1} Cd^{2+} compared with the control group ($P < 0.05$), but at higher Cd^{2+} concentrations (6 and 12 mg L^{-1}), no significant increase in CAT activity was observed. No significant difference in CAT activity was detected in the mantle between the control group and any of the four Cd^{2+}-exposed groups ($P > 0.05$) (**Figure 3A**). CAT activity in the gills of the group exposed to 1.5 mg L^{-1} Cd^{2+} increased significantly compared with the control group, whereas at higher Cd^{2+} concentration, no significant increase in CAT activity relative to the control group was observed.

Increases in SOD activity over the control group were significant for all four tissues in the groups exposed to 1.5 and 3 mg L^{-1} Cd^{2+}, with the gill displaying the highest increase (**Figure 3B**). Lower SOD activity was found in all the four tissues of the group exposed to 12 mg L^{-1} Cd^{2+} compared with all the other Cd^{2+} groups. Furthermore, no significant difference between the SOD activity of this group and that of the control group was observed except for the activity in the foot (**Figure 3B**).

As for GPx activity, Cd^{2+} had both enhancing and inhibitory effects, depending on the tissue. In the case of the hepatopancreas and foot, Cd^{2+} treatment led to a drop in GPx activity compared with the control, whereas in the gill and mantle, GPx activity

