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Changes in Antioxidant Enzyme Activity and Physiological Responses to Cadmium and Tributyltin Exposure in the Ark Shell, *Scapharca Broughtonii*

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Abstract

Cadmium (Cd) and tributyltin (TBT) are common contaminants of marine and freshwater ecosystems, and can induce the formation of reactive oxygen species (ROS). These ROS can, in turn, cause oxidative stress. In the present study, we investigated time-related effects of Cd (0.05 and 0.1 ppm) and TBT (5 and 10 ppb) treatment on antioxidant enzyme activity, i.e., the activity of superoxide dismutase (SOD) and catalase (CAT) in the gills and digestive glands of the ark shell, *Scapharca broughtonii*. In addition, hydrogen peroxide (H_2O_2) concentrations, lysozyme activity, and glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) levels were measured in the hemolymph. We found that Cd and TBT treatment significantly increased antioxidant enzyme mRNA expression and activity in the digestive glands and gills in a time-dependent manner. In response to the Cd and TBT treatments, antioxidant enzymes mRNA expression and activity increased up to day 5 in the digestive glands and then decreased by day 7. In the gills, antioxidant enzymes mRNA expression and activity increased up to day 3 and then decreased by day 5. Likewise, H_2O_2 concentrations significantly increased up to day 5 and then decreased by day 7. Finally, lysozyme activity decreased during the experimental period, whereas GOT and GPT levels were significantly increased in a time-dependent manner. These results suggest that antioxidant enzymes play an important role in decreasing ROS levels and oxidative stress in ark shells exposed to Cd and TBT.

Increasing amounts and varieties of organic and inorganic compounds, chemicals, and heavy metals are entering the aquatic environment and, as a result, are being sequestered in the tissues of aquatic organisms. Bivalve mollusks are especially susceptible to contamination within their respective habitats because of poor mobility as compared with other aquatic species (e.g., fish). Because bivalve mollusks feed via gill filtration, a large amount of contaminants can be absorbed from the environment and may accumulate in the body[1].

Heavy metals such as cadmium (Cd), lead (Pb), and hydrargyrum (Hg) enter the aquatic environment as a result of human activities such as wastewater production, agriculture and mining. These contaminants can be toxic to aquatic organisms even very small amounts and accumulate within the bodies of aquatic organisms. Specially, Cd has adverse physiological effects on the growth, reproduction, and osmoregulation of aquatic organisms[2]. Cd accumulates within organismal tissues and can alter and degrade enzymatic processes and cause cell damage, which may result in cell death[3]. In addition, Cd induces oxidization and generates reactive oxygen species (ROS) that promote oxidative damage[4].

Tributyltin (TBT), an organic compound containing tin, is a highly toxic environmental pollutant that is present in agents that counteract marine biological adhesion[5]. Due to its widespread use as an antifouling agent in boat paints, TBT is a common contaminant of marine and freshwater ecosystems. TBT affects all types of aquatic organisms, in addition to those that adhere to surfaces[6]. Many countries have banned the use of organotin-based antifouling paints due to their toxic, persistent, bioaccumulative, and endocrine disruptive characteristics[6]. In particular, mollusks easily accumulate TBT[6]. Accumulated TBT has been demonstrated to cause impairments in growth, development, and reproduction, enzyme inhibition, reductions or

alterations in protein and DNA synthesis, cytoskeletal alterations, and disruptions in ATP synthesis[7]. In addition, Huang *et al*.[8] reported that TBT exposure generates ROS production and oxidative stress in the Asiatic hard clam, *Meretrix meretrix*.

To maintain homeostasis and prevent oxidative stress caused by heavy metals and other toxic substances, living organisms have evolved antioxidant defense mechanisms[9]. These systems include both enzymatic and non-enzymatic components[9]. Antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione S-transferase (GST). Low molecular weight antioxidant materials include glutathione (GSH), ascorbic acid, metallothionein (MT), and α-tocopherol[10].

SOD and CAT directly inactivate ROS such as superoxide anion ($O_2{\cdot}^-$) and hydrogen peroxide (H_2O_2). Thus, these antioxidant enzyme activities are used as a marker involved in the primary defense against oxidative damage. SOD is a defense element against ROS and removes the $O_2{\cdot}^-$ through the process of dismutation to singlet oxygen (O_2) and H_2O_2 ($2O_2{\cdot}^- + H^+ \rightarrow H_2O_2 + O_2$)[11]. As a metalloenzyme, SOD is generally classified into copper/zinc-SOD (Cu/Zn-SOD), manganese-SOD (Mn-SOD), and iron-SOD (Fe-SOD) based on which metals are bonded to the active sites of the enzyme[12]. Previous studies on SOD activity have examined benzo [a]pyrene exposure in the blood clam, *Scapharca inaequivalvisdisk*[13], and polychlorobiphenyls (PCBs) and heavy metal exposure in the freshwater mussel, *Dreissena polymorpha*[14].

H_2O_2 produced by SOD is sequentially reduced to H_2O and O_2 by CAT[15]. CAT is an oxidoreductase enzyme that breaks down two molecules of H_2O_2 to two molecules of H_2O and O_2 ($2H_2O_2 \rightarrow 2H_2O + O_2$), therefore counteracting the toxicity of H_2O_2[11]. Previous studies on CAT activity have investigated polycyclic aromatic hydrocarbon (PAHs) and organochlorine pesticide exposure in the green-lipped mussels, *Perna viridis*[16], and PCB and heavy metal exposure in the freshwater mussel[14].

