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Ecotoxicological Effect of Single and Combined Exposure of Carbamazepine and Cadmium on Female *Danio rerio*: A Multibiomarker Study

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Abstract: In aquatic environments, organisms are exposed to mixtures of pollutants which may change the toxicity profile of each contaminant, compared to its toxicity alone. Carbamazepine (CBZ) and cadmium (Cd) are among the pollutants that co-occur in aquatic environments. To date, most research about their toxicity towards aquatic vertebrates is based on single exposure experiments. The present study aims to evaluate single and combined effects of CBZ and Cd on biomarkers in female *Danio rerio* (zebrafish) by exposing them to environmentally relevant concentrations of these two pollutants for ten days. Four kinds of biomarkers involved in antioxidant systems, energy metabolism, nervous system, and endocrine disruption, respectively, were studied. Our research results coincided with those of former studies in single exposure experiments. However, the combined exposure of CBZ and Cd exerted different responses from other studies in which these two contaminants were examined alone in zebrafish. The present study evidenced the need to conduct more coexposure studies to enhance the environmental relevance of these experimental results.

Keywords: carbamazepine; cadmium; combined toxicity; biomarkers

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are widely used in daily life throughout the world [1], which lead to their frequent detection in different environments [2–4]. Hence, PPCPs have been recognized as emerging pollutants, whose potential adverse effects on nontarget organisms have drawn more and more attention [5,6].

Carbamazepine (CBZ) has been widely used in the treatment of epilepsy, psychomotor attacks, and trigeminal neuralgia effects [7]. Its annual consumption consists of over 1000 tons [8]. Approximately 30% of oral CBZ dosage is excreted in urine and feces in its unaltered form [8]. In addition, the conventional treatment process in wastewater treatment plants (WWTPs) was found to be invalid for its removal [9,10]. Its continuous discharge and long half-life in water leads to its frequent detection in aquatic systems [11]. The concentration of CBZ in aquatic systems was reported to range from 0.03 to 11.6 µg/L throughout the world [12–15].

Previous studies showed that CBZ could have adverse effects on the health of aquatic organisms. Oxidative stress has been reported as an important impact of CBZ on aquatic organisms [16–18]. Besides, CBZ was also found to cause delay in the first reproduction age in *Daphnia magna* [19], behavioral modification in *Dugesia tigrina* [20], and even endocrine-disrupting effects on *Gobiocypris rarus* [21].

However, most research concerning CBZ toxicity to aquatic organisms has focused on its individual effects. Regarding zebrafish, a very limited number of articles describes the combined effect of CBZ with other pollutants. In fact, organisms are exposed to complex mixtures of pollutants that may interact and exert effects quite different from single-compound exposure [22]. The interactions of multiple stressors and related biochemical responses of exposed organisms may be completely different from the mode of action (MoA) of each single pollutant [23].

PPCPs and metals frequently co-occur in aquatic environments [24–26]. Among these metals, cadmium (Cd) is on the list of priority substances Annex I of the Directive 2013/39/EU. Cd is widely applied in industry, which leads to its rapid release into aquatic systems; this has aroused wide public concerns in recent years [27]. Archary et al. reported that Cd was detected in coastal waters of Kalpakkam on southeast coast of India, with a concentration of $1.6 \pm 0.15 \mu\text{g/L}$ [28]. Jitar et al. mentioned in their paper that the concentration of Cd in water samples of the Romanian coastline of the Black Sea was $0.41\sim 50 \mu\text{g/L}$ [29]. Yiğiterhan et al. reported that the concentration of Cd in sea water ranged from 1.5 to 3.65 ng/L [30]. The adverse effects of single exposure to Cd are a well-known issue [31–33]. However, few studies can be found which investigate the impacts induced by the combination of Cd with PPCPs [34–36].

Despite the available toxicity data for Cd and CBZ [37], to our best knowledge, no research has evaluated their combined adverse effects on zebrafish. In the present study, we aimed to evaluate the single and combined effects of these two pollutants on several biomarkers involved in different biochemical process, such as antioxidant systems, energy metabolism, nervous system, and reproduction/endocrine disruption in zebrafish, by exposing female zebrafish to environmentally relevant concentrations for ten days.

