Bull Environ Contam Toxicol (2017) 98:612–618
DOI 10.1007/s00128-017-2063-0

Anti-oxidative Responses on Hepatic Tissue of Zebrafish (*Danio rerio*) in a Short Duration of Sub-lethal Concentrations of Cadmium Exposure

Su Mei Wu[1] · Li-Hsin Shu[1] · Jia-Hao Liu[1] · Ching Hsein Chen[2]

Received: 29 July 2016 / Accepted: 11 March 2017 / Published online: 18 March 2017
© Springer Science+Business Media New York 2017

Abstract The aim of the present study was to identify whether the responses of oxidative stress in zebrafish liver are similar to those in mammalians upon low doses of Cd^{2+} exposure in short durations. Fish were exposed to 1.78 µM Cd^{2+} (treatment) and 0.0 µM Cd^{2+} (control) for 0, 1, 3, and 6 h. The reactive oxygen species (ROS) and lipid peroxidation (LPO) of hepatic tissues significantly increased after 3 and 6 h of Cd^{2+} exposure, respectively. Antioxidants glutathione peroxidase (*gpx1a*), superoxide dismutase (*sod*), and catalase (*cat*) were up regulated after 1–3 h, and metallothionein isoforms (*smtB and mt2*) increased after 3–6 h of Cd^{2+} exposure. The *caspase-3* and *p53* mRNA expressions significantly increased threefolds after 1 h of Cd^{2+} exposure. Results confirmed that oxidative stress in the hepatic tissue was induced by Cd^{2+} within 3 h. However, anti-oxidative functions immediately up regulated, causing cell apoptosis levels to decrease after 6 h of Cd^{2+} exposure.

Keywords Apoptosis · Cadmium · Metallothionein · Oxidative stress · Zebrafish

Cadmium (Cd) is a non-essential and toxic metal but it contaminates water and sediments in aquatic environments commonly through industrial effluents and wastes, agricultural pesticide runoff, domestic garbage dumps, and mining activities. The Cd accumulation correlated with the duration of exposure and with the ambient Cd concentration in fish. For example, zebrafish larvae were exposed to various concentrations of Cd^{2+} (0.2, 1.0, 5.0, 25, 125 µM) for 3 h. Cadmium contents in larvae appeared dose responses (Matza et al. 2007). The toxicants of Cd^{2+} might have damaged cells, and are generally associated with the formation of reactive oxygen species (ROS) (Company et al. 2004), while the ROS will be degraded by a specific and non-specific system. In a normal functioning cell, the process of generation and degradation of ROS are stable, which is why the steady-state ROS concentration is very low ($<10^{-8}$ M). However, once the balance between ROS generation and degradation is disturbed, it can lead to an increase of ROS called oxidative stress. There are well-characterized mechanisms of cellular tolerance to oxidative stress; both enzymatic and non-enzymatic antioxidants up-regulates to protect itself from the toxicity of the environment (Lushchak 2011). The genes related to enzymatic antioxidants include *sod, cat*, and *gpx*. Like many other vertebrates, fish use both types as antioxidant defense mechanisms to reduce damage from oxidative stress. In addition, we have reported that the *smtB* and *mt2* genes seem to have anti-oxidative stress functions in zebrafish, and both belong to non-enzymes antioxidants (Wu et al. 2015). When the tissues have oxidative stress, it can cause lipid oxidation, DNA damage, and cell apoptosis. Apoptosis related gene expressions such as *p53* and *caspase-3* are both activated after cellular stress or DNA damage (Pyati and Look 2011). Therefore, the present study detects ROS and LPO (lipid oxidation) levels, the protective effects of anti-oxidative enzyme or non-enzyme transcripts, proapoptotic transcription factors (*p53* and *caspase3* mRNA expressions), and apoptosis levels on the hepatic tissue of zebrafish after Cd^{2+} exposure.

✉ Su Mei Wu
sumei@mail.ncyu.edu.tw

[1] Department of Aquatic Biosciences, National Chiayi University, Chiayi 600, Taiwan, Republic of China

[2] Graduate Institute of Biomedical and Biopharmaceutical Sciences, National Chiayi University, Chiayi 600, Taiwan, Republic of China

A cascade of antioxidant responses will be reacted with oxidative stress. The SOD is the enzyme which deals with oxygen toxicity. It can catalyze O_2^- and H^+ into less reactive species, O_2 and H_2O_2 (Ruas et al. 2008). The CAT enzyme can reduce H_2O_2 into water and oxygen to prevent oxidative stress and maintain cell homeostasis. In addition, GPx activity is believed to play an important role in cellular antioxidant defense by using glutathione (GSH) as a reducing agent to reduce H_2O_2 and various hydro-peroxides. Several studies have also examined the role of metallothioneins (MTs), which are non-enzyme antioxidants. From our previous study, it has shown that isoforms of MTs (*smtB* and *mt2*) have a function to defend oxidative stress in the brain, liver, and gills after cold shock (Wu et al. 2015).