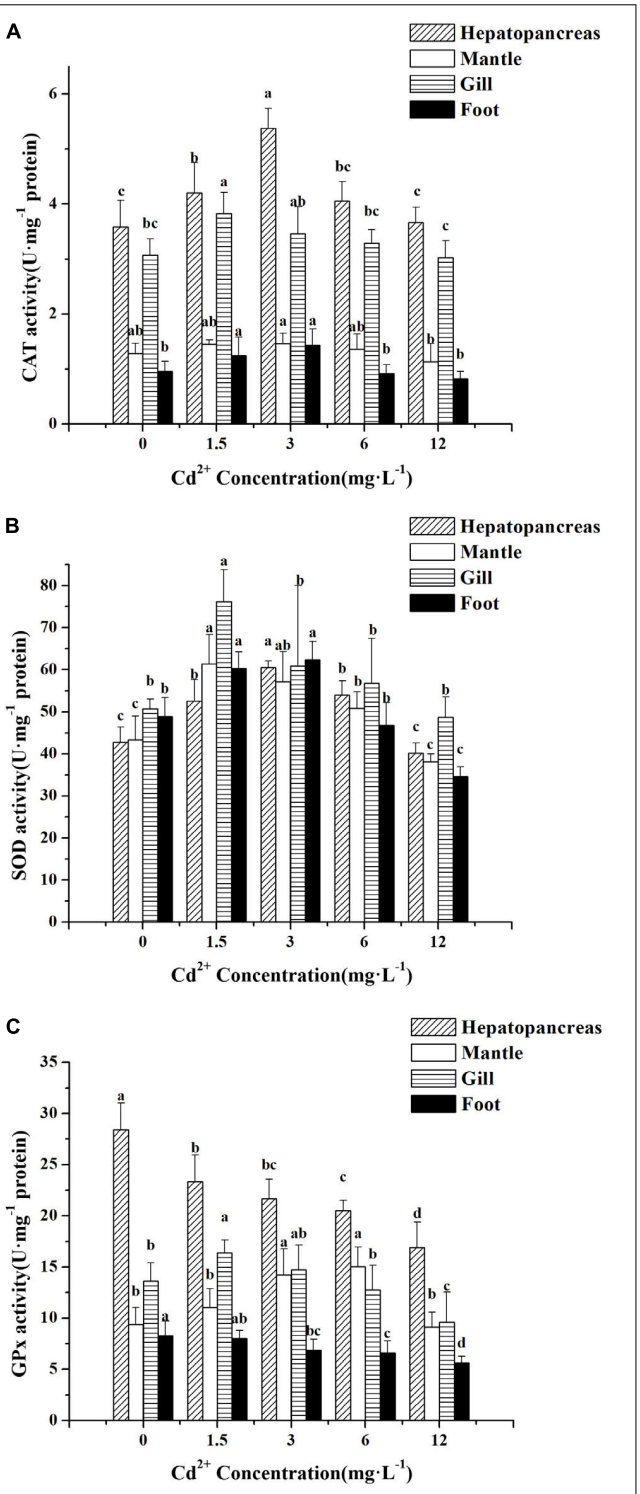

FIGURE 3 | Effect of Cd^{2+} on the activities of antioxidant enzymes in different tissues of *M. meretrix* following Cd^{2+} exposure. Hepatopancreas (twill pillars), mantle (white pillars), gill (horizontal stripes pillars), and foot (black pillars). **(A)** CAT, **(B)** SOD, and **(C)** GPx. Data are the means ± standard errors (SEs) (n = 6). Different letters above the pillars indicate significant ($P < 0.05$) differences among the different Cd^{2+}-treated groups (0, 1.5, 3, 6, or 12 mg L^{-1}) for the same tissue as revealed by Tukey's *post hoc* multiple comparison tests.

tended to increase at lower Cd^{2+} concentrations, which then decreased at higher Cd^{2+} concentrations (**Figure 3C**). In the hepatopancreas, significant decreases in GPx activity relative to the control were also observed for all the Cd^{2+}-exposed groups. As for the other three tissues, not all the differences in GPx activity between the control and Cd^{2+}-exposed groups were significant.

Taken together, the results suggested that exposure of *M. meretrix* to Cd^{2+} could significantly alter the levels of antioxidant activity, but the effect on individual tissues might vary depending on the exact tissue.

Effects of Cd^{2+} on GSH and GSSG Content

The levels of GSH in the hepatopancreas and foot were increased at lower Cd^{2+} concentrations but decreased at higher Cd^{2+} concentrations compared with the control group. A maximum level of GSH was found in the group exposed to 1.5 mg L^{-1} Cd^{2+}, which was significantly different ($P < 0.05$) from the control group and other Cd^{2+}-exposed groups (**Figure 4A**). As for the mantle and gill, the GSH level decreased significantly with increasing Cd^{2+} concentrations, and the highest GSH level was found in the control group, which differed significantly ($P < 0.05$) from all Cd^{2+}-exposed groups. Similar to GSH level, changes in GSSG level in response to Cd^{2+} exposure followed the same trend, but the decreases observed at higher Cd^{2+} concentrations (3–12 mg L^{-1}) were much smaller than those shown by GSH level (**Figure 4B**).

In the hepatopancreas and foot, there were no significant changes in GSH/GSSG ratio compared with the control group when the clams were exposed to low (1.5 mg L^{-1}) Cd^{2+} concentration, but a dose-dependent decrease in GSH/GSSG ratio was observed in clams exposed to higher Cd^{2+} concentrations (3–12 mg L^{-1}) (**Figure 4C**). The ratio of GSH/GSSG in the gill decreased continuously and reached the lowest level in the group exposed to the highest Cd^{2+} concentration (12 mg L^{-1}). However, in the mantle, exposure to lower Cd^{2+} concentrations (1.5–3 mg L^{-1}) did not result in significant changes in GSH/GSSG ratio, although exposure to higher Cd^{2+} concentrations (6 and 12 mg L^{-1}) resulted in a marked reduction in GSH/GSSG ratio.

Thus, cadmium could lead to a general decrease in GSH and GSSG levels as well as GSH/GSSG ratio. However, the GSH level was most affected by higher Cd^{2+} concentrations (6–12 mg L^{-1}).

Regression Analysis Between GSH and GPx or MDA

Regression analysis showed a significant negative correlation between the mean values of GSH and MDA found in the gill (**Table 2**, $P = 0.005$; **Figure 5A**, $R^2 = 0.947$) and mantle (**Table 2**, $P = 0.004$; **Figure 5B**, $R^2 = 0.857$). A significant positive correlation between GSH and GPx in the foot was observed (**Table 2**, $P = 0.018$; **Figure 5C**, $R^2 = 0.881$). There was no significant correlation between GSH and the other oxidative stress indexes.