In bivalves, hemolymph involves in defense mechanisms. Among the hemolymph constituents, lysozyme enzyme participates in inactivation of invading microbes and is generally affected by environmental stress (e.g., heavy metals and organic compounds)[17,18]. Also, GOT and GPT are caused by the inflow of cells into the hemolymph due to tissue damage by environmental contaminants[4].

Therefore, we quantified changes in H_2O_2 concentrations, lysozyme activity, glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) levels in hemolymph, as well as changes in the activity and mRNA expression of antioxidant enzymes (SOD and CAT) by exposure of Cd and TBT. The goal of this study was to identify the antioxidant mechanisms and physiological responses of the ark shell, *Scapharca broughtonii*, to Cd and TBT exposure.

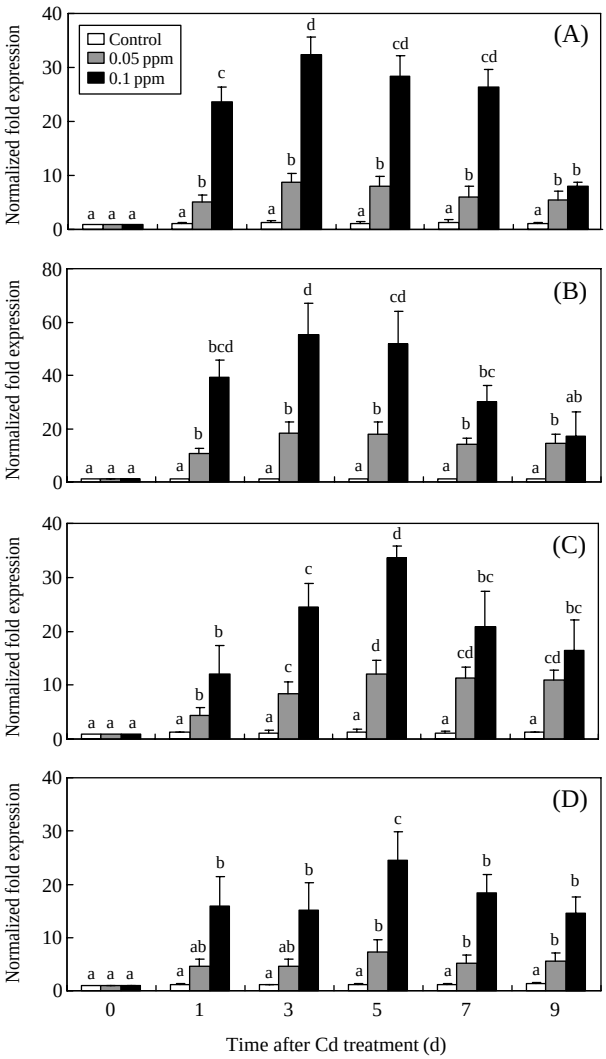

Figure 1. In the gills, SOD (A) and CAT (B) mRNA expression of ark shells treated with 0.05 and 0.1 ppm Cd for 1, 3, 5, 7, and 9 days. In the digestive glands, SOD (C) and CAT (D) mRNA expression of ark shells treated with 0.05 and 0.1 ppm Cd for 1, 3, 5, 7, and 9 days. Values in the same experimental group with dissimilar letters are significantly different ($P < 0.05$) from each other. Values are expressed as means ± S.D. ($n=5$).

Expression Levels of Antioxidant Enzymes Cd Treatments

Expression levels of antioxidant enzymes during Cd exposure were shown in Figure 1. In the gills, SOD mRNA expression increased with time and reached a