The zebrafish (*Danio rerio*) is an excellent model for toxicology, and the liver has been addressed as a target organ for Cd^{2+} intoxication. There is much literature dealing with fish exposure to waterborne Cd^{2+} that shows a variety of accumulation of metal patterns. However, many studies have reported that the liver is the major accumulation organ (Hollis et al. 2001). In addition, the liver also plays an important role in Cd^{2+} metabolism, but it is the most critical organ for detoxification in acute exposures. On the other hand, Cd^{2+} could indirectly generate oxidative stress and free radicals (Cuypers et al. 2010). Thus, much available evidence has suggested that the DNA damage by Cd^{2+} was linked to ROS and inhibition of DNA repair mechanism (Chen et al. 2014). Generally, apoptotic pathways are triggered in zebrafish liver upon Cd exposure. *p53* activation initially triggers the mitochondrial pathway, with the consequent up-regulation of *caspase-9*, and then up-regulation of *caspase-3, -6* and *-7*, to result in DNA damage. Therefore, it is suggested that the *p53* and *caspase-3* mRNA expressions play an important role in the activation and induction of apoptosis. Our previous study also found that oxidative stress was generated by ROS and it induced cell apoptosis on gills and brain tissues of zebrafish after Cd^{2+} exposure (Wu et al. 2016). However, Zhu and Chan (2012) reported that ROS did not seem to be a key factor in Cd^{2+} cytotoxicity in the zebrafish liver cell line. Their data suggested that there is a diversity of effects to impute the use of different cell types, Cd^{2+} doses, and exposure time. Indeed, zebrafish is an excellent model used in various toxicological studies with immense genetic information. However, a few studies have confirmed that the anti-oxidative mechanisms are induced in hepatic tissue upon a low dose of Cd^{2+} exposure within hours. The present study investigates how the antioxidants are induced, and what is the pathological response on hepatic tissue within 6 h in low doses of Cd^{2+} exposure.

Materials and Methods

Sexually mature zebrafish of both sexes were obtained from the Taiwan Zebrafish Core Facility (TZCF, Miaoli, Taiwan). The fish were kept in an aquarium supplied with circulating, dechlorinated, aerated local tap water at 28°C with a 14 h:10 h light and dark photoperiod (Table 1).

According to the preliminary study, and the concentration of 1.78 μM Cd^{2+} is a suitable exposure medium. It was diluted with aerated local tap water from 10 mg/L Cd^{2+} stock solution. Media were analyzed by an atomic absorption spectrophotometer (Z-8000, Hitachi, Japan), using air/acetylene flame for Cd^{2+} analysis. The Cd^{2+} standard solution (Merck, Germany) was used to create a standard curve for the measurements. The addition of certain amounts of the standard solution to the test samples was used for background correction to estimate the matrix effect following the user instructions for the Hitachi spectrophotometer. The detection limit of Cd^{2+} was 10 μg/L. Each experiment used four test tanks, with five male zebrafish in each tank. In experiment 1, four tanks of control groups were used, containing 0 μM Cd^{2+} water, while another four tanks of treatment groups contained 1.78 μM Cd^{2+} water. The fish were immediately anesthetized with 50 mg/L MS222 (Ethyl 3-aminobenzoate methanesulfonate salt; Sigma) after various Cd^{2+} exposure durations, after which the hepatic tissues were excised from the body to determine anti-oxidative factors. In experiment 2, another four test tanks were utilized: one tank contained 0.0 μM Cd^{2+} water (control group), and three tanks contained 1.78 μM Cd^{2+}

Table 1 Primers targeting genes and the endogenous control gene β-actin of zebrafish (*D. rerio*) (Wu et al., 2015)