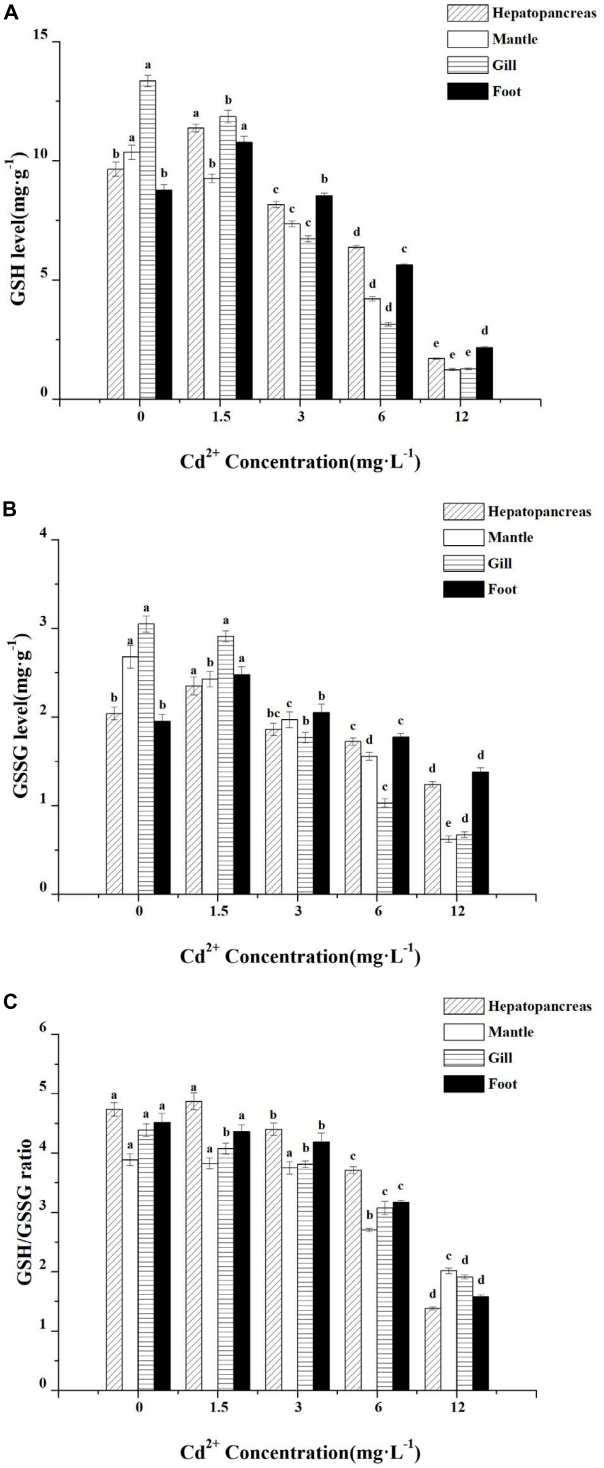

FIGURE 4 | Effect of Cd^{2+} on the levels of glutathione in different tissues of *M. meretrix* following Cd^{2+} exposure. Hepatopancreas (twill pillars), mantle (white pillars), gill (horizontal stripes pillars), and foot (black pillars). **(A)** GSH, **(B)** GSSG, and **(C)** GSH/GSSG ratio. Data are the means ± standard errors (SEs) ($n = 6$). Different letters above the pillars indicate significant ($P < 0.05$) differences among the different Cd^{2+}-treated groups (0, 1.5, 3, 6, or 12 mg L^{-1}) for the same tissue as revealed by Tukey's *post hoc* multiple comparison tests.