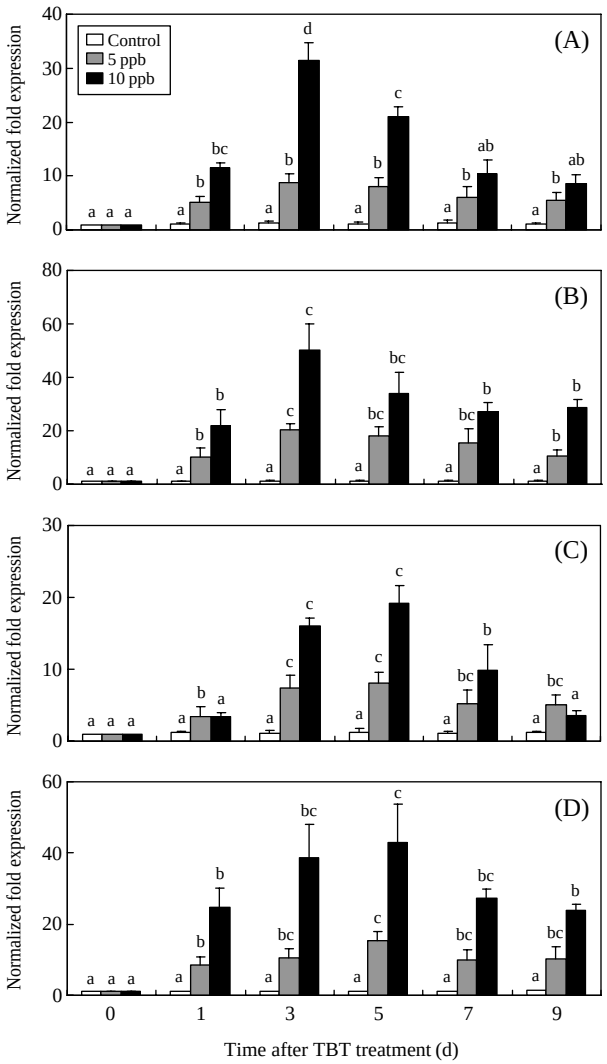

Figure 2. In the gills, SOD (A) and CAT (B) mRNA expression of ark shells treated with 5 and 10 ppb TBT for 1, 3, 5, 7, and 9 days. In the digestive glands, SOD (C) and CAT (D) mRNA expression of ark shells treated with 5 and 10 ppb TBT for 1, 3, 5, 7, and 9 days. Values in the same experimental group with dissimilar letters are significantly different ($P < 0.05$) from each other. Values are expressed as means ± S.D. (n=5).

peak after 3 days of exposure to 0.05 ppm Cd (8.8-fold higher than the control) and 0.1 ppm Cd (32.4-fold higher than the control) and then decreased (Figure 1A). CAT mRNA expression increased with time and peaked after 3 days of exposure to 0.05 ppm Cd (18.2-fold higher than the control) and 0.1 ppm Cd (55.4-fold higher than the control) and then decreased (Figure 1B). In the digestive glands, SOD mRNA expression increased with time and reached a peak after 5 days of exposure to 0.05 ppm Cd (12.0-fold higher than the control) and 0.1 ppm Cd (33.6-fold higher than the control) and then decreased (Figure 1C). CAT mRNA expression increased with time and peaked after 5 days of exposure to 0.05 ppm Cd (7.2-fold higher than the control) and 0.1 ppm Cd (24.5-fold higher than the control) and then decreased (Figure 1D).

Expression Levels of Antioxidant Enzymes TBT Treatments

Expression levels of antioxidant enzymes during TBT exposure were shown in Figure 2. In the gills, SOD mRNA expression increased with time and reached a peak after 3 days of exposure to 5 ppb TBT (8.8-fold higher than the control) and 10 ppb TBT (31.4-fold higher than the control) and then decreased (Figure 2A). CAT mRNA expression increased with time and peaked after 3 days of exposure to 5 ppb TBT (20.4-fold higher than the control) and 10 ppb TBT (50.1-fold higher than the control) and then decreased (Figure 2B). In the digestive glands, SOD mRNA expression increased with time and reached a peak after 5 days of exposure to 5 ppb TBT (8.0-fold higher than the control) and 10 ppb TBT (19.2-fold higher than the control) and then decreased (Figure 2C). CAT mRNA expression increased with time and peaked after 5 days of exposure to 5 ppb TBT (15.4-fold higher than the control) and 10 ppb TBT (43.1-fold higher than the control) and then decreased (Figure 2D).

Activity of Antioxidant Enzymes Cd Treatments

SOD and CAT activities were measured in tissue homogenates of the gills and digestive glands. In the gills, SOD activity showed a significant increase after 3 days of exposure to 0.05 ppm Cd (1.5 ± 0.1 U/mL) and 0.1 ppm Cd (2.1 ± 0.1 U/mL) and then subsequently decreased (Figure 3A). CAT activity increased with time and peaked after 3 days of exposure to 0.1 ppm Cd (23.7 ± 1.2 nM/min/mL); there was no change in CAT activity after exposure to 0.05 ppm (Figure 3B). In the digestive glands, SOD activity significantly increased after 5 days of exposure to 0.05 ppm Cd (1.1 ± 0.1 U/mL) and 0.1 ppm Cd (2.1 ± 0.1 U/mL) and then decreased (Figure 3C). CAT activity increased with time and peaked after 5 days of exposure to 0.1 ppm Cd (25.7 ± 1.2 nM/min/mL); there was no change in CAT activity after exposure to 0.05 ppm (Figure 3D).

Activity of Antioxidant Enzymes TBT Treatments

SOD and CAT activities were measured in tissue homogenates of the gills and digestive glands. In the gills, SOD activity significantly increased after 3 days