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
sod	GGC CAA CCG ATA GTG TTA GA	CCA GCG TTG CCA GTT TTT AG
cat	AGG GCA ACT GGG ATC TTA CA	TTT ATG GGA GAC CTT GG
gpx1a	ACC TGT CCG CGA AAC TAT TG	TGA CTG TTG TGC CTC AAA GC
smt-B	TGC TCC AAA TCT GGA TCT TG	GCA GTC CTT CTT GCC CTT AC
mt-2	AGA CTG GAA CTT GCA ACT GTG G′	CAG CTG GAG CCA CAG GAA TT
p53	GGG CAA TCA GCG AGC AAA	ACT GAC CTT CCT GAG TCT CCA
cps-3	CCG CTG CCC ATC ACT A	ATC CTT TCA CGA CCA TCT
β-actin	CGG AAT CCA CCA AAC CAC CTA	ATC TCC TTC TGC ATC CTG TGA

water (treatment group). TUNEL assay and flow cytometry was then used on each fish to detect key parameters of apoptosis, in addition to measuring apoptosis related genes expressions. All experiments were repeated three times. The MasterPure™ RNA Purification Kit (Epicentre, WI, USA) was used for total RNA extraction. Finally, the mRNA pellets were precipitated with 0.1 mg glycogen, 1/10 the volume of 3 mM NaOAc, and 95% ethanol, and stored at −20°C for cDNA synthesis. A first-strand cDNA library for both tissues was then constructed according to the protocol of the SMART™ cDNA Library Construction Kit (Clontech, USA) for RT-PCR analysis. Quantitative real-time PCR (qPCR) was carried out using a SYBR Green dye-based assay (Qiagen, Hilden, Germany) with an ABI Prism 7500 Sequence Detection System (Perkin-Elmer, Applied Biosystems, Wellesley, MA, USA). We followed Choi et al. (2007) in selecting primers targeting *sod, cat*, and *gpx1a*, and in selecting primers targeting *smtB* and *mt2* (Wu et al. 2015). The internal control gene *β-actin* was designed using the Primer Express 2.0 software (Applied Biosystems). All *q*PCR procedures followed our previous study (Wu et al. 2015). The standard curve of each gene was confirmed to be in a linear range with *β-actin* as an internal control. In addition, the *β-actin* Ct levels were also evaluated to ensure that there were not variably expressed housekeeping genes in liver tissue after 0–6 h of Cd^{2+} exposure in the preliminary study. Within *q*PCR reactions, triplicate reactions were performed for each sample, including *β-actin* (internal control) and target genes. To ensure data precision, *q*PCR data were accepted only when the variation Ct values were <1 in triplicate reactions of each gene within the whole set of experiments. Dissociation curves analysis was used to ensure that there was only one product. A negative control without template was transcribed for each primer pair. The hepatic tissues (5 mg) were excised from the body, and individually homogenized in a homogenization buffer either with 500 µL of Extract R-Saturated methanol solution for the LPO assay (Holliwell 1996), or with 200 µL of L-012 (8-Amino-5-chloror-7-phenylpyrido(3,4-d)pyridazine-1,4-(2H,3H)dione sodium salt) (Wako, Japan) for the ROS assay (Imada et al. 1999). Briefly, L-012 was dissolved into ddH_2O to a final concentration at 20 mM, and then diluted with an appropriate buffer [50 mM Tris–HCl (pH 7.5)] to be an assay that has a concentration of 4 µM for O_2^- radical measurements. The homogenates were incubated with 4 µM of L-012 reaction medium for 10 min under a dark space, and then centrifuged at 12,000×*g* for 3 min, after which 100 µL of supernatants was used to measure the relative light units (RLU) with a luminescence instrument (Berthold, Lumat LB9507, Germany). Cell death was detected using TUNEL assays to label DNA strand breaks. The TUNEL assay was performed on 5 µM sections and reacted with an ApopTag® Peroxidase In Situ Apoptosis Detection Kit S7100 (Millipore, USA). The positive control sample was treated with DNase solution (1 U/µL) for 5 min at room temperature, while negative control staining was performed without active TdT but was incubated with proteinase K digestion to control the nonspecific incorporation of nucleotides or the nonspecific binding of enzyme conjugates. In addition, Cd^{2+} induced apoptosis in zebrafish hepatocytes was determined by flow cytometry (FACScan, Becton Dickinson, CA, USA). Results were aanalyzed by two-way ANOVA with tukey's multiple comparison. Data were given as mean ± S.D. Significance difference was set at $p < 0.05$.