DISCUSSION

Cd Bioaccumulation in Different Tissues

Among the benthic species, bivalves are characterized by their ability to accumulate very high levels of metals, which can reflect the bioavailability of metal contaminants in the water environments (Huo et al., 2012; Chan and Wang, 2018; Chen et al., 2020). Depending on whether the uptake is directly from the water or via the ingested food, the properties of the biological barriers such as the gills, mantle, and gut wall that separate the animals from their surrounding medium can lead to varying degrees of specific metal distribution in the different tissues (Huo et al., 2012; Marasinghe Wadige et al., 2017; Jing et al., 2019). The levels of Cd^{2+} accumulated in the four tissues of *M. meretrix* examined differed markedly, both within and among groups (**Table 1**), demonstrating that the accumulation of Cd^{2+} in *M. meretrix* was tissue-dependent. Similar to many aquatic animals, such as *Tegillarca granosa* (Huo et al., 2012), *Hyridella australis* (Marasinghe Wadige et al., 2017), and *Eriocheir sinensis* (Cheng et al., 2018), the gill was the major target for Cd^{2+} accumulation. As mentioned before, the gills are the primary target organ of metal contaminants and both as a site of metal uptake and as an important reservoir for metal storage, which could be one of the main causes of higher Cd bioaccumulation in the gill (Cheng et al., 2018; Zhen et al., 2018). Zhu et al. (2018) found that Cd can alter the gill morphology of *Scylla paramamosain*, which can result in osmoregulation dysfunction, lower oxygen uptake, and even hypoxic stress. Domouhtsidou and Dimitriadis (2000) found that toxic metals can cause the gill filaments of *Mytilus gallolprovincialis* Lamarck to rupture, thus affecting the transport of toxic metal to the other tissues. Zhen et al. (2018) found that Cd^{2+} can destroy the structure of gill epithelial cells in *M. meretrix* and deprive these cells of their normal physiological functions. The higher level of Cd^{2+} found in the gill of *M. meretrix* (**Table 1**) suggested that Cd might influence the functions of the gill, such as gas exchange, ion transport, waste excretion, osmoregulation, and transport of Cd^{2+} to other tissues. On the other hand, our data revealed significantly ($P < 0.05$) lower Cd^{2+} level in the hepatopancreas compared with the other tissues examined (**Table 1**). However, other investigators have reported different results for bivalves. For example, Wang et al. (2009) found the order of Cd^{2+} level in *Patinopecten yessoensis* raised in a typical mariculture area in northern China to be hepatopancreas > gill > sexual gland > mantle > muscle and suggested that it could be related to the induction of MT since the hepatopancreas is the main tissue for the synthesis of MT. In a different study, García-Navarro et al. (2017) demonstrated that *Mytilus galloprovincialis* exposed to Cd^{2+} for seven days exhibited a significantly higher Cd^{2+} concentration in the digestive gland than in the gill. However, Jing et al. (2019) found that Cd concentrations in the different tissues of *Anodonta woodiana* can display different orders when exposed to 0.168 mg L^{-1} Cd^{2+} (digestive gland > gills > mantle > visceral mass > foot) and to 0.675 mg L^{-1} Cd^{2+} (gills > digestive gland > mantle > visceral mass > foot) for 28 days. Additionally, Cd concentrations in the gills and digestive gland of the mussel *Perna canaliculus* were found to exhibit different orders as a result of different exposure methods (Chandurvelan et al., 2015). Metal accumulation in the tissues of aquatic organisms is dependent upon the exposure time, exposure method, and exposure dose as well as other factors, such as temperature, age, interaction with other metals, water chemistry, and the metabolic activity of the organism (Wang et al., 2009; Huo et al., 2012; Min et al., 2016; Jing et al., 2019). Thus, differences in Cd^{2+} accumulation level in the hepatopancreases, gill, mantle, and foot of *M. meretrix* may be due to the different physiological functions and responses of these tissues to acute toxicity. The higher levels of Cd^{2+} found in the gill, mantle, and foot compared with the hepatopancreases could be attributed to the location of these tissues, which is in direct contact with the external water. For example, the mantle has a relatively large contact area with water, allowing easier adsorption or passive diffusion of Cd from the water. This could have led to the highest increase of 18,510% in the mantle of the 12 mg L^{-1} Cd^{2+}-exposed groups compared with the control group. Similar results have also been demonstrated for *A. woodiana* (Jing et al., 2019) and *Mytilus edulis* (Zhang et al., 2015). It has been demonstrated that shellfish can absorb both dissolved and particulate heavy metals. Dissolved metals are absorbed mainly by direct adsorption occurring on the animal surface (gill, mantle, and foot), while particulate metals tend to be absorbed more by the digestive organs along with the food (Chandurvelan et al., 2015; García-Navarro et al., 2017; Jing et al., 2019). Previous studies have shown that in invertebrates, Cd^{2+} concentration in the gill is higher than in the hepatopancreas following acute exposure (Huo et al., 2012; Zhang et al., 2015; Marasinghe Wadige et al., 2017), but in chronic exposure, the final concentration of Cd^{2+} in the gill was found to be lower than that in the digestive gland after its absorption, excretion, and transfer to other tissues (Chandurvelan et al., 2012; Jing et al., 2019). Jing et al. (2019) also suggested that Cd bioaccumulation in the gills could reflect short-term Cd pollution, and Cd bioaccumulation in the digestive glands might indicate longer Cd exposure. The higher concentrations of Cd^{2+} in the digestive gland or gill could be explained by the redistribution of cadmium among the tissues before its excretion (Zhang et al., 2015). Ghosh et al. (2020) found that the bioaccumulation process of metal in a specific tissue or organ of *Lamellidens marginalis* is temporary (gills, alimentary canal, and foot) and may be transported to the next tissue or organ in a series that may be the site of depuration or sequestration or excretion (anterior and posterior adductor muscles, hepatopancreas, and kidney). García-Navarro et al. (2017) suggested that Cd can quickly saturate the gill surface as a result of the direct contact and holding of Pb, Cd, and Cu, which would accelerate the distribution of Cd to the digestive gland.