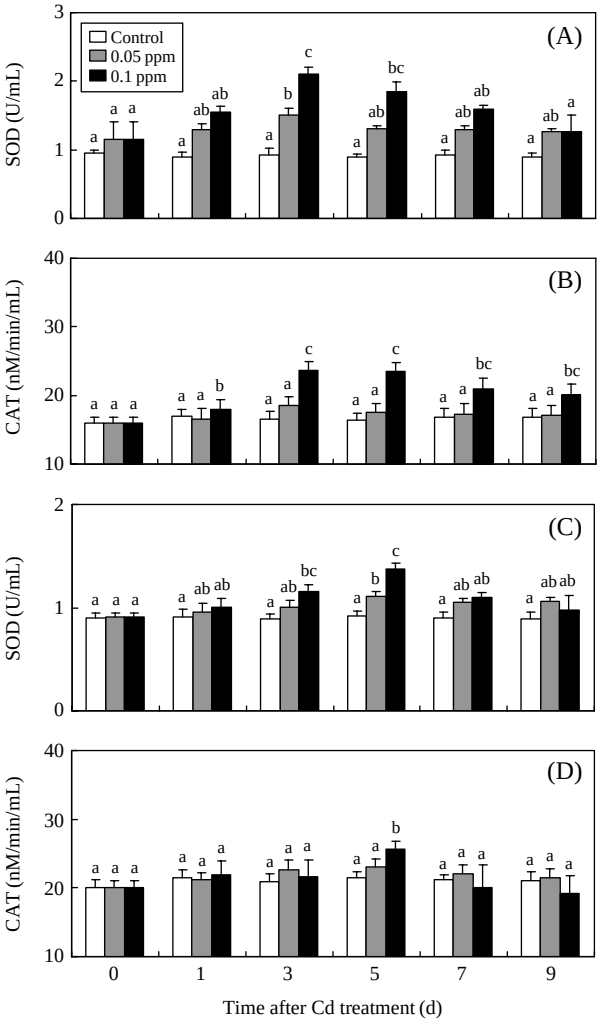

Figure 3. In the gills, SOD (A) and CAT (B) activity of ark shells treated with 0.05 and 0.1 ppm Cd for 1, 3, 5, 7, and 9 days. In the digestive glands, SOD (C) and CAT (D) activity of ark shells treated with 0.05 and 0.1 ppm Cd for 1, 3, 5, 7, and 9 days. Values in the same experimental group with dissimilar letters are significantly different ($P < 0.05$) from each other. Values are expressed as means ± S.D. (n=5).

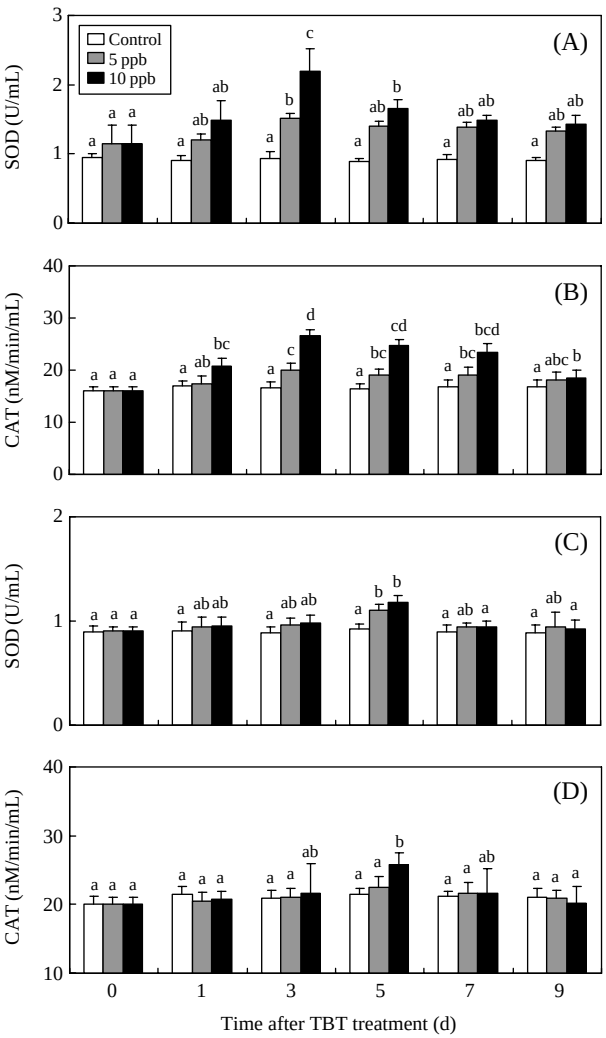

Figure 4. In the gills, SOD (A) and CAT (B) activity of ark shells treated with 5 and 10 ppb TBT for 1, 3, 5, 7, and 9 days. In the digestive glands, SOD (C) and CAT (D) activity of ark shells treated with 5 and 10 ppb TBT for 1, 3, 5, 7, and 9 days. Values in the same experimental group with dissimilar letters are significantly different ($P < 0.05$) from each other. Values are expressed as means ± S.D. (n=5).

of exposure to 5 ppb TBT (1.5 ± 0.1 U/mL) and 10 ppb TBT (2.2 ± 0.3 U/mL) and then decreased (Figure 4A). CAT activity increased with time and peaked after 3 days of exposure to 5 ppb TBT (20.0 ± 1.2 nM/min/mL) and 10 ppb TBT (26.6 ± 1.2 nM/min/mL; Figure 4B). In the digestive glands, SOD activity significantly increased after 5 days of exposure to 5 ppb TBT (1.1 ± 0.1 U/mL) and 10 ppb TBT (1.2 ± 0.1 U/mL) and then decreased (Figure 4C). CAT activity increased with time and peaked after 5 days of exposure to 10 ppb TBT (25.8 ± 1.7 nM/min/mL); there was no change in CAT activity after exposure to 5 ppb (Figure 4D).

H_2O_2 Concentrations

Hemolymph H_2O_2 concentrations significantly increased following Cd and TBT treatment in a dose- and time-dependent manner. H_2O_2 concentrations increased with time and peaked after 5 days of exposure to 0.05 ppm Cd (18.2 ± 2.6 nM/mL), and 0.1 ppm Cd (25.3 ± 1.5 nM/mL; Figure 5A). For TBT treatments, H_2O_2 concentrations increased significantly after 5 days of exposure to 5 ppb TBT (18.1 ± 3.1 nM/mL), and 10 ppb TBT (25.8 ± 1.5 nM/mL; Figure 5B). Maximal responses in H_2O_2 concentration were observed at 5 days and then decreased until day 9.