2. Materials and Methods

2.1. Chemicals

Carbamazepine (CBZ) Xilong, Guangzhou, China), $\text{CdCl}_2 \cdot 5/2\text{H}_2\text{O}$ (Xilong, Guangzhou, China), NaCl (Xilong, Guangzhou, China), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Sinopharm, Shanghai, China), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Lingfeng, Shanghai, China), NaHCO_3 (Lingfeng, Shanghai, China), KCl (Lingfeng, Shanghai, China). The catalase (CAT) kit (KeyGEN, Nanjing, China), the lactate dehydrogenase (LDH) kit (KeyGEN, Nanjing, China), and the Bradford protein quantitation assay kit (KeyGEN, Nanjing, China), Acetylcholinesterase (AChE) kit (Jiancheng, Nanjing, China), the superoxide dismutase (SOD) kit (Jiancheng, Nanjing, China) The vitellogenin (VTG) kit (Enzyme-linked Biotechnology, Shanghai, China), the estradiol (E2) kit (Enzyme-linked Biotechnology, Shanghai, China) were purchased before experiment.

2.2. Zebrafish Husbandry

Mature wild-type female zebrafish (Tübingen line, YSY, Nanjing, China) were purchased and maintained in the laboratory for a month before exposure. Every tenth zebrafish was placed in a 3 L tank containing a 2 L culture medium (0.6 g/L NaCl). During this period, they were maintained at $28 \pm 0.5 \text{ }^\circ\text{C}$ with a 14/10 h light and dark cycle and fed three times daily with live baby brine shrimp for five minutes. Then, 75% of the culture medium was renewed after feeding.

2.3. Exposure Protocol

After acclimation, a total of 140 zebrafish were submitted to ten-day exposure. Every tenth zebrafish was randomly placed in a 3 L tank containing a 2 L chemical exposure solution, evaluating their responses to seven treatments with two replicates for each group on separate occasions: control (without Cd and CBZ); Cd1 (0.1 µg/L Cd); Cd2 (10 µg/L Cd); CBZ1 (0.1 µg/L CBZ); CBZ2 (10 µg/L CBZ); Comb1 (0.1 µg/L Cd + 0.1 µg/L CBZ); Comb2 (10 µg/L Cd + 10 µg/L CBZ). These concentrations were chosen according to realistic environmental concentrations. After feeding, 75% of the exposure medium was renewed. Feeding was stopped 24 h before the harvest. After exposure, zebrafish were killed on ice and stored at −80 °C for further study.

2.4. Biomarkers

The homogenized sample (the whole fish) in 0.6% NaCl, using a hand-held cell crusher (MS FastPrep-1, Santa Ana, California, America), was centrifuged at 8500 rpm for 10 min at 4 °C. The supernatant was collected. Measurement of biomarkers are briefly described as follows:

2.4.1. Antioxidant Enzymes

The enzyme activity was assessed following the instructions provided by the commercial kits. One SOD activity unit was expressed as the amount of enzyme corresponding to the SOD inhibition rate of 50% in the reaction system. The activities of CAT were expressed as the number of micromolar H₂O₂ consumed per minute per milligram of protein.

2.4.2. Biomarker Concerning Energy Metabolism

The LDH activity was determined following the instruction provided by the commercial kit. One LDH activity unit was defined as 1 mmol pyruvic acid being produced in the reaction system after 1 g of tissue protein reacted with the substrate at 37 °C for 15 min.

2.4.3. Biomarker in the Nervous System

AChE hydrolyzes acetylcholine to produce choline and acetic acid. The amount of choline hydrolyzed can be reflected by the activity of AChE. AChE activity was indirectly assessed using choline content that was quantitatively determined by colorimetric determination following the instructions from the commercial kit.

2.4.4. Biomarkers Reflecting Reproduction/Endocrine Disruption:

E2 and VTG concentration were tested by using commercial enzyme-linked immunosorbent assay (ELISA) commercial kits. The absorbance (OD value) was measured at a wavelength of 450 nm using a microplate reader to calculate the sample concentration.

2.5. Statistical Analyses

All data were analyzed with PASW Statistics 18, including the focus on one-way ANOVA for all kinds of biomarkers. The value $p < 0.05$ indicates that the difference was significant at a 0.05 level, indicated by "*"; $p < 0.01$ indicates that the difference was significant at 0.01 level, indicated by "**"; $p < 0.001$ indicates that the difference was significant at the 0.001 level, indicated by "***". All the graphics were generated with the help of Origin 8.0.

3. Results

3.1. Mortality

After ten days of exposure, none of the tested conditions induced mortality.

