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Effects of copper exposure on the hatching status and antioxidant defense at different developmental stages of embryos and larvae of goldfish *Carassius auratus*

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HIGHLIGHTS

- We developed a response model for fish embryos and larvae to copper exposure.
- Copper affects fish embryos & larvae in concentration- and time-dependent patterns.
- SOD and CAT activities were enhanced after 120 h in goldfish embryos.
- Serious toxicity to fish embryo manifests beyond tolerant copper threshold value.
- Copper concentration to kill pathogen should be less than 0.4 mg L^{-1}.

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ABSTRACT

This study aims to assess the effects of copper exposure on hatching status and antioxidant defense at different stages of embryos and larvae of goldfish *Carassius auratus*. In this study, day-old embryos were randomly grouped after fertilization and then exposed to copper concentrations of 0, 0.1, 0.4, 0.7, and 1.0 mg L^{-1}. Copper-exposed fish embryos were sampled every 24 h to determine superoxide dismutase (SOD), and catalase (CAT) activities, as well as malondialdehyde (MDA) content. In addition, cumulative mortality and larval deformity were also investigated. The findings showed that cumulative mortality and larval deformity rate increased gradually with copper concentration increase. SOD and CAT activities were inhibited at higher copper concentrations. At a lower concentration (0.1 mg L^{-1}), SOD activity increased in larvae, whereas CAT activity showed no significant change ($p > 0.05$). MDA, as the lipid peroxidation product, gradually accumulated in embryos and larvae with increasing copper concentration and the extension of exposure time. At 0.4 mg L^{-1} and more, copper toxicity was shown in embryos and larvae. In conclusion, copper-exposed effects on hatching status and antioxidant defense in *C. auratus* embryos and larvae showed concentration- and time-dependent patterns. The biochemical parameters in this study can be used as effective indicators for evaluating the responses of copper-exposed fish embryos. In addition, this study demonstrates that 0.4 mg L^{-1} copper (corresponding to 1 mg L^{-1} copper sulfate), used to kill parasites in aquaculture, is not safe concentration, because it can result in toxicity to larvae. Therefore, the copper concentration to kill pathogen should be less than 0.4 mg L^{-1} for *C. auratus*.

1. Introduction

Copper, as an important micronutrient of organism, is necessary to meet physiological demands, such as serving as cofactor for many different enzymes (Burke and Handy, 2005). However, at higher concentrations, copper may become extremely toxic (De Boeck et al., 2004). The copper levels in lake and river range from 0.2 μg L^{-1} to 30 μg L^{-1}, but in contaminated water, the concentrations rise to above 100 μg L^{-1} (Craig et al., 2007). The increasing copper concentrations in water mainly result from effluents from industries, such as mining, and agricultural practices, such as using copper pesticide (Liu et al., 2010). In aquaculture, copper sulfate is widely used as a therapeutic agent to control algae and some pathogens in water (Chen et al., 2006; Liu et al., 2010), the concentration of which is generally from 0.7 mg L^{-1} to 1 mg L^{-1}. Currently, increasing copper levels are often observed in aquaculture, which can result in toxicity to fish by interfering with physiological function (Vutukuru et al., 2006). The effect of copper sulfate on fish has been studied exhaustively, and susceptibility to copper varies depending on fish species (Croke and McDonald, 2002). Copper toxicity also varies

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significantly among freshwater fish species (De Boeck et al., 2004; Eyckmans et al., 2011). For example, the copper lethal concentration of 50% for 96 h (LC50 96 h) is 0.2 mg L^{-1} for rainbow trout (*Oncorhynchus mykiss*), 0.65 mg L^{-1} for common carp (*Cyprinus carpio*), and 1.5 mg L^{-1} for gibel carp (*Carassius auratus gibelio*) (Eyckmans et al., 2011). Other studies also suggested that the genus *Carassius* shows a relatively higher tolerance to copper compared to the other freshwater fish (Schjolden et al., 2007). Therefore, copper toxicity to fish shows difference with the variation of fish species, life stages, and environmental factors.