Responses of Metallothionein and Other Biomarkers to Cd Exposure

The biomarker approach has been extensively employed as an indicator of early biological changes caused by toxic metal pollution (Chan and Wang, 2018). Several studies have shown that formation of oxygen free radicals or ROS would increase in

response to toxic metal exposure, and these can act on membrane lipids, leading to lipid peroxidation (Sandbichler and Höckner, 2016; Zheng et al., 2016; Lin et al., 2017). The level of MDA, a secondary product of lipid peroxidation, is commonly used as an indicator of oxidative stress (Chan and Wang, 2018). The MDA levels in the different tissues of *M. meretrix* increased significantly with rising Cd^{2+} concentrations (**Figure 1**), indicating Cd^{2+}-induced lipid peroxidation in the clam by raising the levels of ROS. Increased MDA level has been shown to result in the saturation of antioxidant systems, leading to oxidative damage (Lin et al., 2017; Haque et al., 2018; Cui et al., 2020). This is also consistent with the work reported by Xia et al. (2016), which shows that Cd^{2+} can induce significant apoptosis and oxidative stress in the hepatopancreas of *M. meretrix*, even at concentrations far below the LC_{50}. An early event to prevent free Cd^{2+} from interfering with cellular metabolism is by chelation of Cd^{2+} via cysteine-rich molecules, such as MTs or GSH (Nair et al., 2015; Chan and Wang, 2018; Gu et al., 2019). Besides, the induction of antioxidant enzymes might also form an important protective mechanism for minimizing oxidative damage linked to pollution in the environments. These enzymes might also serve as important biomarkers for detecting toxic metal contaminants (Wang et al., 2010; Min et al., 2016; Chen et al., 2020).