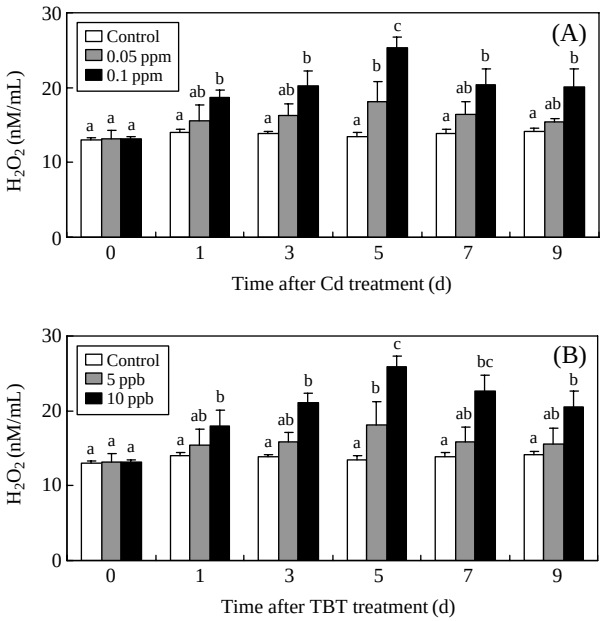

Figure 5. Effects of Cd and TBT exposure on hydrogen peroxide concentrations in the hemolymph of ark shells treated with 0.05 and 0.1 ppm Cd (A), and 5 and 10 ppb TBT (B) for 1, 3, 5, 7, and 9 days. Values in the same experimental group with dissimilar letters are significantly different ($P<0.05$) from each other. Values are expressed as means ± S.D. ($n=5$).

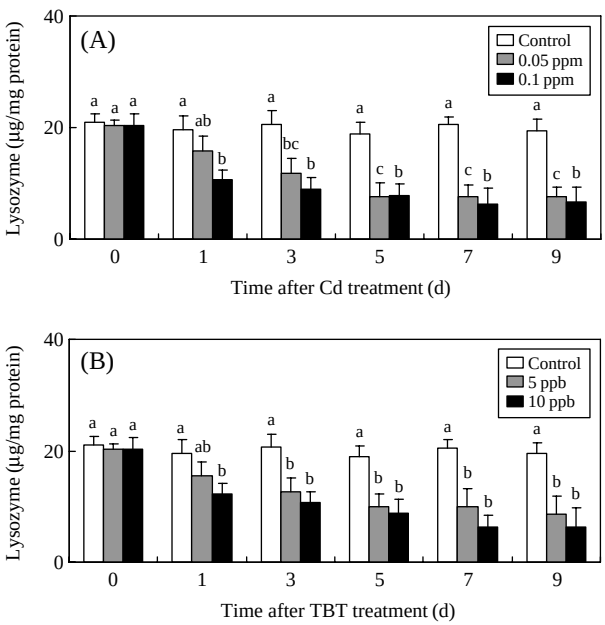

Figure 6. Effects of Cd and TBT exposure on lysozyme activity in the hemolymph of ark shells treated with 0.05 and 0.1 ppm Cd (A), and 5 and 10 ppb TBT (B) for 1, 3, 5, 7, and 9 days. Values in the same experimental group with dissimilar letters are significantly different ($P<0.05$) from each other. Values are expressed as means ± S.D. ($n=5$).

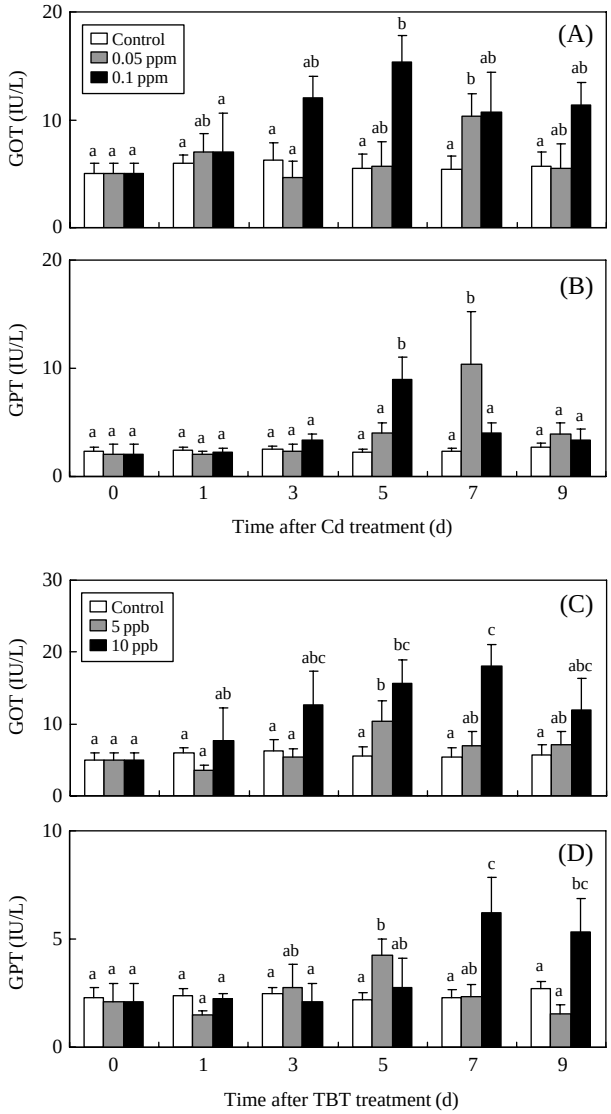

Figure 7. GOT (A) and GPT (B) levels in the hemolymph of ark shells treated with 0.05 and 0.1 ppm Cd. GOT (C) and GPT (D) levels in the hemolymph of ark shells treated with 5 and 10 ppb TBT. Values in the same experimental group with dissimilar letters are significantly different ($P<0.05$) from each other. Values are expressed as means ± S.D. ($n=5$).