Copper is widely distributed in various waters and exists in different chemical forms. Among them, ionic copper has been suggested as one of the most toxic forms to freshwater fish (Meador, 1991). The impact of copper in the aquatic environment is complex and depends on physicochemical characteristics of the water (Mazon and Fernandes, 1999; Tao et al., 1999; Takasusuki et al., 2004). Alkalinity, hardness, temperature, and pH strongly influence copper speciation and copper toxicity in water (Erickson et al., 1996; Tao et al., 2001, 2000). Elevated waterborne copper levels could affect embryonic development (Johnson et al., 2007; Jezierska et al., 2009), juvenile growth (Liu et al., 2010), ionic regulation (McGeer et al., 2000), endocrine modification (Teles et al., 2005), metabolic enzyme activity (Monteiro et al., 2005; Liu et al., 2010), and antioxidant system (Sampaio et al., 2008; Shao et al., 2012). Especially, ascertaining the effect on copper-exposed fish embryo is very important for understanding copper toxicity to embryonic development and improving hatching rate. Although some primary studies on copper effect on fish embryonic survival have been carried out (McKim et al., 1978; Rice et al., 1980; Shazili and Pascoe, 1986; Scudder et al., 1988), controversial results have been obtained. Rice et al. (1980) reported that the life stage most sensitive to copper for the northern anchovy (*Engraulis mordax*) is the embryonic stage, standing prior to blastopore closure. However, some studies suggested that the embryo shows a blunt response to copper exposure by preventing copper ions from penetrating the egg membrane at a specific copper concentration (McKim et al., 1978; Shazili and Pascoe, 1986; Scudder et al., 1988; von Westernhagen, 1988). Thus, the larval stage may be more sensitive to copper toxicity compared to the embryos, presumably due to the protection of the hardened egg chorion in water (von Westernhagen, 1988; Weis and Weis, 1991). The ability of fish embryos and larvae to accumulate heavy metals is well-documented (Finn, 2007). It has suggested that heavy metal exposure before the egg chorion hardens shows greater toxicity to embryo health. Therefore, the further studying copper exposure effect on fish embryo using the multiple biochemical parameters is still needed for understanding copper toxicity to fish embryo.

The high reactivity of copper in tissue can propel the redox reactions in physiological metabolism to form reactive oxygen species (ROS), a process known as the Fenton reaction (Craig et al., 2007). A certain level of ROS is necessary for organism to perform physiological function, but excess ROS will induce oxidative stress and interfere with cellular function by lipid peroxidation, DNA damage, and protein carbonylation. Therefore, maintaining the balance between producing and scavenging ROS is essential in organism. Superoxide dismutase (SOD, EC1.15.1.1) and catalase (CAT, EC1.11.1.6) are protective ROS-scavenging enzymes that play important roles in maintaining the dynamic balance of ROS. SOD can convert superoxide anions into hydrogen peroxide, which can be further decomposed into nontoxic oxygen and water by CAT eventually (Atli and Canli, 2007). Once the ability of antioxidant enzymes for removing ROS is weakened, the surplus ROS will accumulate in the cell, and incur irreversible oxidative stress. Malondialdehyde (MDA) is the final product of lipid peroxidation (LPO) from oxidative stress, which is often used to assess the lipid peroxidation status of cell membrane systems (Alexandrova and Bochev, 2005). In the current study, the influence of waterborne copper on the antioxidant defense was studied to elucidate copper toxicity to developmental embryos and larvae.

The goldfish (*Carassius auratus*) is a very popular ornamental fish species in China. In recent years, the cultivation of *C. auratus* has developed rapidly because of its marketability. However, increasing copper concentrations in water and the widespread application of copper sulfate in controlling aquatic pathogens have increasingly caused embryo abortion in *C. auratus*, as well as larvae abnormality and mortality (Wang, 2011). Therefore, ascertaining the effects of copper exposure on biochemical parameters and embryonic development in the early life stage of *C. auratus* are essential for understanding copper toxicity to fish embryo. In the current study, fertilized eggs of *C. auratus* exposed to different copper concentrations for various time lengths, were used to analyze biochemical parameters (SOD, CAT, and MDA). Moreover, embryo–larval cumulative mortality and larval deformity rate were evaluated at different copper concentrations. The objectives of this study are (1) to assess the influence of copper exposure on the embryo and larvae of *C. auratus*; (2) to investigate the validity of biochemical indices as biomarkers to indicate copper toxicity to fish embryo and larvae; and (3) to evaluate the potential risk of copper exposure to larvae health based on the integrated responses of biochemical parameters. In addition, this research attempts to provide some valuable data and guidelines for effectively killing parasites, attenuating copper stress on fish embryo and larvae, and then improving larval quality in aquaculture.