Metallothioneins are non-enzymatic proteins that bind metals with their cysteine thiol groups (–SH), and this binding can detoxify toxic metals and regulate the metabolism of trace elements (Amiard et al., 2006; Zhu et al., 2018). Binding of Cd^{2+} to MT can lower the availability of free *in vivo* metal ions, thus controlling the intracellular levels of Cd^{2+} and reducing its toxicity (Lavradas et al., 2014; Chan and Wang, 2018). Cadmium is a known MT inducer, but sensitivity to Cd appears to vary depending on the particular tissue and the duration of exposure (Gu et al., 2019). In *M. meretrix*, the level of *MT* mRNA in different tissues could be induced by Cd^{2+} (**Figure 2**), suggesting that the increase in *MT* mRNA level could have led to more Cd^{2+} being sequestered by MT. The upregulation of *MT* genes by a low dose of Cd^{2+} appears to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor (Amiard et al., 2006). Similar results have been obtained in previous studies, in which increase in *MT* mRNA level was found to occur in a dose- and duration-dependent manner in *Charybdis japonica* (Pan and Zhang, 2006), *Mactra veneriformis* (Fang et al., 2010), and *E. sinensis* (Chen et al., 2019). Gu et al. (2019) found that different durations of Cd^{2+} exposure led to differences in *MT* expression in the various tissues (ganglion, dorsal tissue, hepatopancreas, and abdominal tissue) of *Onchidium reevesii* and suggested that *MT* expression level can reveal a clear effect of heavy metal stress that is time- and Cd concentration-dependent. The expression of *MT* was significantly correlated with Cd^{2+} bioaccumulation in the tissues (**Table 1**, **Figure 2**), consistent with a role of Cd^{2+} detoxification for *MT*. However, such a positive correlation between *MT* expression and tissue Cd^{2+} accumulation only occurred up to a certain external Cd^{2+} concentration. This might be due to the physiological disturbance caused by the strong toxicity exerted by the high concentration of Cd^{2+} within the body, or that the concentration of Cd^{2+} in the body exceeded the capability of the MT-mediated detoxification pathway and started to affect the biosynthesis of *MT* mRNA. Many studies have reported that the synthesis of *MT* mRNA or MT protein is stimulated to deal with the increasing amount of ROS generated by the cadmium stress, suggesting that the *MT* gene could serve as a sensitive biomarker for Cd exposure and potentially other heavy metals (Cenov et al., 2018; Zhu et al., 2018). Previous studies looking at the induction of MT by toxic metals in *O. reevesii* (Gu et al., 2019) and *E. sinensis* (Chen et al., 2019) have also suggested that MT possibly plays a role in the scavenging of ROS generated in response to the oxidative damage induced by Cd, a phenomenon that is supported by our data. However, the regulatory mechanism of *MT* gene expression requires more in-depth study to better understand the roles of the MTs in Cd detoxification.

TABLE 2 | Regression analysis of the mean data between GSH and GPx or MDA in different tissues of *M. meretrix*.

	GPx	MDA
Hepatopancreas GSH	$F_{1,3} = 5.312$, $P = 0.105$	$F_{1,3} = 6.461$, $P = 0.085$
Gill GSH	$F_{1,3} = 3.755$, $P = 0.148$	$F_{1,3} = 53.840$, $\mathbf{P = 0.005}$
Foot GSH	$F_{1,3} = 22.235$, $\mathbf{P = 0.018}$	$F_{1,3} = 2.586$, $P = 0.206$
Mantle GSH	$F_{1,3} = 0.013$, $P = 0.915$	$F_{1,3} = 62.872$, $\mathbf{P = 0.004}$

The bolded values means significant correlation.