Results and Discussions

Data showed that the ROS level has a significant difference after 3 h of Cd^{2+} exposure, and LPO levels were significantly higher than the control after 6 h of Cd^{2+} exposure (Fig. 1). Some enzymatic anti-oxidative factors increased following Cd^{2+} exposure. *sod* mRNA levels significantly increased after 3 h, *gpx1a* and *cat* mRNA levels increased by 9.5- and 4.5-folds at 1 h after exposure to Cd^{2+}, respectively. The *cat* mRNA levels rose again after 6 h of Cd^{2+} exposure (Fig. 2). The non-enzymatic antioxidants such as *smtB* and *mt2* mRNA levels also evidently increased during 3–6 h of Cd^{2+} exposure. We suggested that the Cd^{2+} contents might be higher than the control group upon 3 h of Cd^{2+} exposure. Furthermore, the *mt2* mRNA levels were 26–31-fold, and the *smtB* mRNA levels increased 61-fold at 3 h, but only raised 4.6-folds at 6 h after Cd^{2+} exposure (Fig. 3). The *p53* and *caspase-3* mRNA relative expressed significantly increased after 1 h of Cd^{2+} exposure. Furthermore, the *p53* mRNA expression increased again after 6 h (Fig. 4). Results from both the TUNEL assay and the flow cytometry exhibited a similar pattern. The cell apoptosis levels both were showed the highest apoptosis levels at 3 h after Cd^{2+} exposure, respectively. However, it gradually recovered after 6 h of Cd^{2+} exposure (Figs. 5, 6). The concentration of 1.78 µM Cd^{2+} was used in the present study, which was just only 5% the level of LC_{50} for adult zebrafish. It was a sub-lethal concentration Cd^{2+} exposure. The past study also reported that oxidative stress was induced by sub-lethal concentrations of Cd^{2+} exposure for 6–36 h in goldfish. It focused on the antioxidant functions of GPx and MT (Choi et al. 2007). In addition, Liu et al. (2011) reported that the cellular oxidative stress was induced by Cd^{2+} exposure, and there were some responses that could determine antioxidant capacity, LPO stimulation, and tissue damage on zebrafish. Although the biochemical and toxicological properties of Cd^{2+} have been widely studied on sub-lethal concentration of Cd^{2+} exposure, the effects of low concentration (5% of 96 h/LC50 Cd^{2+}) and

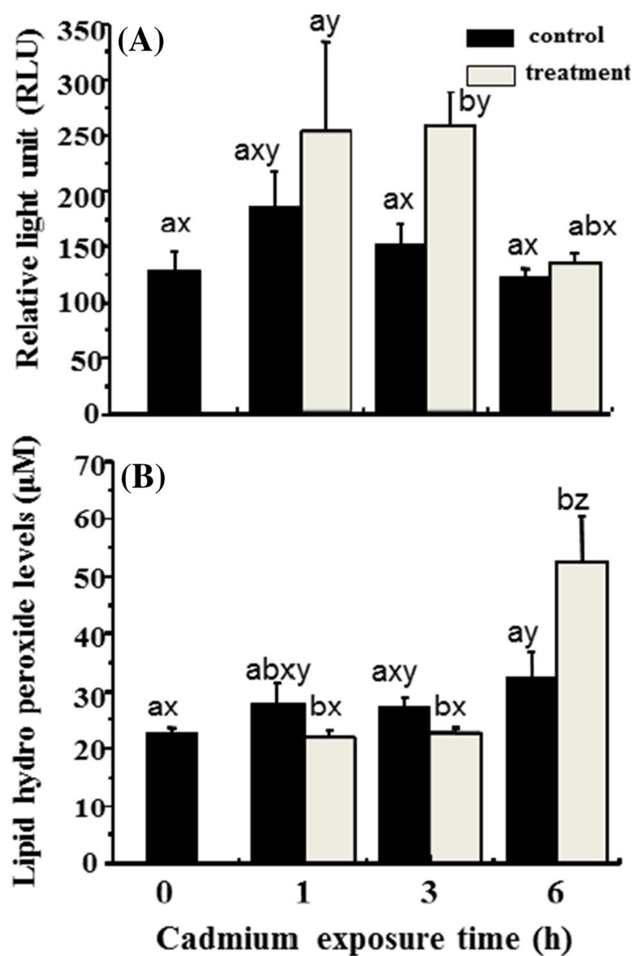

Fig. 1 Comparison of ROS (**a**) and LPO (**b**) levels in hepatic tissue after 0, 1, 3, and 6 h of exposure to 0.0 μM (control) or 1.78 μM Cd^{2+} (treatment). Data are given as mean ± S.D. (n = 5), and data were analyzed by two-way ANOVA with Turkey's comparison. *Different superscript letters* indicate a significant difference ($p < 0.05$) between dosages (*ab*) and among times of treatment (*xyz*)

