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Effects of Single and Joint Subacute Exposure of Copper and Cadmium on Heat Shock Proteins in Common Carp (*Cyprinus carpio*)

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Abstract Copper (Cu) and cadmium (Cd) are the most common heavy metals that are easily detected in aquatic environments on a global scale. In this paper, we investigated the messenger RNA (mRNA) and protein levels of HSPs (HSP60, HSP70, and HSP90) in the liver of the common carp exposed to Cu, Cd, and a combination of both metals by real-time quantitative PCR and Western blot. The results indicated that in each exposure group, the mRNA levels of HSP60, HSP70, and HSP90 were increased significantly compared to the corresponding controls after 96 h of exposure ($P < 0.05$). A significant increase was observed in the HSP70 protein level in the high-dose Cu group and all of the Cd groups. Significant increases were also observed in the protein levels of HSP60 and HSP90 in the high combination group and the low combination group, respectively. These results indicated that the dynamics of HSP expression observed in the common carp support the role of HSPs as biochemical markers in response to environmental pollution and provided valuable insights into the adaptive mechanisms used by the common carp to adapt to the challenges of stressful environments.

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Introduction

Heavy metals emissions, which pose serious threats to humans and other organisms, have been noted as a problem for several decades already. Cu and Cd, two representative heavy metals, have toxic effects on a variety of life forms, particularly on aquatic organisms. Various chemical forms of Cu and Cd make their way into water bodies from different sources, such as agricultural runoff and industrial sewage discharges (Bodin et al. 2013; Venugopal et al. 2009). Cu is a widely utilized metal in electrical components, the automobile industry, and in domestic appliances, and more frequently, it has been used as an efficient antimicrobial surface (Elguindi et al. 2011). Cd is one of the most toxic environmental and industrial pollutants (Templeton and Liu 2010). Many studies on Cd toxicity have shown that Cd preferentially localizes in hepatocytes and causes various adverse effects, mainly the accumulation, histopathological and cellular changes, the enhancement of lipid peroxidation, modulation of mitochondrial function, and DNA chain break (Toman et al. 2005). Therefore, the toxicity of these two heavy metals to aquatic species has been studied by a number of authors. For instance, Zhang et al. confirmed that the heavy metals Cd and Cu are toxic to *Exopalaemon carinicauda*, a commercially important species in China (Zhang et al. 2014). Even at low

concentrations, Cu can produce toxic effect to zebra fish (Paris-Palacios et al. 2000). The pollution of water and diet are responsible for the exposure of fish to Cu and Cd, and these toxic metals can be detected in a variety of tissues (Pierron et al. 2011; Reynders et al. 2008). The toxicity of heavy metals is increasingly threatening the survival of humans and other organisms, particularly aquatic animals.

Heat shock proteins (HSPs) are typically used as suitable biomarkers of exposure to various challenges, such as increased temperature, tissue damage, toxicant exposure, hunger, and virus infection (Eder et al. 2009; Farcy et al. 2007; Madach et al. 2008; Rhee et al. 2011; Zugel and Kaufmann 1999). Under adverse stress conditions, diverse types of HSPs are produced. HSPs have a pivotal role in protein synthesis by preventing non-native protein aggregation, facilitating folding of newly synthesized proteins, stabilizing and refolding damaged proteins, and targeting non-native or aggregated proteins to specific degradative pathways (Manchado et al. 2008; Ranford et al. 2000). Among the heat shock protein family, HSP70 has been suggested to be the most highly conserved and largest member (Gupta and Singh 1994) and is expressed in response to the various stressors, such as pesticides (Xing et al. 2013). A change in HSP70 expression could serve as a biological marker for judging cell physiological function and the ability to cope with stress (Gutsmann-Conrad et al. 1999). HSP90, another common cytosolic chaperone, participates in orchestrating the folding of various proteins yet, is insufficient to accomplish the refolding of denatured proteins. Accordingly, other chaperones, for instance HSP70, are essential to achieve this mission (Csermely et al. 1998; Vogel et al. 2006). In contrast, HSP60 plays an important role in the protein-folding system and has the abilities to respond to the stress responses that can disturb cellular homeostasis (Cechetto et al. 2000; Seveso et al. 2014). Much of the research in recent years has examined the role of HSP60 as an important component in the development of inflammation and immunity in response to bacterial and viral infections in shrimp (Huang et al. 2011). The ability of organisms to secrete HSPs in response to environmental challenges has been widely recognized (Feder and Hofmann 1999; Parsell and Lindquist 1993). In conclusion, HSPs have received increasing attention because of their roles as biochemical markers and have become appropriate subjects of the study of transcriptional regulation, stress response, and molecular evolution (Lindquist 1986; Morimoto 1998; Srivastava 2002).