GSH, a major intracellular antioxidant in living organisms, can be used as an indicator to evaluate the free radical-scavenging capacity of an organism. It can serve as a potential biomarker for trace metals when coupled with lipid peroxidation (Chan and Wang, 2018). Reduced glutathione is involved in cellular protection by relieving the effect of oxidative stress (caused by heavy metals) through forming oxidized glutathione (GSSG) and metal complexes (Lavradas et al., 2014). Our data suggested that GSH might play a role in the initial response to Cd^{2+} stress by chelating with Cd^{2+} ions, as demonstrated by its decreased level at higher Cd^{2+} concentrations (**Figure 4A**). This is in agreement with previously published data, in which the accumulation of Cd^{2+} can form a GSH–metal complex, resulting in the depletion of cellular GSH (Nair et al., 2015; Xia et al., 2016; Yao et al., 2020). At higher Cd^{2+} concentrations (3–12 mg L^{-1}), the significantly lower levels of GSH in the gill and mantle compared with the foot and hepatopancreas might indicate partial toxicity caused by Cd^{2+}. Regression analysis also showed a significant negative correlation between the mean values of MDA and GSH in the gill and mantle (**Figures 5A,B**). This could mean that the antioxidant capacity might not be adequate to rid the system of the oxidative stress induced by Cd^{2+}, consequently leading to redox imbalance in the cells (Dabas et al., 2014), as a concomitant increase in MDA amount was observed in both tissues (**Figure 1**). The lack of change observed for the GSH/GSSG ratio upon exposure to low Cd^{2+} concentration (1.5 mg L^{-1}) (**Figure 4C**) suggested that adaptive processes might have been activated to maintain a state of redox balance. However, under high Cd^{2+} concentrations, a significant decrease in GSH/GSSG ratio occurred, and this could be the cause of high lipid peroxidation occurring in *M. meretrix* exposed to high Cd^{2+} concentration (**Figure 1**).

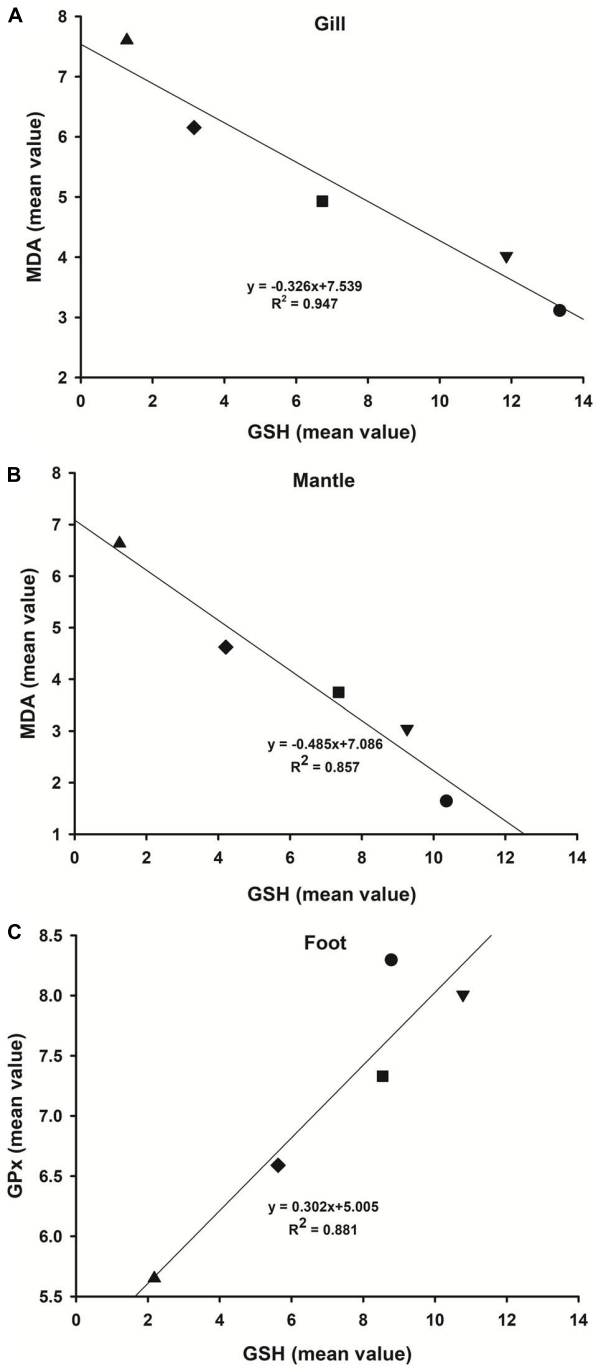

FIGURE 5 | Regression analysis of the mean data of GSH and MDA or GPx in the various tissues of Cd^{2+}-exposed *M. meretrix*. GSH and MDA in the gill **(A)** and mantle **(B)**. GSH and GPx in the foot **(C)**. Symbols in the plot denote control or one of the Cd^{2+}-treated groups.