2. Materials and methods

2.1. Copper exposure and sampling of fish embryos and larvae

Fertilized eggs of *C. auratus* were obtained by artificial insemination. They were subsequently collected and randomly grouped with approximately 1500 fertilized eggs per group. The eggs were incubated in 2 L polyethylene tanks with approximately 1 L water at a light regimen of 12 h light/12 h dark. Water temperature was controlled at 13 ± 1.5 °C. The day-old fertilized eggs, presenting at the stage of gastrulation, were exposed to sublethal copper ion concentrations of 0.1, 0.4, 0.7, and 1.0 mg L^{-1}. The assigned copper concentrations were prepared by diluting copper sulfate stock solution (Copper sulfate pentahydrate, Sigma–Aldrich, US). The nominal concentrations in the experiment were designated based on the copper ion LC_{50} concentration of 1.6 mg L^{-1} for three-day-old embryos during the pre-experimental procedure. All experiments were conducted in triplicate. Three-fifths of the incubating solution in the tank was exchanged every 24 h using the same concentration copper solution. In the experiment, the nominal copper concentrations of 0, 0.1, 0.4, 0.7, and 1.0 mg L^{-1} are corresponding to the average copper concentrations of 0, 0.1 ± 0.01, 0.4 ± 0.02, 0.7 ± 0.05, and 1.0 ± 0.08 mg L^{-1} measured using an atomic absorption spectrophotometer (Varian SpectrAA 220 FS, USA) at time points of 72, 144, and 216 h. The embryos exposed to copper were sampled at 24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 h (at 168 h, almost all embryos hatched into larvae). During embryonic development, the dead eggs were removed promptly from the tanks and counted. The work described in this study was carried out in accordance with the requirements for animal experiments.

2.2. Observations on cumulative mortality and deformity rate

Observations on the mortality of embryo–larvae and larval deformity were performed under an optical anatomical microscope. The criterion for embryo mortality is the opacity of the em-

bryo, and for larval mortality is the failure to respond to a prod with a polished glass rod (Rice et al., 1980). The dead embryos or larvae were promptly removed and recorded. Cumulative mortality during embryo and larvae development was determined at different time points. Larval deformity was also observed under an optical anatomical microscope. The criterion for larval deformity is the appearance of vertebral deformity (scoliosis) or tail curvature. The larval deformity rate was counted at time point of 192 h copper exposure (approximately 24 h after embryos hatched into larvae).

2.3. Crude extract preparation

Approximately 100 embryos were sampled respectively from different groups, placed on a filter paper to absorb any surplus moisture, and then stored in Eppendorf tubes at −80 °C until biochemical assays. In preparing the crude extract, embryos from each tank were randomly divided into three parts, and homogenized respectively at a ratio of 1:4 (1 g embryos:4 mL 0.9% sodium chloride solution). Homogenization was carried out using 15–20 strokes of a hand-driven Teflon Potter. All operations were performed on ice. The homogenate was centrifuged at 11 130 g at 4 °C for 10 min. The supernatant was divided into aliquots and then stored at −20 °C until these were used for measuring SOD, and CAT activities, as well as MDA content. All measurements were conducted within 1 week.

2.4. Determination of biochemical parameters

SOD activity was measured based on the method described by Orbea et al. (2002), in which the degree of inhibition of the reduction of cytochrome *c* by superoxide radicals that are generated by the xanthine–xanthine oxidase system at 550 nm was determined. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 50 μM hypoxanthine, 1.87 mU mL^{-1} xanthine oxidase, and 10 μM cytochrome *c*. One unit of SOD activity is defined as the inhibition of 50% of cytochrome c reduction in 1 g protein (U g^{-1} Pr). Catalase activity was determined using the molybdate colorimetric method (Goth, 1991;Cheng and Meng, 1994) using 1 μmol H_2O_2 decomposed in 1 g protein per minute in a reaction solution defined as a unit of CAT activity (U g^{-1} Pr). MDA content was determined based on the method described by Okhawa et al. (1979). The principle of this method is based on the chemical reaction between MDA and 2-thiobarbituric acid, which yields a red product with an absorption peak at 532 nm.

The protein concentration of the crude extract of *C. auratus* embryos or larvae was determined using the method described by Bradford (1976), whereby bovine serum albumin (BSA, AMRESCO) was the standard.

2.5. Statistical analysis

The data are presented as mean + standard deviation (M + SD).Statistical analyses were conducted using software SPSS19.0. Results were initially tested for normality by Shapiro–Wilk test and equality of variance by Levene test. If necessary, data were log-transformed to fulfill normality and equality of variance criteria. Afterwards, significant differences between the different groups were tested with one way ANOVA, followed by post-hoc Tukey test. If data were not sufficient to perform parametric analysis, non-parametric Kruskall–Wallis ANOVA and its post-hoc test were used. The level of statistical significance was set at $p < 0.05$.The making of figure was implemented in the Microsoft Excel 2010.