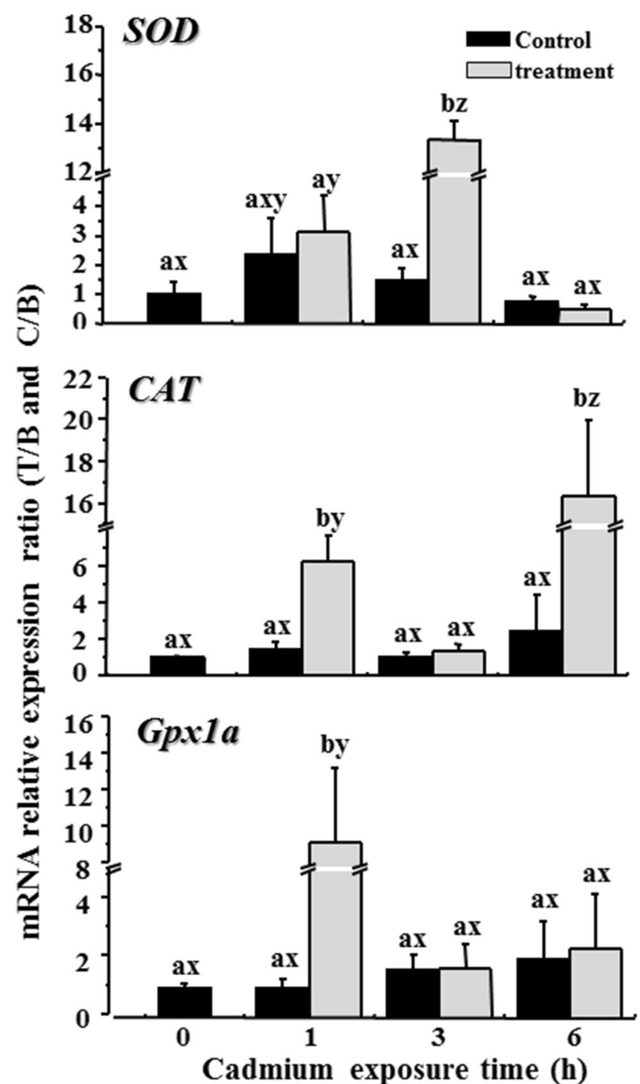

Fig. 2 Comparison of three target gene mRNA relative expressions (*sod, cat, gpx1a*) ratio in hepatic tissues after 0 (B: blank), 1, 3, and 6 h of exposure to 0.0 (C: control) or 1.78 mM Cd^{2+} (T: treatment). Data are given as mean ± S.D. (n = 5), and data were analyzed by two-way ANOVA with Turkey's comparison. *Different superscript letters* indicate a significant difference ($p < 0.05$) between dosages (*ab*) and among times of treatment (*xyz*)

in short durations (within 6 h) of Cd^{2+} exposure on relevant antioxidant parameters of in vivo studies on hepatic tissues were lacking. The present study included two oxidative stress levels (ROS and LPO), responses of enzymatic- and non-enzymatic antioxidants expressions, cell apoptosis, and apoptosis related genes (*caspase-3* and *p53*). The results could identify the primary oxidative responses of fish once the environment is already contaminated with Cd^{2+}.

Antioxidant enzymes such as *sod, cat, gpx1a*, and antioxidant materials with low molecular weight such as *smtB* and *mt2* have been found rich in the brain and liver of zebrafish (Wu et al. 2015). ROS is produced naturally during oxygen metabolism and it includes superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO^-), and singlet oxygen ($^1O_2$). The ROS level, *sod*, and *mt2* mRNA expressions are also found to be higher at 1 h of transfer than the initial point in control group. That may be specifically the "oxygen and ROS metabolism" and responses to oxidative stress since the handling stress increased oxygen metabolism, and caused the ROS level increased. In addition, it shows that the ROS levels significantly increased 3 h after exposure to Cd^{2+}, but it was capable of returning to its initial state after 6 h. According to Lushchak (2011) oxidative stress has three types. The results evidently show the fish under an acute oxidative stress, and it might be induced by sub-lethal concentrations of Cd^{2+} exposure. It occurs when ROS, especially HO^-, attack polyunsaturated fatty acids leading to a chain reaction, and severe injury of plasma membranes cause abnormal functions