3.2. Antioxidant Enzymes

The SOD activity, depicted in Figure 1A, increased sharply compared to the control group when exposed to 0.1 µg/L Cd, and continued to increase when exposed to 10 µg/L Cd. When exposed to CBZ, the SOD activity in female zebrafish increased slightly in comparison with the control group. When exposed to the combination of Cd and CBZ, the SOD activity increased sharply in the 0.1 µg/L Cd + 0.1 µg/L CBZ group, even higher than in the 0.1 µg/L Cd group, and the increasing combination concentration did not lead to the continual increase of SOD activity. As shown in Figure 1B, CAT activity was reduced by Cd exposure and was suppressed in the CBZ exposure group at low concentration, but was induced in this group at a high CBZ concentration. The data from the combined treatments suggested that the combined exposure of Cd and CBZ exerted less effects on CAT activity compared to single Cd exposure.

Figure 1. (A) Superoxide dismutase (SOD) activities and (B) the catalase (CAT) activities in female zebrafish after ten days of exposure to cadmium (Cd), carbamazepine (CBZ), and Cd + CBZ. The results of one-way ANOVA are indicated at the significance levels * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.3. Biomarker in the Nervous System

The AChE activity is depicted in Figure 2, showing that its activity decreased in all single exposure groups, and was induced in the combined exposure groups.

Figure 2. Acetylcholinesterase (AChE) activity in female fish after ten days of exposure to Cd, CBZ, and Cd + CBZ. The results of one-way ANOVA are indicated at the significance levels * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.4. Biomarker Concerning Energy Metabolism

The LDH activity is depicted in Figure 3, and was suppressed by Cd exposure.

Figure 3. Lactate dehydrogenase (LDH) activity in female fish after ten days of exposure to Cd, CBZ, and Cd + CBZ. The results of one-way ANOVA are indicated at the significance levels * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.5. Biomarkers Reflecting Reproduction/Endocrine Disruption

As shown in the Figure 4, VTG content and E2 decreased sharply in Cd exposure groups, and both showed similar tendencies.

Figure 4. (A) Vitellogenin (VTG) content and (B) Estradiol (E2) content in female fish after ten -days of exposure to Cd, CBZ, and Cd + CBZ. The results of one-way ANOVA are indicated at the significance levels * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

4. Discussion

Cd was found to induce reactive oxygen species (ROS) production and oxidative stress in zebrafish at a nonlethal concentration, which can enhance SOD activity to eliminate the ROS radicals [38]. When exposed to CBZ, the SOD activity in female zebrafish increased slightly in comparison with the control group. Similar trends of SOD activity displayed in previous studies neutralized the impact of increased ROS generation in rainbow trout after chronic CBZ treatment [39]. When exposed to the combination of Cd and CBZ, the SOD activity increased sharply in the 0.1 $\mu\text{g/L}$ Cd + 0.1 $\mu\text{g/L}$ CBZ group, even higher than in the 0.1 $\mu\text{g/L}$ Cd group. Interestingly, the increasing combination concentration did not lead to the continual increase of SOD activity. Almeida et al. studied the effects of single and combined exposure of CBZ and Cd of *R. philippinarum*, and found that SOD activity increased when exposed to the 0.5 $\mu\text{g/L}$ Cd + 1.0 $\mu\text{g/L}$ CBZ compared to the control group, but was lower than the single-exposed Cd (0.5 $\mu\text{g/L}$) and CBZ (1.0 $\mu\text{g/L}$) groups, respectively [35]. In that research, no further combination concentrations were investigated. Some other studies investigated the effects induced by the coexposure of Cd and organic pollutants including PPCPs, showing that Cd effects on the aquatic organisms may depend on the type of organic pollutants present in aquatic systems [34,37,40].

CAT activity was reduced by Cd exposure, which may be because it was consumed by the degradation of the hydrogen peroxide produced during the superoxide disproportionation under oxidative stress [39,41]. CAT activity in mice also had similar tendencies after Cd exposure for seven days [42]. CAT activity was suppressed in the CBZ exposure group at a low concentration, but was induced in the group at high concentration. The data from the combined treatments suggests that the combined exposure of Cd and CBZ exerted weaker effects on CAT activity compared to single Cd exposure, indicating that the presence of the CBZ attenuated the Cd effects.

As previously mentioned, the combined effects of Cd with CBZ in zebrafish have not been studied. However, some researchers evaluated the effects of PPCPs combined with Cd or other metals in bivalves. For example, Almeida et al. observed that weaker effects on CAT activity in *R. philippinarum* were exerted by combined exposure of 0.5 µg/L Cd + 1.0 µg/L CBZ than by single exposure of 0.5 µg/L Cd [35]. Gonzalez-Rey et al. observed that Cu combined with PPCPs (ibuprofen, diclofenac, and fluoxetine) exerted weaker effects on CAT activity in mussel gills than single exposure by PPCPs [26].