Lysozyme Activity

Hemolymph lysozyme activity was significantly lower in ark shells treated with Cd and TBT as compared with controls. Lysozyme activity decreased with time and reached the lowest point after 9 days of exposure to 0.05 ppm Cd (7.6 ± 1.4 μg/mg protein) and 7 days of exposure to 0.1 ppm Cd (6.3 ± 2.9 μg/mg protein; Figure 6A). With TBT treatment, lysozyme activity decreased with time and reached the lowest point after 9 days of exposure to 5 ppb TBT (8.7 ± 3.2 μg/mg

protein) and 7 days of exposure to 10 ppb TBT (6.3 ± 2.1 μg/mg protein; Figure 6B).

GOT and GPT

Hemolymph GOT and GPT levels during Cd and TBT treatments are shown in Figure 7. In the Cd treatment groups, GOT and GPT levels significantly increased after 7 days of exposure to 0.05 ppm (GOT, 10.3 ± 2.1 IU/L; GPT, 10.3 ± 4.9 IU/L) and after 5 days of exposure to 0.1 ppm (GOT, 15.3 ± 2.5 IU/L; GPT, 9.0 ± 2.0 IU/L). In the TBT treatment groups, GOT and GPT levels significantly increased after 7 days of exposure to 10 ppb TBT (GOT, 18.0 ± 3.0 IU/L; GPT, 6.2 ± 1.6 IU/L) and after 5 days of exposure to 5 ppb TBT (GOT, 10.3 ± 2.9 IU/L; GPT, 4.3 ± 0.8 IU/L).

Discussion

To identify the antioxidant mechanisms and physiological responses of the ark shell to Cd and TBT exposure, we investigated time- and dose-effects of these contaminants on antioxidant enzyme mRNA expression and activity in the gills and digestive glands. Additionally, we quantified changes in H_2O_2 concentrations, lysozyme activity, and GOT and GPT levels in the hemolymph.

Because the gills and digestive glands are important target organs for contaminant accumulation, these tissues were chosen to investigate antioxidant defense mechanisms against contamination[19]. The gills were chosen because they represent the main site of contamination uptake, which occurs via a direct route of exposure, and they have a large exchange area that is in contact with the surrounding environment. The digestive gland is the major site of xenobiotic uptake and is involved in most biotransformation processes and redox-cycling generation[19].

In bivalves, it has been reported that Cd exposure increases ROS production, e.g., H_2O_2, in the interior of the body[4]. ROS produced as a result of Cd exposure can cause oxidative stress, which may lead to cell membrane damage, DNA damage, lipid peroxidation, protein denaturation, and the inhibition of enzyme activation[4].

We found that the mRNA expression and activity of antioxidant enzymes increased in the gills and digestive glands with Cd treatment (Figures 1 and 3). In addition, H_2O_2 concentrations increased in the hemolymph as Cd exposure time and dose increased (Figure 5A). These results suggest that Cd exposure increases the formation of ROS. In response, the mRNA expression and activity of antioxidant enzymes may increase to detoxify the ROS produced as a result of Cd exposure. Jo *et al*.[4] reported that in the Pacific oyster, *Crassostrea gigas*, exposure to Cd leads to increases in H_2O_2 concentrations and of SOD, CAT and GPX mRNA expression as compared with controls. This increase may due to an increase in the defensive mechanisms of the antioxidant system as a result of ROS production exerted by Cd. Funes *et al*.[20] demonstrated that antioxidant enzyme activity is higher in metal polluted areas as compared with clean areas by comparing levels of SOD, CAT, GPX, and GST activity in Portuguese oysters, *Crassostrea angulata*, and Mediterranean mussels, *Mytilus galloprovincialis*, from different sampling sites. These increases may represent a protective mechanism against metal pollution-induced the production of ROS. These results suggest that, to protect itself from ROS, the ark shell increases its antioxidant defense capacity by increasing antioxidant enzymes mRNA expression and activity concurrent with increases in Cd dose and exposure time.