3. Results

3.1. Cumulative mortality of C. auratus embryo and larvae and larval deformity under copper exposure

Cumulative mortality was counted at different developmental periods of embryos and larvae of *C. auratus* exposed to copper, as indicated in Fig. 1. Compared to the control, the embryos and larvae showed no significant difference in cumulative mortality at 0.1 mg L^{-1} of copper concentration at the various time points, but presented a significant increase at 0.4 mg L^{-1} after 168 h, as well as at 0.7 and 1.0 mg L^{-1} after 72 h. The larval deformity rate was assessed at 192 h, which increased gradually with increasing copper concentrations (Fig. 2) and was significantly higher at 0.4, 0.7, and 1.0 mg L^{-1} of copper concentrations compared to the control.

3.2. SOD activity changes in C. auratus embryo and larvae under copper exposure

SOD activity changes in *C. auratus* embryo and larvae with prolonged exposure time at different copper concentrations showed different response patterns (Fig. 3). Compared to that in the control, SOD activity was obviously induced at 0.1 mg L^{-1} at 192, 216, and 240 h ($p < 0.05$). However, it was significantly inhibited at 0.4 mg L^{-1} at 216 and 240 h; at 0.7 mg L^{-1} at 192, 216, and 240 h; and at 1.0 mg L^{-1} after 48 h. Only for the control, SOD activity also varied with the developmental process, which decreased before 120 h, compared to that at 24 h (the first sampling time), and reached a trough with a significance at 120 h ($p < 0.05$). Subsequently, SOD activity gradually increased with exposure extension, and showed a significant increase at 240 h. At 0.1 mg L^{-1} of copper concentration, SOD activities indicated the similar change pattern with those in the control, but were significantly higher at 192, 216, and 240 h than at 24 h ($p < 0.05$). With respect to 0.4 mg L^{-1} exposure, it significantly decreased at 120 and 240 h; for 0.7 mg L^{-1} exposure, at 96, 120, 144, 216, and 240 h; for 1.0 mg L^{-1} exposure, at 72, 96, 120, 144, 192, 216, and 240 h. SOD activity, compared to that at 120 h, significantly increased in the control after 192 h ($p < 0.05$) and at 0.1 mg L^{-1} copper after 168 h ($p < 0.05$). At 0.4 mg L^{-1}, no significant change was observed. At 0.7 and 1.0 mg L^{-1}, SOD activity was only significantly higher at 168 h. SOD activity, compared to that at 168 h, significantly increased in the control at 240 h and at 0.1 mg L^{-1} copper at 216 and 240 h ($p < 0.05$), while significantly decreased at 0.7 and 1.0 mg L^{-1} at 240 h ($p < 0.05$).

3.3. CAT activity changes in C. auratus embryo–larvae under copper exposure

CAT activity changes during *C. auratus* embryo and larvae with prolonged exposure at different copper concentrations showed similar change patterns (Fig. 4). Compared to the control at the same time point, CAT activity significantly decreased at 0.4 mg L^{-1} of copper concentration at 192, 216 and 240 h; at 0.7 mg L^{-1} after 48 h; and at 1.0 mg L^{-1} at all stages ($p < 0.05$). Compared to that of the 24 h exposure at the same copper concentration, CAT activity gradually decreased before 120 h, and reached a minimum value at 120 h ($p < 0.05$), then increased at 168 h. After 168 h, CAT activity continuously increased in the control, whereas it gradually went down at 0.4, 0.7, and 1.0 mg L^{-1} of copper concentrations. Of the various time points after 120 h, CAT activity significantly increased only at 240 h in the control compared to that at 120 h ($p < 0.05$), but no remarkable change was observed in exposure groups ($p > 0.05$). With respect to the various time points after

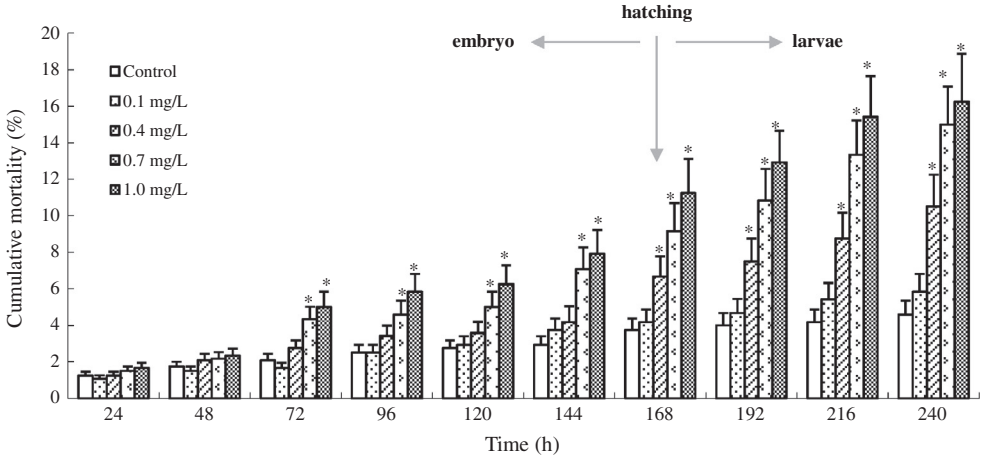


Fig. 1. Cumulative mortality of the embryo–larvae of *C. auratus* at different time points of copper exposure. Values are presented as mean + standard deviation (M + SD; $n = 3$). * Significant difference compared to the control ($p < 0.05$).