AChE is a good candidate that is responsible for the hydrolysis of the neurotransmitter acetylcholine, which plays an important role in the nervous system [43]. AChE could be a target for pollutants, resulting in the inhibition of its activity, failure of synaptic transmission, and muscle overstimulation [44,45]. Thus, AChE activity has been used as a biomarker to evaluate the neurotoxicity of pollutants. Pan et al. studied the AChE activity in zebrafish gill, suggesting that Cd can inhibit the AChE activity in zebrafish: the higher the Cd concentration; the higher the inhibition degree of AChE activity [46]. This result was similar to the behavior response based on behavior strength, suggesting that inhibition exerted by Cd can induce a decrease of swimming behavior, loss of coordination, and other kinds of behavior change of zebrafish [47]. Juhel et al. observed that exposure to CBZ led to a significant reduction of AChE activity in *Perna viridis* [21]. Santos et al. observed that exposure to 10 µg/L CBZ led to slight reduction of AChE activity in zebrafish [47].

Interestingly, the present study showed that AChE activity in zebrafish was enhanced when exposed to combined stress of Cd and CBZ. The increase of AChE activity has been related to the production of free radicals [48] and to the apoptotic processes occurring in the organism [49,50]. AChE activity increase leads to fast acetylcholine degradation and downregulation of acetylcholine receptors, influencing the cognitive functions of the organism [51].

Metabolic regulation plays a very important role in environmental stress tolerance, since supplying enough energy to meet the energy demand is crucial for survival [52]. To assess the alterations in the pathways of cellular energy production induced by toxicants, LDH was quite often used, since it is the terminal enzyme of anaerobic glycolysis located in the cytoplasm [53]. Some previous studies showed that heavy metals can influence the LDH activity. For example, Pandey et al. found that mercury exposure can reduce the LDH activity in gill of *L. marginailis* [54]. Akintunde et al. found that LDH activity in rat can be reduced by mixed-metal exposure, including Pd, Cd, Co, and Cr [55].

In the present study, we found that exposure to Cd can deplete LDH activity, which potentially indicates the increase of energy costs associated with raised metabolic regulatory demands [56]. LDH activity was induced by CBZ exposure and the combined exposure of CBZ and Cd, suggesting that increased use of the anaerobic pathways for producing energy is likely to require additional energy to deal with this stress factor. The increase in use of this pathway under pollution stress has been observed in several organisms exposed to contaminants [57–59].

VTG is an egg yolk precursor that is normally produced in oviparous females, which provides energy for embryonic development in adult female ovipara [60]. VTG has been considered as a sensitive biomarker for monitoring estrogenic activity in the environment [61], since it responds to estrogens circulating in blood plasma [60–62].

In the present study, VTG content and E2 decreased sharply in Cd exposure groups. For the case of VTG, since Cd has a strong affinity with estrogen receptor [63], we hypothesize that the binding of Cd with this receptor might reduce the available binding sites for estradiol, resulting in the reduction

of VTG content. This hypothesis needs further experimentation to verify. For the case of E2, Cd is likely to completely abolish the E2 induction of the receptor gene and the estrogen receptors [64].

CBZ inhibited the VTG content and E2 in female zebrafish gradually. Some previous studies showed that CBZ has the potential to disrupt the endocrine system. For example, Aguirre-Martínez et al. found that VTG expression was reduced after 21 days of exposure to CBZ [65]. Hammill et al. found that parent exposure to CBZ can disrupt the sexual differentiation in the F1 offspring [66]. The reduction of VTG content in female fish is presumably due to the decrease in E2 [67], which agrees with the results in the present study (Figure 4A). Interestingly, the VTG and E2 content in female fish were also reduced by the combined exposure of Cd + CBZ, but are lower than those in the CBZ single exposure group. The detailed mechanism needs further research.

5. Conclusions

The present study implies that the combination of CBZ and Cd exerted different responses from those of the contaminants alone in female zebrafish. Exposure to environmentally relevant concentrations of CBZ, Cd, and their mixture can alter enzymes involved in the antioxidant system, energy metabolism, nervous system, and endocrine system disruption. This study could help gain a broad view on the risk assessment of these two pollutants, and highlights the need for a better understanding of the coeffects of environmental PPCPs and heavy metals on nontarget organisms. Measuring biomarkers is a common and efficient way to illustrate the toxic effect of pollutant exposure. To further verify these effects, other evidence, such as transcriptomics, proteomics, and histopathology, could be used in future studies.

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