The common carp (*Cyprinus carpio* L.), a species of *Cyprinidae*, is an economically important freshwater fish in aquaculture and is widely used as an experimental animal for the aquatic risk assessment of threatening contaminants (Wang et al. 2007). In addition, common carp are convenient to obtain and easy to feed. Toxicant evaluation in common carp could increase the understanding of the reaction and regulation mechanisms in response to heavy metals. As is widely known, the liver is one of the major organs targeted by heavy metals toxicity, and most toxicology research has focused on this organ (Bartosiewicz et al. 2001; Vetillard and Bailhache 2005). To further investigate the potential toxic effects of copper and cadmium and to evaluate the potential of HSPs as molecular biomarkers in environmental monitoring, we measured the expression levels of three representative members of the HSP family (HSP60, HSP70, and HSP90) in the liver of the common carp by real-time quantitative PCR and Western blot analysis.

Materials and Methods

Fish

Common carp with an average weight of 45.3 g were purchased from Harbin National Aquafarm, Heilongjiang Province, China. Animals were maintained in the 220 L laboratory tanks with continuous aeration. The carp were acclimatized to the experimental environment for 2 weeks. Water temperature was maintained at 20 ± 1 °C, the dissolved oxygen was 7 ± 0.20 mg L^{-1}, the pH was 7.3 ± 0.3, and the water hardness was 15.50 mmol L^{-1}. The photoperiod was 12 h light and 12 h dark. Commercial food was given once a day until satiation. The water physicochemical conditions and the animal breeding conditions did not change until the toxicity test was complete. Experiments were performed according to the European Communities Council Directive (86/609/EEC) and were approved by a local ethics committee.

Experimental Design

The experimental animals were divided into the following seven groups as follows: two Cu^{2+} treatment groups (0.05 and 0.1 mg L^{-1}), two Cd^{2+} treatment groups (0.63 and 1.26 mg L^{-1}), two combination treatment groups (0.045 and 0.09 mg L^{-1}), and one water control group. Each treatment group contained 30 fish and two replicates. The combination of both metals was composed of a 1:1 mass ratio of Cu^{2+} and Cd^{2+}. CuSO$_4$·5H$_2$O (AR) and CdCl$_2$·2.5H$_2$O (AR) were purchased from Sigma-Aldrich Chemical Co. (USA). The concentrations used in the present study are approximately one fourth and one half of the 96-h LC$_{50}$. The 96-h LC$_{50}$ of Cu^{2+} and Cd^{2+} for common carp has been reported to be 0.20 mg L^{-1} Cu and 2.52 mg L^{-1} Cd (Kuz'mina 2011; Mukherjee et al. 1992): We verified these concentrations. The 96-h LC$_{50}$ of the combination was obtained from a preliminary experiment (unpublished data).

Five fish from each group were randomly collected at 24, 48, 72, and 96 h post-exposure to Cu^{2+}, Cd^{2+}, and their combination. The fish were euthanized with sodium pentobarbital. The livers of common carp were quickly collected,

immediately frozen in liquid nitrogen, and stored at −80 °C until RNA isolation.

Primer Design

To design primers, we used the fish HSP60, HSP70, HSP90, and β-actin mRNA GenBank sequences with an accession numbers of BC068415, AY035309.1, AF068773, and AF057040. β-actin, a house-keeping gene, was used as an internal reference. Primers (Table 1) were designed using the Oligo 6.0 Software (Molecular Biology Insights, Cascade, CO) and synthesized by Invitrogen Biotechnology Co. Ltd. in Shanghai, China.

Gene Expression Analysis

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Shanghai, China). The dried RNA pellets were resuspended in 50 μL of diethylpyrocarbonate-treated water. The concentration and purity of the total RNA were determined spectrophotometrically at 260/280 nm. First-strand complementary DNA (cDNA) was synthesized from 5 μg of total RNA using Oligo-dT primers and SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen, Shanghai, China). Synthesized cDNA was diluted five times with sterile water and stored at −80 °C.