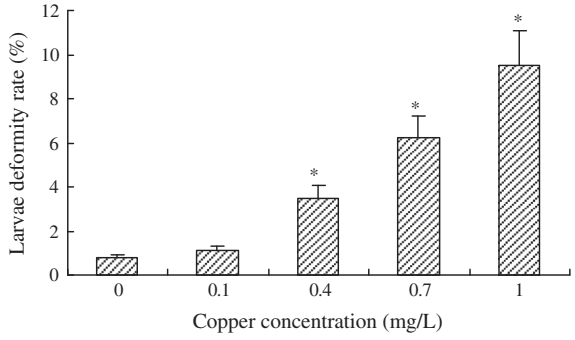
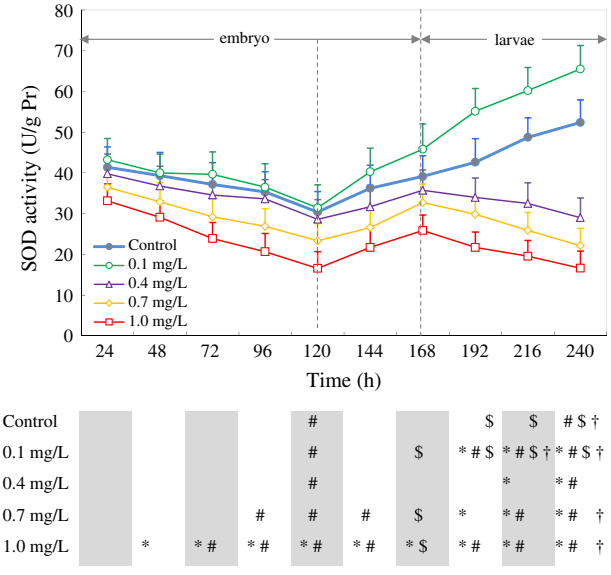
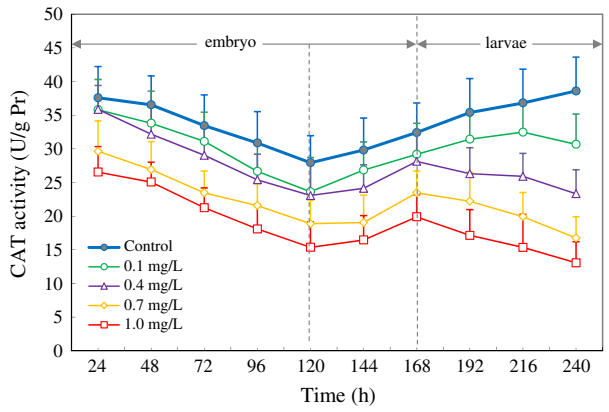


Fig. 2. Deformity rate of *C. auratus* larvae hatching from embryos exposed to copper. Values are presented as M + SD ($n = 3$). *Significant difference compared to the control ($p < 0.05$).



	24	48	72	96	120	144	168	192	216	240
Control					#			$	$	# $ †
0.1 mg/L					#		$	* # $	* # $ †	* # $ †
0.4 mg/L					#				*	* #
0.7 mg/L			#		#	#	$	*	* #	* # †
1.0 mg/L		*	* #	* #	* #	* #	* $	* #	* #	* # †

Fig. 3. Changes in SOD activity in the development of *C. auratus* embryo–larvae exposed to copper. Values are presented as M + SD ($n = 3$) in U g^{-1} Pr. * Significant difference compared to the control at the same time point ($p < 0.05$); # significant difference compared to the 24 h exposure at the same copper concentration ($p < 0.05$); $ significant change after 120 h compared to the 120 h exposure at the same concentration ($p < 0.05$); † significant change after 168 h compared to the 168 h exposure ($p < 0.05$).



	24	48	72	96	120	144	168	192	216	240
Control							#			$
0.1 mg/L					#	#	#			
0.4 mg/L					#	#	#	* #	* #	* #
0.7 mg/L		*	*	* #	* #	* #	*	*	* #	* # †
1.0 mg/L	*	*	*	* #	* #	* #	*	* #	* #	* # †

Fig. 4. Changes in CAT activity in the development of *C. auratus* embryo–larvae exposed to copper. Values are presented as M + SD ($n = 3$) in U g^{-1} Pr. * Significant difference compared to the control at the same time point ($p < 0.05$); # significant difference compared to the 24 h exposure at the same copper concentration ($p < 0.05$); $ significant change after 120 h compared to the 120 h exposure at the same concentration ($p < 0.05$); † significant change after 168 h compared to the 168 h exposure ($p < 0.05$).