In mollusks, antioxidant enzymes also respond to excessive toxic metal stress by eliminating excessive ROS (Cong et al., 2012; Chan and Wang, 2018), and hence, antioxidant enzymes are also recognized as important biomarkers which can reveal the early effects of toxic metal contamination (Liu and Wang, 2016; Cui et al., 2020). Endogenous antioxidants such as SOD, CAT, and GPx are key components of the antioxidant defense system that protects organisms against oxidative stress (Xia et al., 2016; Lin et al., 2017; Chen et al., 2020). Our results showed that lower Cd^{2+} levels in the tissues of *M. meretrix* could activate the antioxidant protective mechanisms to cope with the potential oxidative stress inflicted by the increasing levels of $O_2^{-\cdot}$ and H_2O_2, observations that have been reported by studies conducted with other mollusks (Chandurvelan et al., 2015; Liu and Wang, 2016; Yao et al., 2020). However, higher Cd^{2+} levels in the tissues might suppress SOD and CAT activities through the binding of Cd^{2+} to the active sites of these enzymes or the extra ROS generated could have reduced the scavenging capability of both SOD and CAT, ultimately leading to cytotoxicity (Xia et al., 2016; Cui et al., 2020). For GPx activity, a significant upregulation was observed in the gill and mantle at lower Cd^{2+} concentrations (**Figure 3C**), and this could mean that GPx probably participated in the active detoxification of Cd via various biochemical mechanisms, such as the decomposition of H_2O_2 to O_2 and H_2O or the conversion of lipid hydroperoxides into less toxic hydroxyl products. However, a significant downregulation of GPx activity was observed in the gill and mantle at higher Cd^{2+} concentrations and in the hepatopancreas and foot of all Cd^{2+}-exposed groups. Glutathione peroxidase activity is dependent on the level of selenium, which might be different in the different tissues of *M. meretrix*. By binding to Cd^{2+}, the active site (Se–Cys) of GPx is altered, and this might inactivate the enzyme (Loro et al., 2012; Xia et al., 2016). Regression analysis between GSH and GPx in the foot (**Figure 5C**) suggested a role for both GSH and GPx in the elimination of peroxides and ROS, thereby providing another line of defense against oxidative damage. The antioxidant function of GSH is also due to its participation as an abundant redox buffer in the cells, where it could serve as a substrate for GSH-related enzymes (Cui et al., 2020).

CONCLUSION

Overall, the bioaccumulation of Cd^{2+} in *M. meretrix* was found to display a concentration-dependent trend with significant differences among different tissues exposed to the same Cd^{2+} concentration. This observation would support speculation that people who continue to consume cadmium-loaded clams in their diets could risk exposing themselves to Cd toxicity in the long term. Change in the level of MDA of the clams might provide a useful biomarker for Cd^{2+} contamination due to their constant increase with increasing Cd^{2+} concentrations in the water, and the gill might be a good tissue to detect the early biological responses in a Cd^{2+}-contaminated environment. In the detoxification aspect, at low concentrations of Cd^{2+}, the combined action of MT and GSH could lead to more sequestration of free Cd^{2+} inside the organism. At the same time, enhanced levels of CAT, SOD, and GPx activities would help to neutralize the toxic effect of Cd^{2+} and to protect the membrane lipids against oxidative stress. The continuous increase in Cd^{2+} concentration in the environment would lead to the depletion of GSH, triggering the MT-based

mechanism, which could further sequester the Cd^{2+}, presumably through the formation of MT-Cd^{2+} complexes. However, the precise mechanism associated with the detoxification of Cd^{2+} toxicity by MT is a subject of further study.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

YH and HT were responsible for the experimental design, carrying out the experiments, data processing, and manuscript writing. JJ and MF performed the sample collection, sample processing, and extraction. AC and XY provided experimental guidance and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.