The reverse transcription reaction (40 μL) consisted of the following components: 10 μg of total RNA, 1 μL of Moloney murine leukemia virus reverse transcriptase, 1 μL of RNAase inhibitor, 4 μL of deoxynucleoside triphosphate, 2 μL of Oligo-dT, 4 μL of dithiothreitol, and 8 μL of 5× reverse transcriptase buffer. The reverse transcription was performed according to the manufacturer's instructions (Invitrogen).

Real-Time Quantitative Reverse Transcription PCR

Real-time quantitative reverse transcription PCR was used to detect the expression of the HSP60, HSP70, HSP90, and β-actin gene in the liver using SYBR Premix ExTaq (Takara, Shiga, Japan) and real-time PCR. Reaction mixtures were incubated in the ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The program was 1 cycle at 95 °C for 30 s, 40 cycles at 95 °C for 5 s, and 40 cycles at 60 °C for 34 s. Dissociation curves were analyzed by Dissociation Curve 1.0 Software (Applied Biosystems) for each PCR reaction to detect and eliminate possible primer-dimer and nonspecific amplification. The mRNA relative abundance was calculated according to the method of Pfaffl (Pfaffl 2001).

Western Blot Analysis

One hundred milligrams of liver tissue was homogenized in 800 μL of ice-cold grind buffer (20 mmol L^{-1} Tris–HCl, pH 7.4, 2 mmol L^{-1} EDTA, 2 mmol L^{-1} EGTA, 1 mmol L^{-1} PMSF, 30 mmol L^{-1} NaF, 30 mmol L^{-1} sodium pyrophosphate, 0.1 % SDS, 1 % Triton X-100, and protease inhibitor cocktail). The sample was then centrifuged for 10 min at 10, 000 g at 4 °C, and supernatant was collected. Protein content was measured according to Bradford's procedure (Bradford 1976). Equal amounts of total protein (40 μg/condition) were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions on 15 % gels. Separated proteins were then transferred to nitrocellulose membranes using a tank transfer for 2 h at 100 mA in Tris-glycine buffer containing 20 % methanol. Membranes were blocked with 5 % skim milk for 24 h and incubated overnight with diluted primary antibody against HSP60, HSP70, and HSP90 (1:1000, production of polyclonal antibody by the College of Veterinary Medicine, Northeast Agricultural University) followed by a horseradish peroxidase (HRP) conjugated secondary antibody against rabbit IgG (1:1000, Santa Cruz Biotechnology, USA). To verify equal loading of samples, the membrane was incubated with monoclonal β-actin antibody (1:1000, Santa Cruz Biotechnology, USA), followed by a HRP-conjugated goat anti-mouse IgG (1:1000). The signal was detected by an X-ray film (TransGen Biotech Co., Beijing, China). The optical density (OD) of each band was determined by the Image VCD

Table 1 Gene-specific primers used in the real-time quantitative reverse transcription PCR

Gene	Accession number	Primer sequence
HSP60	BC068415	Forward 5′-CGCTCAGGTGGCTACTATTTC-3′
		Reverse 5′-ACACCCTTACGACCGACTTTC-3′
HSP70	AY035309.1	Forward 5′-TGAGAACATCAACGAGCCCA-3′
		Reverse 5′-TTGTCAAAGTCCTCCCCACC-3′
HSP90	AF068773	Forward 5′- CTTGAGGAAGGCGAGAAAC −3′
		Reverse 5′-ATCCTCCCAGTCATTGCTT-3′
β-actin	AF057040	Forward 5′-GATGGACTCTGGTGATGGTGTGAC-3′
		Reverse 5′-TTTCTCTTTCGGCTGTGGTGGTG-3′

gel imaging system, and the relative abundance of HSP60, HSP70, and HSP90 protein levels were expressed as the ratios of the OD of these proteins to that of β-actin.

Statistical Analyses

The K-S test was used to verify normal distribution in the data. The data that met the normal distribution and showed no significant difference (>5 % significance level) was used for further analysis. Statistical analyses on all data was performed using the GraphPad Prism 5.0 computer software. Differences between the means were assessed using Tukey's honestly significant difference test for post hoc multiple comparisons. All data were expressed as the mean ± SD, where $P < 0.05$ was considered significantly different.