168 h, CAT activity significantly decreased only at 240 h at 0.7 and 1.0 mg L^{-1} compared to that at 168 h ($p < 0.05$).

3.4. MDA content changes in C. auratus embryo and larvae under copper exposure

MDA content changes in *C. auratus* embryo and larvae with prolonged exposure at various copper concentrations showed varying change patterns (Fig. 5). For the control and 0.1 mg L^{-1} exposure groups, MDA initially increased and then decreased. For the 0.4, 0.7, and 1.0 mg L^{-1} exposure groups, MDA gradually accumulated with increasing exposure time. Compared to that in the control at the same time point, MDA accumulated significantly at 0.4 mg L^{-1} after 192 h, at 0.7 mg L^{-1} after 96 h, and at 1.0 mg L^{-1} after 48 h. Compared to that at 24 h, MDA accumulated significantly in the control at 120, 144, 168, and 192 h, at 0.1 mg L^{-1} cop-

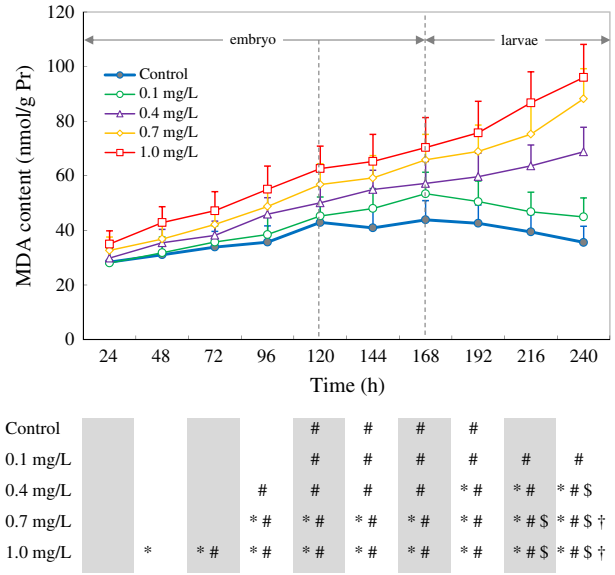


Fig. 5. Changes in MDA content in the development of *C. auratus* embryo–larvae exposed to copper. Values are presented as M + SD ($n = 3$) in nmol g^{-1} Pr. * Significant difference compared to the control at the same time point ($p < 0.05$); # significant difference compared to the 24 h exposure at the same copper concentration ($p < 0.05$); $ significant change after 120 h compared to the 120 h exposure at the same concentration ($p < 0.05$); † significant change after 168 h compared to the 168 h exposure ($p < 0.05$).

per after 120 h, at 0.4 and 0.7 mg L^{-1} after 96 h, and at 1.0 mg L^{-1} after 48 h ($p < 0.05$). Compared to that at 120 h at the same copper concentration, MDA content significantly increased at 0.4 mg L^{-1} at 240 h; and at both 0.7 and 1.0 mg L^{-1} at 216 and 240 h ($p < 0.05$). Compared to that at 168 h at the same copper concentration, MDA significantly increased at 0.7 and 1.0 mg L^{-1} only at 240 h ($p < 0.05$).

4. Discussion

4.1. Mortality and abnormality of embryo and larvae of C. auratus under copper exposure

In recent years, fish embryos have been popularly considered as a biological indicator for assessing the potential risk of heavy metal accumulation on fish (Burton, 1991; Cao et al., 2009; Barjhoux et al., 2012). The embryos and larvae of fish have been used to evaluate effectively the effect of contamination on hatchability, development, and growth, which are considered critical endpoints for monitoring (Burton, 1991). In this study, no significant change in the cumulative mortality of embryos and larvae was observed at 0.1 mg L^{-1} in relation to the control. It was suggested that 0.1 mg L^{-1} Cu is too low to result in mortality for fish embryos and larvae. The cumulative mortality significantly increased at 0.4 mg L^{-1} after 168 h of exposure (at the moment, embryos had hatched into larvae), while for 0.7 and 1.0 mg L^{-1}, obviously went up after 72 h of exposure (at the moment, still at the embryonic stage). The larval deformity rate was significantly higher at 0.4, 0.7, and 1.0 mg L^{-1} compared to the control. Copper concentrations of 0.4 mg L^{-1} and above could be toxic to the embryo–larvae. At 0.4 mg L^{-1} of copper concentration, the cumulative mortality of embryo–larvae significantly increased after 168 h. At this time point, fish embryos had hatched into larvae, and a higher mortality appeared. Therefore, at 0.4 mg L^{-1} of copper concentration, goldfish larvae could not cope with copper intoxication, and showed the sensitivity to copper exposure. The previous studies, examined copper sensitivity at various life stages of freshwater fish, have revealed that the larval stage is the most sensitive to copper (McKim et al., 1978; Shazili and Pascoe, 1986; Scudder et al., 1988; von Westernhagen, 1988), and suggested the hypothesis that the hardened egg membrane could hamper the penetration of copper ions into the embryos. In this study, it probably supports the hypothesis mentioned above in some degree. However, Rice et al. (1980) found that the embryonic stage of the northern anchovy, *E. mordax*, was more sensitive to copper compared to the larval stage. Therefore, further studies on copper toxicity to fish embryo–larvae need to be carried out to understand the mechanisms of copper action.