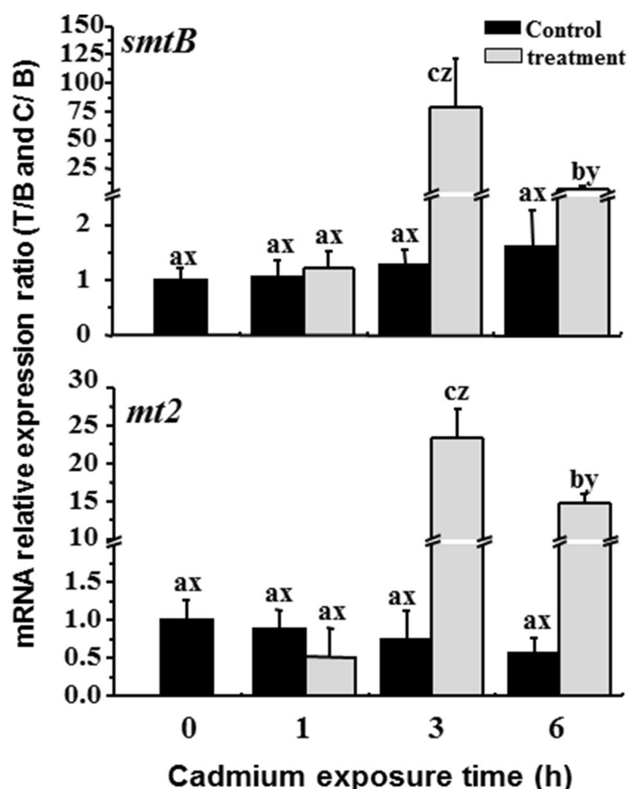

Fig. 3 Comparison of two target gene (*smtB* and *mt2*) mRNA relative expression ratio in hepatic tissues after 0 (B: blank), 1, 3, and 6 h of exposure to 0.0 µM (control) or 1.78 µM Cd^{2+} (treatment), with 0 being the control. Data are given as mean ± S.D. (n = 5), and data were analyzed by two-way ANOVA with Turkey's comparison. *Different superscript letters* indicate a significant difference ($p < 0.05$) between dosages (*abc*) and among times of treatment (*xyz*)

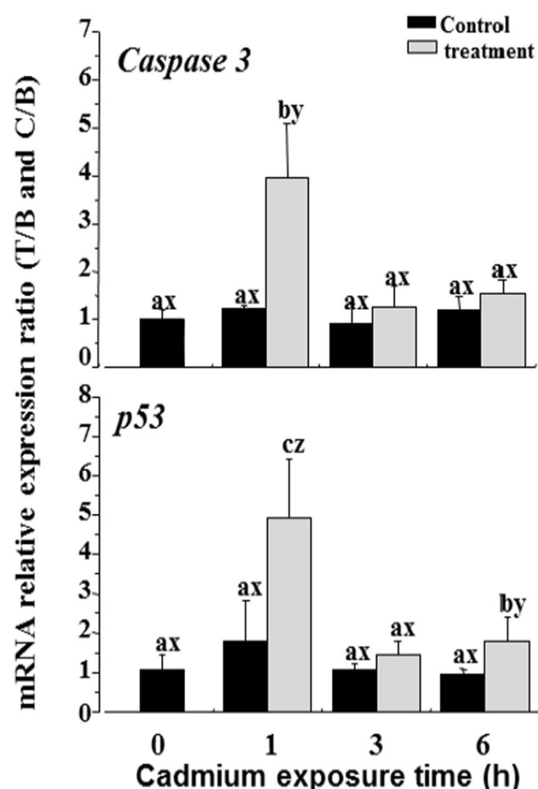

Fig. 4 Comparison of the mRNA relative expression ratio of *caspase 3* and *p53* in hepatic tissues at 0 (B: blank), 1, 3, and 6 h after 0.0 µM (control) or 1.78 µM Cd^{2+} (treatment) exposure. Data are given as mean ± S.D. (n = 3), and data were analyzed by two-way ANOVA with Turkey's comparison. *Different superscript letters* indicate a significant difference ($p < 0.05$) between dosages (*abc*) and among times of treatment (*xyz*)

and loss of membrane integrity. LPO, malonedialdehyde and 4-hydroxy-2-Nonenal, are all indicators of oxidative stress levels. The LPO levels significantly increased following ROS at 6 h of Cd^{2+} exposure (Fig. 1). In addition, we found that the LPO level significantly decreased at 3 h after Cd^{2+} exposure, which also had a similar response profile in gills (Wu et al. 2016). Generally, there is a cascade of antioxidant responses: SOD can catalyze $O_2^{\cdot}$ and H^+ into less reactive species, O_2 and H_2O_2 (Ruas et al. 2008); CAT can reduce H_2O_2 into water and oxygen to prevent oxidative stress; and GPx is playing a role in detoxification and removing hydrogen peroxide from cells by GSH oxidation. The present results indicated that the *cat* and *gpx1a* mRNA expressions had higher activity levels than in the control at 1 h after Cd^{2+} exposure, but the *sod* mRNA levels significantly increased at 3 h after Cd^{2+} exposure. The results equitably explain that the fish might have demonstrated a compensative physiological function since the fish increased oxygen metabolism upon Cd^{2+} exposure. In addition, the *cat* mRNA expression significantly increased following the increase of the *sod* mRNA expression 3 h after exposure to Cd^{2+}, which might have increased in order to reduce H_2O_2 into water and oxygen (Fig. 2). Our previous studies have found that *mt2* mRNA can be significantly induced upon Cd^{2+} exposure in embryo, juvenile, and adult zebrafish. However, *smtB* mRNA was only evidently raised in adult zebrafish after Cd^{2+} exposure. In addition, black goby, goldfish, shark, and rainbow trout, all treated with Cd^{2+}, had higher levels of *MT* mRNA than the control (Choi et al. 2007). It is evident that exposure to Cd^{2+} significantly increases MT expressions in various species and tissues with some differences depending on dosage and exposure time. The past studies compared their tissue-specific gene expressions, and found that the *mt1* and *mt2* were different tissue specific expressions after various doses of Cd^{2+} exposure. Thus, the present study was to inspect *mt2* and *smtB* expressions. Results showed that the *smtB* mRNA levels significantly increased over 61-fold than the control after 3 h of Cd^{2+} exposure. However, it just appeared 4.6-fold higher than the control after 6 h of Cd^{2+} exposure. The *mt2* mRNA expression was 31.4- and 26.1-fold higher than the control after 3 and 6 h of Cd^{2+} exposure, respectively