Result

The Expression of HSP60 in Liver

The effects of Cu^{2+}, Cd^{2+}, and their combination on HSP60 mRNA and protein levels in common carp are shown in Figs. 1a, 2a and 3a. After exposure to Cu^{2+} and Cd^{2+} alone and combination, no significant differences were found in HSP60 mRNA levels in each exposure group compared with the control group. However, as time progressed, HSP60 mRNA levels increased significantly ($P < 0.05$), except for the Cu low-dose group (CuL) and the combination low-dose group (cL), and reached their peak values at 96 h. In contrast, the upregulation of the HSP60 protein level was only observed in two groups that were exposed to a combination of Cu^{2+} and Cd^{2+} (Fig. 2a). HSP60 protein levels were not significantly increased at 96 h in the other groups.

The Expression of HSP70 in Liver

The effects of Cu^{2+}, Cd^{2+}, and the Cu^{2+}/Cd^{2+} combination on HSP70 mRNA and protein levels in common carp were investigated (Figs. 1b, 2b and 3b). The HSP70 mRNA transcript level increased significantly ($P < 0.05$) compared to the control groups at the end of the exposures. The HSP70 protein level concurrently decreased in each exposure group after the 24 h exposure ($P < 0.05$) and increased significantly in the high-dose Cu group and all of the Cd groups after 96 h of exposure. HSP70 values peaked in all of the treatment groups at 96 h (Fig. 2b).

The Expression of HSP90 in Liver

The effects of Cu^{2+}, Cd^{2+}, and the Cu^{2+}/Cd^{2+} combination on HSP90 mRNA and protein levels in common carp were investigated (Figs. 1c, 2c and 3c). The
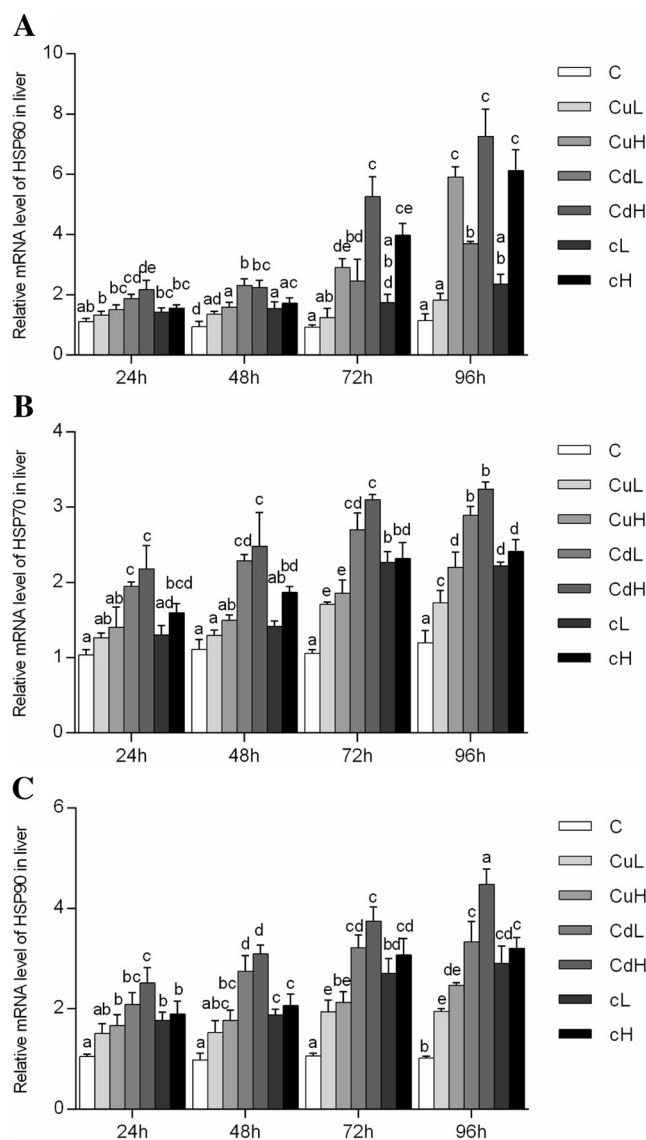