4.2. Changes of antioxidant enzymes and lipid peroxidation under copper exposure

Organisms have an antioxidant defense system comprising antioxidant enzymes and endogenous antioxidants that counter oxidative stress due to surplus ROS (Winston and Di-Giulio, 1991). Copper exposure could alter antioxidant enzyme activity and result in oxidative stress and lipid peroxidation in various organs and in the enterocytes of fish (Jiang et al., 2011). SOD serves as one of the most important scavengers of free radical. It initially responds to oxygen radicals against oxidative stress. Jiang et al. (2011) suggested that SOD activity is depressed upon copper exposure. Decreased SOD activity may indicate toxicity associated with copper-induced ROS stress. In the present study, SOD activity was significantly induced at 192 h and longer (namely, after yolk sac period of larval development) at 0.1 mg L^{-1} of copper concentration, whereas no significant difference was observed before 192 h. SOD activity in embryo is apparently insensitive to copper exposure at low concentrations. In the larvae, however, SOD activity was induced to increase ROS removal. This result is in agreement with a report that demonstrated increased SOD activity in fish liver during copper exposure (Sampaio et al., 2008). It also supported the suggestion that 0.1 mg L^{-1} Cu could not result in mortality for fish embryos and larvae. At more than 0.4 mg L^{-1} of copper exposure, SOD activity was inhibited in concentration- and time-dependent patterns. At 0.4 and 0.7 mg L^{-1} of copper exposures, the embryos still showed a higher tolerance. It is postulated that the hardened egg membrane of the embryo blocks the penetration of copper ions, thus reducing copper toxicity, but this suggestion needs to be supported by the further studies.

With regard to the control, SOD activity decreased gradually with developmental time before 120 h, and attained the lowest value with the significant decrease at 120 h. Then, activity gradually increased, and got to a significant increase at 240 h. It has been demonstrated that SOD activity changes dynamically during embryonic development, and copper stress apparently increases the complexity of SOD regulation in embryos.

David et al. (2008) reported that CAT is an essential defense factor against the potential toxicity of hydroxyl radicals. In the current study, CAT activity significantly decreased at 0.4 mg L^{-1} at 192 h and longer (larval stage). The responses of CAT to copper exposure in the larval stage were more sensitive than those during the embryonic stage. It is consistent with the result that cumulative mortality was higher in larvae than in embryos at 0.4 mg L^{-1}. At 0.7 and 1.0 mg L^{-1}, CAT activity was obviously inhibited. This finding is in agreement with the previous study result that CAT activity was inhibited in the liver and gill of juvenile *Pagrosomus major* at copper exposures of 0.5, 0.7, and 1.5 mg L^{-1}. In addition, the inhibition of CAT activity is concentration-dependent (Dai et al., 1998). Liu et al. (2006) found that CAT activity was inhibited at copper ion concentrations of higher than 0.01 mg L^{-1} in the hepatopancreas of goldfish. Vutukuru et al. (2006) reported that CAT activity continuously decreases within 96 h in juvenile *Esomus danricus* at 0.14 and

1.4 mg L^{-1} of copper ion concentrations. According to a recent study (Liu et al., 2010), CAT activity is also inhibited at 0.15 and 0.3 mg L^{-1} of copper concentrations at 15 d exposure in the liver of *Synechogobius hasta*. These results are consistent with the observations of the present study that CAT activity can be inhibited under copper exposure. Decreased CAT activity presumably results from the direct damage of copper on enzyme structure or the reduced synthesis of CAT (Liu et al., 2006). Shen et al. (2007) found that peroxisome membrane is damaged under copper exposure, the permeability of the membrane increases and eventually results in the decreased CAT activity.