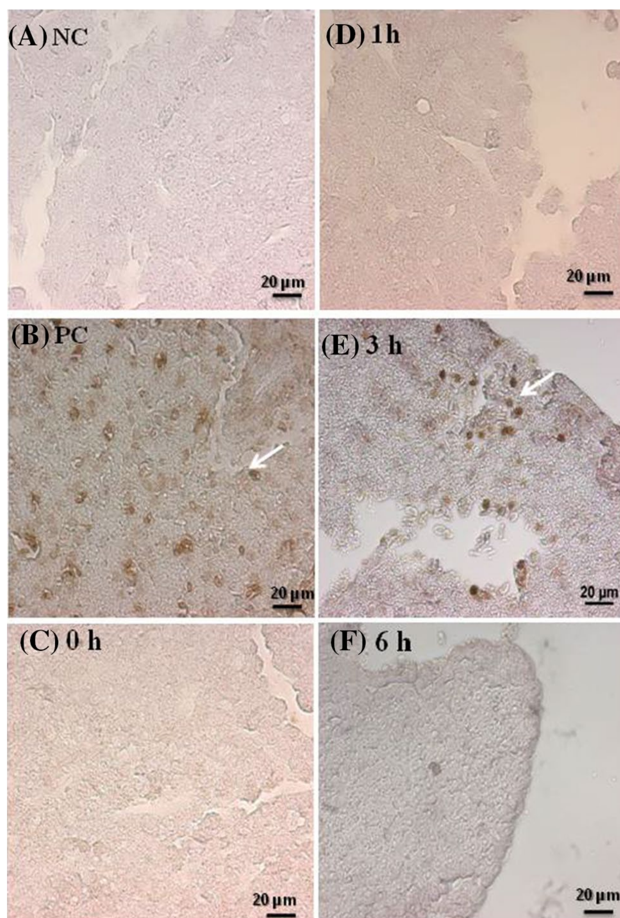

Fig. 5 Cell apoptosis (*arrow*) of hepatic tissue in zebrafish is shown in **a** NC: negative control, **b** PC: positive control; and after **c** 0, **d** 1, **e** 3, and **f** 6 h of Cd^{2+} exposure. Magnification ×50

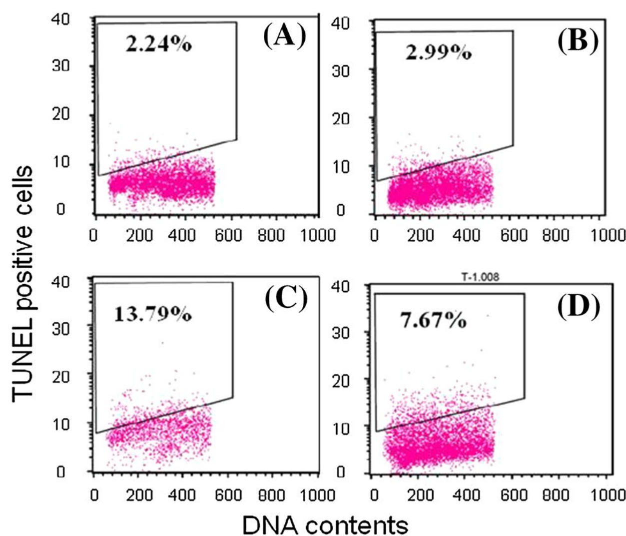

Fig. 6 The values in each *panel* indicate the percentages of apoptotic cells. The hepatic cells of zebrafish were determined with flow cytometer counter at **a** 0, **b** 1, **c** 3 and **d** 6 h after Cd^{2+} exposure