Fig. 1 Effects of Cu^{2+}, Cd^{2+}, and their combination on the mRNA levels of HSP60, HSP70, and HSP90 in the liver of the common carp. **a** Effects of Cu^{2+}, Cd^{2+}, and their combination on the mRNA level of HSP60 in the liver. **b** Effects of Cu^{2+}, Cd^{2+}, and their combination on the mRNA level of HSP70 in the liver. **c** Effects of Cu^{2+}, Cd^{2+}, and their combination on the mRNA level of HSP90 in the liver. The mean value of the control group was set to 1. Each value represents the mean ± SD of five individuals. *Different letters within each series* indicates significant difference in the relative expression of the target genes ($P < 0.05$). (*C* control group, *CuL* Cu low-dose group, *CuH* Cu high-dose group, *CdL* Cd low-dose group, *CdH* Cd high-dose group, *cL* combination low-dose group, *cH* combination high-dose group)

HSP90 mRNA transcript level increased significantly ($P < 0.05$) compared to the control group at the end of the exposure and reached peak values in each group at 96 h. The HSP90 protein level only significantly increased in the high-dose combination group at the end of exposure ($P < 0.05$), and no significant difference was observed in the other groups compared with the control group (Figs. 2c).
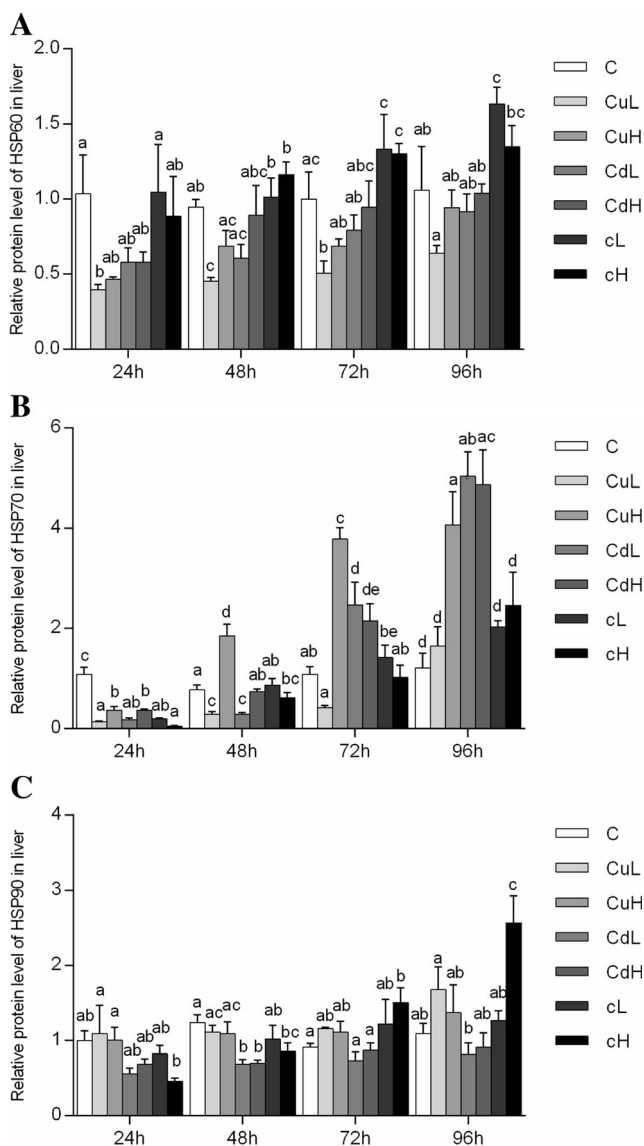
Fig. 2 Effects of Cu²⁺, Cd²⁺ and their combination on the protein levels of HSP60, HSP70, and HSP90 in the liver of the common carp. **a** Effects of Cu²⁺, Cd²⁺, and their combination on the relative protein level of HSP60 in the liver. **b** Effects of Cu²⁺, Cd²⁺, and their combination on the relative protein level of HSP70 in the liver. **c** Effects of Cu²⁺, Cd²⁺, and their combination on the relative protein level of HSP90 in the liver. The mean value of the control group was set to 1. Each value represents the mean ± SD of five individuals. *Different letters within each series* indicates significant differences in the relative expression of the target genes ($P < 0.05$). (*C* control group, *CuL* Cu low-dose group, *CuH* Cu high-dose group, *CdL* Cd low-dose group, *CdH* Cd high-dose group, *cL* combination low-dose group, *cH* combination high-dose group)

Fig. 3 The western blot results of **a** HSP60, **b** HSP70, and **c** HSP90 in the liver of common carp exposed to Cu²⁺, Cd²⁺, and their combination (*C* control group, *L* 4^{-1} 96-h LC$_{50}$ concentration group, *H* 2^{-1} 96-h LC$_{50}$ concentration group)