Rice and Weeks[21] demonstrated that TBT affects ROS modulation and stimulates the production of superoxide anions ($O_2^{\cdot -}$) and hydroxyl radicals ($OH\cdot$). In the present study, TBT exposure leads to increase antioxidant enzymes mRNA expression and activity in the gills and digestive glands (Figure 2 and 4). Further, H_2O_2 concentrations increased as TBT concentration and exposure time increased (Figure 5B). TBT has the highest toxicity levels than other contaminants (Cu, Hg, and ammonia) comparing the median lethal concentration[22]. In addition, it has been reported that the effects of TBT exposure generate ROS and induce oxidative stress[8]. In the Pacific oyster, TBT exposure induces the production of ROS and increases SOD mRNA expression, which facilitates ROS removal[23]. In cuvier, *Sebasticus marmoratus*, TBT exposure also induces the production of ROS and increases antioxidant enzyme activity[24]. Therefore, we conclude that to protect against ROS caused by TBT exposure, ark shells may increase antioxidant enzymes mRNA expression and activity as TBT exposure time and dose increase. Additionally, for both Cd and TBT treatment groups, the mRNA expression and activity of antioxidant enzymes peaked at an earlier exposure time in the gills as compared with the digestive gland. The gills may represent the main barrier involved in contamination uptake via direct routes of exposure[19]. It is suggested that the gills developed an antioxidant defense system in advance of the digestive gland.

In the present study, we hypothesized that Cd and TBT exposure would increase ROS production in the ark shell. To counter this, SOD provides a first-line of defense against $O_2^{\cdot -}$. Because SOD mRNA expression and activity increases, H_2O_2 concentrations are high in the hemolymph, CAT mRNA expression and

activity increases to reduce H_2O_2 concentrations. Additionally, when CAT mRNA expression and activity decreases, there is a concurrent decrease in hemolymph concentrations of H_2O_2. These results suggest that H_2O_2 concentrations are regulated by the CAT.

In bivalves, changes in hemolymph constituents caused by heavy metal contamination, various environmental factors, and pathogenic agents have been reported[25]. Among the changes observed in hemolymph constituents, lysosomal enzymes are generally affected by environmental stress (e.g., heavy metals and organic compounds)[17,18]. In this study, lysozyme activity decreased as Cd and TBT exposure time and dose increased (Figure 6). In some bivalve species, the immune system, including lysosomal enzyme activity, is affected by the production of ROS such as $O_2^{\cdot -}$, H_2O_2, and $OH^{\cdot}$[26]. Chen *et al*.[27] demonstrated that acute temperature challenges increase ROS concentrations and SOD activity in scallops, whereas lysosomal activity decreases. This result suggests that ROS produced under stress affect aspects of immunity. Thus, we conclude that Cd and TBT exposure increases ROS concentrations and antioxidant enzyme activity; however, contamination and ROS affect immunity and decrease lysozyme activity. Therefore, we suggest that in response to Cd and TBT exposure, the antioxidant system is upregulated, but continuative stress caused by contamination affects immunity and decreases lysozyme activity.

Among the changes in hemolymph constituents, increases in GOT and GPT levels are generally caused by the inflow of cells into the hemolymph. These cells are separated due to tissue damage by environmental contaminants[4]. Vaglio and Landricina[28] reported that Cd exposure increases GOT and GPT levels in serum of gilthead bream, *Sparus aurata*. Additionally, Yoshizuka *et al*.[29] reported that TBT exposure increases GOT and GPT levels in the rat, *Rattus rattus*. In the ark shell, we found that Cd and TBT exposure increased GOT and GPT levels as exposure time and dose increased (Figure 7). Therefore, the significant increases in GOT and GPT levels in the hemolymph of the ark shell observed after exposure to Cd and TBT may be a result of tissue damage.

In conclusion, it is assumed that Cd and TBT exposure increases the production of ROS such as H_2O_2 in the ark shell. To counter this stress, SOD provides a first-line of defense against $O_2^{\cdot -}$. Secondly, CAT activates to reduce H_2O_2. Because lysozyme activity decreased in the hemolymph throughout the experiment, we suspect that contamination and ROS negatively affect immunity and decrease lysozyme activity. The observed increases in GOT and GPT indicate that tissue damage occurred in the ark shell as a result of contaminants and ROS.

Materials & Methods

Experimental Animal

We used ark shells (average shell length: 65.7 ± 5.3 mm; height: 40.1 ± 3.1 mm; weight: 85.3 ± 10.9 g) obtained from the Yongwon in Jinhae (Gyeongnam, Korea). Ark shells were placed in 50 L circulating filter tanks in the laboratory at a density of 50 specimens per tank. During the experimental period, water temperature and salinity were maintained at 20 ± 1°C and 35 ppt, respectively. Animals were kept in a photoperiod of 12 h light/12 h dark and were fed a mixture of microalgae (*Chaetoceros* sp., and *Isochrysis galbana*; 1 : 1).

Cd and TBT Treatments

After acclimatization, 30 ark shells were transferred to 50 L plastic aquaria filled with 1 μm filtered natural seawater (control), Cd-treated seawater, or TBT-treated seawater (experimental groups). For the Cd treatment groups, Cd was added to the water as $CdCl_2 \cdot 2.5H_2O$ (Kanto Chemical) at a dissolved Cd^{2+} concentration of either 0.05 or 0.1 ppm. For the TBT treatment groups, TBT was added to the water as TBTO (Tributyltin (IV) oxide; Riedel-de Haen) at a dissolved TBT concentration of either 5 or 10 ppb. Ark shells were exposed to these treatments for 9 days. During this time the water was changed daily and resupplied with the corresponding treatment. Hemolymph and tissues (digestive glands and gills) were sampled from five randomly selected ark shells after 0, 1, 3, 7, and 9 days of treatment.