(Fig. 3). From these results, Cd^{2+} toxicity, at least in part, induces ROS formation, activation of *p53* and *caspase-3*, and causes cell apoptosis. In fact, the early increases in the *p53* mRNA expression observed in the present study is suggestive of its involvement in apoptosis activation, since TUNEL-positive cells became more evident soon after 2–3 h of Cd^{2+} exposure (Figs. 5, 6). They appear to have a time and dose dependent response, which *p53* and *caspase-3* mRNA levels increased 2.8- and 3.2-fold at 1 h of Cd^{2+} exposure, respectively. The apoptosis percentage was 2.99% at 1 h, but it was 13.79% and 7.67% at 3 and 6 h, respectively. Conclusion, antioxidants including *sod, cat, gpx1a, mt2* and *smtB* interacted to defend against oxidative stress within several hours. Therefore, even though there was an up-regulation of *p53* and *capase-3* levels after 1 h of Cd^{2+} exposure, they recovered to its normal state afterwards. Zebrafish liver seems had a powerful anti-oxidative system, and it was created promptly after Cd^{2+} exposure, which resulted in reduced levels of cell apoptosis.

Acknowledgements This work was supported by grants (NSC101-2311-B-415-001-MY3) from the National Science Council, and in part supported by National Chiayi University, Taiwan.

References

Chen yy, Zhu JY, Chan KM (2014) Effects of cadmium on cell proliferation, apoptosis, and proto-oncogene expression in zebrafish liver cells. Aqua Toxicol 157:196–206

Choi CY, An KW, Nelson ER, Habibi HR (2007) Cadmium affects the expression of metallothionein (MT) and glutathione peroxidase (GPx) mRNA in goldfish, *Carassius auratus*. Comp Biochem Physiol 145C:595–600

Company R, Serafim A, Bebianno MJ, Cosson R, Shillito B, Fiala-Medioni A (2004) Effect of cadmium, copper and mercury on antioxidant enzyme activities and lipid peroxidation in the gills of the gills of the hydrothermal vent mussel *Bathymodiolus azoricus*. Mar Environ Res 58:377–381

Cuypers A, Plusquin M, Remans T, Jozefczak M, Keunen E, Gielen H (2010) Cadmium stress: an oxidative chellenge. Biometals 23:927–940

Hollis L, Hogstrand C, Wood CM (2001) Tissue-specific cadmium accumulation, metallothionein induction, and tissue zinc and copper levels during chronic sublethal cadmium exposure in juvenile rainbow trout. Arch Environ Contam Toxicol 41:468–474

Holliwell B (1996) Antioxidants: the basics-what they are and how to evaluate them. Adv Pharm 38:3–20

Imada I, Sato EF, Miyamoto M, Ichimori Y, Minamiyama Y, Konaka R, Inoue M (1999) Analysis of reactive oxygen species generated by neutrophils using a chemiluminescence probe L-012. Anal Biochem 271: 53–58

Liu XJ, Luo Z, Li CH, Xiong BX, Zhao YH, Li XD (2011) Antioxidant responses hepatic intermediary metabolism, histology and ultrastructure in *Synechogobius hasta* exposed to waterborne cadmium. Ecotoxicol Environ Saf 74:1156–1163

Lushchak VI (2011) Adaptive response to oxidative stress: bacteria, fungi, plants and animals. Comp Biochem Physiol 153C:175–190

Matza CJ, Trebleb RG, Kronea PH (2007) Accumulation and elimination of cadmium in larval stage zebrafish following acute exposure. Ecotoxicol Environ Saf 66:44–48

Pyati UJ, Look T (2011) Analysis of cell death in zebrafish. In: Reed JC, Green DR (eds) Apoptosis: physiology and pathology. Cambridge University Press, New York, pp 412–421

Ruas CBG, Carvalho CDS, de Araujo HSS, Espindola ELG, Pernandes MN (2008) Oxidative stress biomarkers of exposure in the blood of cichlid species from a metal-contaminated river. Ecotoxicol Environ Saf 71:86–93

Wu SM, Liu JH, Shu LH, Chen CH (2015) Anti-oxidative responses of zebrafish (*Danio rerio)* gill, liver and brain tissues upon acute cold shock. Comp Biochem Physiol 187A:202–213

Wu SM, Shu LH, Liu JH (2016) Anti-oxidative functions of *mt2* and *smtB* mRNA expression in the gills and brain of zebrafish (*Danio rerio*) upon cadmium exposure. Fish Physiol Biochem. doi:10.1007/s10695-016-0251-1

Zhu JY, Chan KM (2012) Mechanism of cadmium-induced cytotoxicity on the ZFL zebrafish liver cell line. Metallomics 4:1064–1076