Discussion

In general, nearly all organisms living in the aquatic ecosystem, particularly fish, are inevitably faced with the threat of various environmental stressors that can induce biochemical, physiological, and histological alterations. Heavy metals, particularly Cu and Cd, have been considered one of the major pollutants in aquatic environments. Prior t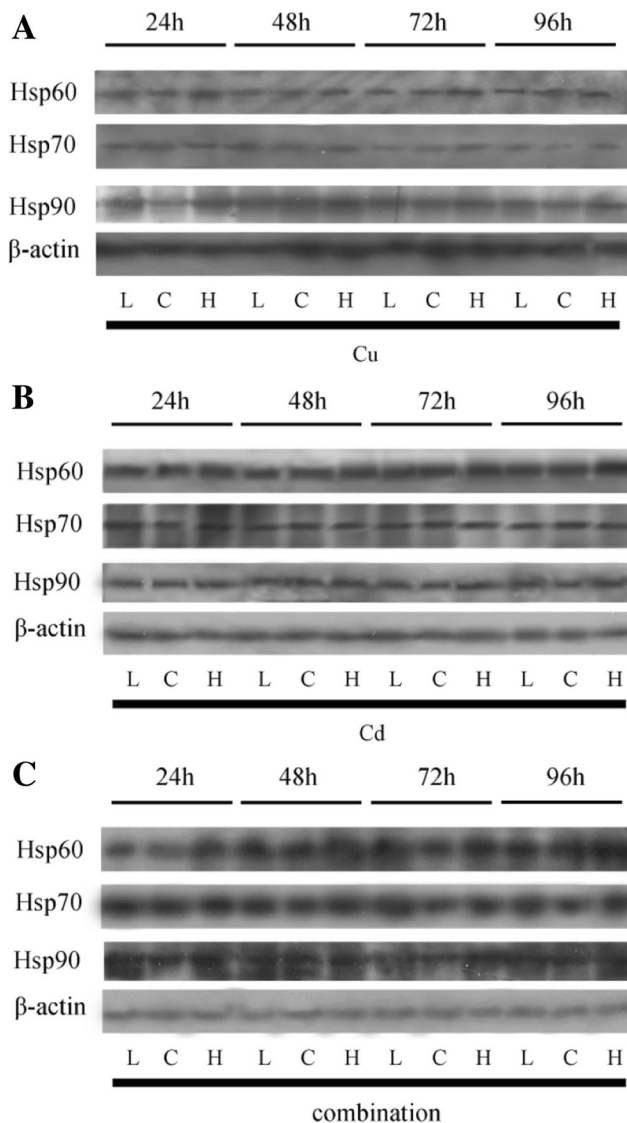o this study, the toxic effects of Cd and Cu in fish had been studied in rainbow trout *Oncorhynchus mykiss*, cyprinidae fish *Tanichthys albonubes*, and gilthead seabream *Sparus aurata* (Jing et al. 2013; Schwartz et al. 2004; Souid et al. 2013), and these authors advocated the necessity to use different fish models for testing metal toxicity. The common carp, one of the Chinese Four Family Carps, possesses various advantages as a research model, for example, they are tolerant and convenient to maintain. In addition, the common carp is regarded as one of the major breeding fish in paddy field fish culture. Unfortunately, according to many research results, the contamination of Cd and Cu in rice was serious, particularly in China (Cao and Hu 2000; Fu et al. 2008; Hang et al. 2009; Sun et al. 2007). These circumstances aroused our attention about the risk assessment of Cu and Cd exposure in common carp. To the best of our knowledge, this is

the first study to assess the effects of Cu, Cd, and their combination on common carp via real-time quantitative PCR and Western blot analysis, which have been suggested as sophisticated methods only appropriate for laboratory experiments (Quiros et al. 2007). In this study, we found that Cd, Cu, and their combination induce the overexpression of HSP60, HSP70 and HSP90 in the liver of common carp.