Quantitative Real-time PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression of antioxidant enzymes (SOD and CAT) using total RNA extracted from digestive glands and gills of control and experimental groups. With 2.5 μg of total RNA as a template, cDNA were synthesized using M-MLV reverse transcriptase (Bioneer). First-strand cDNA synthesis was conducted using oligo-d(T)15 primer (Promega). Primers for QPCR were designed with reference to known SOD (GenBank accession no. GQ229480), CAT (GQ422471) and 28S rRNA (AB101599) gene sequences of ark shell as follows: SOD forward primer, 5′-CATTCAGTTGCAGGCAGCCATAAG-3′; SOD reverse primer, 5′-GTGACAAATTCCTCCAGGATCAAGTC-3′; CAT forward primer, 5′-CTTTGCTGAAGTGGAACAAATTGC-3′; CAT reverse primer, 5′-CATCTTGTCTGGGCTGGCTTC-3′; 28S rRNA forward primer, 5′-

AAACACGGACCAAGGAGTCT-3′; and 28S rRNA reverse primer, 5′-AGGCTGCCTTCACTTTCATT-3′. QPCR amplification was conducted using a Bio-Rad MiniOpticon™ System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad), according to the manufacturers' instructions. QPCR was undertaken by denaturation at 95˚C for 5 min; followed by 40 cycles of denaturation at 95˚C for 20 s and annealing at 55˚C for 20 s. As an internal control, experiments were duplicated with 28S rRNA, and all data are expressed as the change with respect to the corresponding 28S rRNA calculated threshold cycle (Ct) levels.

Enzyme Activity Assays

SOD activity was assessed by measuring the reduction of cytochrome c via the xanthine oxidase/xanthine methods of Crapo *et al*.[30] and using a superoxide dismutase assay kit (Cayman Chemical). 1 g of tissue was homogenized in 10 mL of cold 20 mM HEPES buffer (pH 7.2, 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose). The sample was centrifuged at 1,500 × g for 5 min at 4˚C and removed the supernant for assay and stored on ice. 200 µL of radical detector (Cayman Chemical), 20 µL of xanthine oxidase (Cayman Chemical), and 10 µL of sample were added per well to flat bottom 96 well microtitre plates. The plate was incubated on a shaker for 20 min at room temperature. The absorbance was readed at 450 nm using a plate reader. Concentrations are expressed as U/mL.

CAT activity was measured using a catalase assay kit (Cayman Chemical) and the spectrophotometric methods outlined by Wheeler *et al*.[15], which are based on the enzymatic reaction of CAT with methanol in the presence of H_2O_2. 1 g of tissue was homogenized in 10 mL of cold buffer (50 mM potassium phosphate, pH 7.0, 1 mM EDTA). The sample was centrifuged at 10,000 × g for 15 min at 4˚C and removed the supernant for assay and store on ice. 100 µL of assay buffer (Cayman Chemical), 30 µL of methanol, and 20 µL of sample were added per well to flat bottom 96 well microtitre plates. The plate was incubated on a shaker for 20 min at room temperature. 30 µL of potassium hydroxide (Cayman Chemical) and 30 µL of Purpald (Cayman Chemical) were added to the each well. The plate was incubated on a shaker for 10 min at room temperature. 10 µL of potassium periodate (Cayman Chemical) was added to the each well. The plate was incubated on a shaker for 5 min at room temperature. The absorbance was readed at 540 nm using a plate reader. Concentrations are expressed as nM/min/mL.

H_2O_2 Assays

H_2O_2 concentrations were measured using the modified methods of Nouroozzadeh *et al*.[31] and a peroxidetect kit (Sigma). 20 µL whole hemolymph in marine anticoagulant (MAC, 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 50 mM EDTA, 0.45 M NaCl, pH 7.5) was added per well to flat bottom 96 well microtitre plates. Plates were incubated at room temperature for 20 min to allow hemocytes to settle and adhere. A working color reagent was prepared by mixing 100 mL distilled water containing 100 mM sorbitol and 125 µM xylenol orange (Sigma) with 1 mL 25 mM ferrous ammonium sulphate prepared in 2.5 M sulphuric acid (Sigma). 200 µL of this reagent was then added to each well and allowed to incubate at room temperature for 1 h. Absorbance was read at 560 nm and concentrations of H_2O_2 were interpolated from a standard curve. Concentrations are expressed as nM/mL.

Lysozyme Activity Assays

Lysozyme activity was quantified in hemolymph according to the methods described by Santarém *et al*.[32]. Briefly, pooled hemolymph was centrifuged at 780 × g for 10 min and the supernatant was collected. Hemolymph was then frozen and stored at −80˚C until use. To quantify lysozyme activity, 10 µL hemolymph was added to 200 µL of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma) in 66 mM phosphate buffer, pH 6.2. Decreases in absorbance (ΔA/min) were then continuously recorded at 450 nm for 5 min at 20˚C in a 96 well plate. The average decrease in absorbance per min was determined for each enzyme solution, and a standard curve of enzyme concentration vs. ΔA/min was constructed. One unit of lysozyme was defined as the amount of enzyme producing activity equivalent to 1 µg lysozyme in the conditions described above. Results are expressed as µg lysozyme/mg protein.

Plasma Parameters Analysis

Plasma GOT and GPT were examined using a biochemistry autoanalyzer (Hitachi).

Statistical Analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One way ANOVA followed by Tukey *post hoc* test was used to compare differences in the data ($P < 0.05$). Values are expressed as mean ± S.D.

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