In the present study, in contrast with the 24 h exposure, CAT activity decreased with increasing developmental time before 120 h at all copper concentrations. The minimum CAT activity was observed at 120 h, after which, it increased until 168 h. Beyond 168 h, CAT activity continuously increased in the control, whereas it gradually decreased at 0.4, 0.7, and 1.0 mg L^{-1} of copper concentrations. It has been addressed that CAT activity is suppressed at higher copper concentrations during the larval stage.

The decreases in SOD and CAT activities indicate a reduced ability to remove ROS. When organisms cannot effectively scavenge superoxide anions via SOD and H_2O_2 via CAT, surplus ROS accumulate in the cell and then induce oxidative stress, eventually causing lipid peroxidation and MDA accumulation. In this study, MDA accumulated according to concentration- and time-dependent patterns. For the control, with the developmental time, MDA initially increased and then decreased; for the 0.1 mg L^{-1} copper exposure, it also showed the similar change pattern with that in the control, and no significant difference was observed between the two groups at the same developmental time. Thus, no significant effect of copper exposure on embryo–larvae was exhibited at lower copper concentrations. It strongly supports the suggestion that 0.1 mg L^{-1} copper exposure shows no significant effect on cumulative mortality of embryo–larvae. However, at 0.4, 0.7, and 1.0 mg L^{-1}, MDA gradually accumulated with prolonged exposure. This finding is in agreement with the previous result showing increased MDA content in the muscle of *Labeo rohita* under copper sulfate exposure (Jena et al., 2009). At 0.4 mg L^{-1} of copper exposure, MDA accumulated significantly during the larval stage (after 192 h), in contrast with the control, whereas no remarkable difference was observed during the embryonic stage, indicating that egg membrane has a certain protection. However, beyond a copper concentration threshold, the protective ability of the egg membrane decreased. Therefore, severe copper toxicity to embryos was exhibited at higher copper concentrations (MDA accumulated significantly at 0.7 mg L^{-1} after 96 h and at 1.0 mg L^{-1} after 48 h). Previous studies proposed that the accumulated ROS under copper exposure could result in oxidative stress, damage CAT structure, and inhibit CAT activity (Sampaio et al., 2008). Therefore, the inhibition of CAT activity in this study might result from damage from the increased ROS. Jiang et al. (2011) reported that the SOD activity of *C. carpio* enterocytes exposed to copper is depressed in a concentration-dependent pattern. When SOD and CAT activities are inhibited, oxidative stress and lipid peroxidation occur in fish, as described in this study. This finding is in accordance with that of a previous study demonstrating that waterborne copper exposure of $\geqslant$0.6 mg L^{-1} induces oxidative stress and results in lipid peroxidation in Jian carp (*C. carpio*) (Jiang et al., 2011). In the present study, in the control, MDA content showed no significant change after 120 h. It was demonstrated that antioxidant enzyme activity gradually increased, after a specific stage of embryonic development, to enhance ROS removal with the improvement of antioxidant defense ability. The same result was also observed at the lower copper concentration (0.1 mg L^{-1}).

5. Conclusions

In this study, the effects of copper exposure on hatching status and antioxidant enzyme activities, as well as lipid peroxidation in *C. auratus* embryos, proceed in concentration- and time-dependent patterns. The cumulative mortality of embryo–larvae gradually increased with increasing copper concentration and prolonging exposure time. The larval deformity rate also increased gradually with increasing copper concentration. At 0.1 mg L^{-1} of copper concentration, no significant effect was observed in cumulative mortality and larval deformity. With regard to antioxidant enzyme activities at 0.1 mg L^{-1}, no significant change in CAT activity was shown, whereas SOD activity was significantly induced in larvae. Therefore, a certain antioxidant enzyme can be stimulated to increase the ability to remove surplus ROS at low copper concentrations (0.1 mg L^{-1}). This suggestion is consistent with the observations of cumulative mortality and larval deformity. Once beyond the threshold value of tolerant copper concentrations, the adjustable ability decreases, resulting in oxidative stress and MDA accumulation. Therefore, at higher copper concentrations and for longer exposure time, SOD and CAT activities are significantly inhibited, and MDA significantly accumulates, indicating that oxidative stress is incurred. In this study, a copper concentration of 0.4 mg L^{-1}, showed the slight effects on the embryos, whereas severely affected the larvae. Therefore, it was indicated that 0.4 mg L^{-1} of copper concentration (corresponding to 1 mg L^{-1} copper sulfate, used to kill pathogen in aquaculture) is not safe for *C. auratus* larvae, although little impact was shown on the embryo. In addition, the biological parameters investigated in this study can be used as the effective biomarkers to assess the effects of copper exposure and potential health risk to *C. auratus* embryo–larvae.

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