Over the past several years, multiple HSP families have been identified and shown to be responsive to many forms of stresses in the common carp (Gao et al. 2007; Mukhopadhyay et al. 2003). However, scant data exists on the molecular information of HSPs in common carp and their expression responses against environmental stressors, particularly their protein levels. In the current study, we compared and clarified the effects of Cu and Cd alone and in combination on the expression of HSPs in mRNA expression levels and protein levels. No significant difference was observed among all control groups at each time point in the expression of all genes as determined by real-time PCR and Western blot. Therefore, we used a single water control group as our reference group for all other exposure groups. β-actin was used as the internal control gene. There was no mortality observed in control groups or in exposure groups. The results reveal that HSP60, HSP70, and HSP90 mRNA transcript levels are generally increased compared to their control counterparts after a 96-h exposure ($P < 0.05$). The changes indicate that the exposure of Cu and/or Cd leads to stress on the common carp.

One of the known reaction mechanisms the organisms developed in response to the stressors was the induction of HSPs. Prior studies have shown that the expression of HSPs was affected in a wide variety of aquatic organism in response to heavy metals stressors including Cu and Cd. Qian et al. reported that cadmium exposure increased the expression of four HSP genes (HSP60, HSP70, HSC70, and HSP90) of the Pacific white shrimp (Qian et al. 2012). Feng et al. reported that $CuSO_4$ treatment (0, 25, 50, 100, and 200 μM) resulted in a dose-dependent elevation in HSP70 expression at 24 and 48 h post-exposure in rainbow trout hepatocytes (Feng et al. 2003). The mechanisms by which Cd produces toxic effects involves interference with the homeostasis of essential metals at different levels, as the long half-life of Cd allows it to interfere with many metal-dependent proteins (Nzengue et al. 2011). HSPs are involved as molecular chaperones in protein folding/unfolding, translocation, and degradation of proteins and in the protection against a wide range of environmental stressors. The upregulation of HSPs in liver reflected the cellular requirement for more HSPs to repair denatured proteins and might be a mechanism for the organism to increase their stress threshold and self-protection. This definitely demonstrated the significance of the liver in metabolism and that heat shock protein family members widely participate in various physiological functions.

It is obvious that HSP60 protein levels of the high- and low-dose combination groups, after the 96 h exposure, increased significantly compared with the control groups ($P < 0.05$). The HSP70 protein level increased significantly at the high-dose Cu group and all of the Cd groups after a 96-h exposure compared with the control groups ($P < 0.05$). In particular, the protein level of HSP90 only significantly increased in the high-dose combination groups ($P < 0.05$). Many studies have demonstrated that metal pollutants modulate HSP70 expression in fish (Dang et al. 2010; Iwama et al. 2004). For example, Cu and Cd were found to induce the expression of HSP70 in the fathead minnow, yellow perch, and rainbow trout (Feng et al. 2003; Pierron et al. 2009; Sanders et al. 1995). Our results verified and supplemented the abovementioned conclusion. The defense mechanisms of organisms were different following exposure to various concentrations of heavy metals. When carp were exposed to a high dose of heavy metals, other defense mechanisms would be activated.

An interesting phenomenon that drew our attention was that the protein expression of expression was not consistent with their mRNA levels following exposure to Cu, Cd, and their combination. We found that HSP60 protein levels were decreased in the low-dose Cu group (CuL) after a 24-h exposure. The HSP70 protein levels were reduced at all exposure groups at 24 h, while their mRNA concentrations were increased. Under normal circumstances, in both bacteria and eukaryotes, the cellular concentration of protein is closely related to the abundance of its corresponding mRNA (Vogel and Marcotte 2012). On the contrary, accumulating research has demonstrated different processes in various experimental models under the condition of stress (Greenbaum et al. 2003; Gygi et al. 1999). The study of Laia Quirós revealed that there was no significant correlation ($P > 0.05$) in the liver from the freshwater fish *Barbus graellsii* between metallothionein levels and the expression of the corresponding gene (Quiros et al. 2007). This may be because the proteins are damaged or degraded, or it may be the result of resource regulation. However, a series of linked processes are essential to the maintenance of protein level. When an animal is faced with environmental stress, the exact response mechanism remains unclear.

In conclusion, the present experiments showed that exposure to Cu and Cd alone and in combination produced toxic effects and led to stress on common carp. By detecting the expression of HSP60, HSP70, and HSP90 at different treatment doses and over different time periods, we demonstrated that the HSP60, HSP70, and HSP90 genes participate in the response to heavy metals, which supplements the knowledge of HSPs as biochemical markers. The results of the HSP expression levels may provide insight into environmental risk assessment. Owing to the extensive use of pesticides in agriculture, our subsequent research will focus on the risk assessment of the combined effect of heavy metals and pesticides in aquatic organisms.

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