Effects of EGCG and Chlorpyrifos on the Mortality, AChE and GSH of Adult Zebrafish: Independent and Combination

Rong Zhang*, Jian Zhang, Qian Gao and Nichun Guo
School of Resources and Environment, Anhui Agricultural University, 230036, No. 130, Changjiang West Road, Hefei, China.
* Corresponding author email: rongzhang@ahau.edu.cn

Abstract. Chlorpyrifos is a neurotoxic agent and also causes oxidative stress in the body. EGCG is a typical strong antioxidant and has been reported to be neuroprotective. Our study investigated the mortality, the activity of acetylcholinesterase (AChE) in the brain and glutathione (GSH) in the liver of the adult Zebrafish in present of Chlorpyrifos and EGCG independent and combination. The results indicated that after the addition of EGCG, the mortality of zebrafish induced by Chlorpyrifos was reduced and the activity of AChE and glutathione (GSH) inhibited by Chlorpyrifos in zebrafish was significantly increased, which demonstrated that EGCG inhibited the toxicity Chlorpyrifos to zebrafish. The inhibition was dependent on the concentration of EGCG and Chlorpyrifos, which was not shown a gradual change trend but a complex situation.

Keywords: EGCG, Chlorpyrifos, Acetylcholinesterase, Oxidative Stress, Zebrafish.

1. Introduction
Chlorpyrifos (CPF) is widely used in agricultural production [1, 2] and is sensitive to the fish [3-6]. Chlorpyrifos bond with cholinesterase by covalent [7, 8] resulting in the accumulation of acetylcholine in the nervous system [8, 9]. They causes irreversible inhibition of AChE, which leads to neuronal overstimulation and death [9]. Yen, et al [10] used zebrafish to examine how exposures to Chlorpyrifos affected zebrafish’s acetylcholinesterase (AChE) activity. Their tests show that 300 nM (≈ 0.1 mg/L) Chlorpyrifos exposure over 5 days inhibited AChE activity by over 80%. Richendrfer et al [8] observed that Larvae treated with 0.1 mM (≈ 35.0mg/L) Chlorpyrifos had decreased more than 30% of levels of AChE. Recent researches showed that Chlorpyrifos could destroy oxygen free radical balance and form oxidative stress [3, 11, 12]. The GSH and GST as the important antioxidants substances in liver stressed by Chlorpyrifos were investigated extensively. Wang et al [13] studied the effect of Chlorpyrifos on the antioxidative enzymes (CAT), superoxide dismutase (SOD), reactive oxygen species (ROS) generation and GSH contention in zebrafish. The researches indicated that Chlorpyrifos resulted in a significant increase of ROS generation in the zebrafish. Therefore, Chlorpyrifos not only inhibits AChE, but also has an impact on the redox physiological processes.

Besides the AChE and GSH, the assessment of changes in the amount and the express of protein can also explain a certain damage of body from Chlorpyrifos. Chlorpyrifos causes the toxic effects on the brain size [8] and the tissue of liver of zebrafish [12]. Gomez et al [14] evaluated the effect of Chlorpyrifos exposure on metabolic profiles of zebrafish muscle and observed the induction of muscle exhaustion as well as the oxidative stress and the general disruption of neurotransmitter metabolism. Liu et al [15] investigated that the protein
expression profiles of zebrafish embryos under Chlorpyrifos stress and provided the data that help to understand the functions and the molecular mechanisms of those proteins in zebrafish embryos' response to Chlorpyrifos exposure. Topal et al [12] reported that Chlorpyrifos inhibited glucose-6-phosphate dehydrogenase (G6PD) enzyme activity in liver and gill, which might impede the synthesis of protein. Many authors postulated that Chlorpyrifos disturb cellular redox processes, and change the activities of antioxidant enzymes [11, 15, 16]. Studies have indicated that the oxidative stress may also play a role in the regulation and activity of AChE. Rodriguez & Rubio [16] evaluated the joint effects of an antioxidant, vitamin C (VC), and Chlorpyrifos, on AChE activity in zebrafish embryos after 72 h exposure as well as the oxidative stress by measuring the quantification of total glutathione. This topic aroused us to be interested in the effects of natural antioxidants on the biotoxicity of Chlorpyrifos. We expect to understand the effect of the stronger natural antioxidant than that of VC although VC by itself was not observed altered the AChE activity in the zebrafish embryos [16].

Epigallocatechin-3-gallate (EGCG) is known for its intensive antioxidant property which is 10 times than that of VC. EGCG can scavenge the ROS in vivo and in vitro [17, 18]. It provides both short and long-term protection against oxidative stress through a variety of mechanisms [17] and has the function of protecting the nervous system of rats [18]. Many features of zebrafish (Danio rerio) have made it become a model system for the examination of environmental toxicology[14]. It also help to determine the critical neuro-developmental processes impacted by organophosphorus pesticides, Chlorpyrifos as an instance [10, 19-21].

According to our knowledge, there are no data indicated the actions of EGCG, and the combined effects of EGCG and Chlorpyrifos on the mortality, AChE, the GSH and the proteins of zebrafish. To obtain an expected result, we adopted the adult zebrafish which are relatively stable in the nervous and hepatic physiological systems, analyzed the effects of Chlorpyrifos and EGCG independent and combination and evaluated changes of acetylcholinesterase, the glutathione activity and protein quality in adult zebrafish. The purpose was to observe a protective effect of EGCG on AChE inhibited by Chlorpyrifos and resistant to the oxidative stress from Chlorpyrifos.

2. Materials and Methods

2.1. Chemicals and Materials

CPF (Purity > 99%) and EGCG (Purity ≥97.0%) were purchased from Sigma Aldrich. Stock solutions of CPF were prepared by dissolving it in acetone. The commercial kits for determining glutathione (GSH) contents, total protein and AChE activities were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Other reagents were analytic purity and purchased from Tianjin Chemical Company (Tianjin, China). Solution for exposed test was diluted with distilled water to add into the water.

2.2. Zebrafish Maintenance

The Adult zebrafish were obtained from a local pet store and maintained in glass aquariums (60×30×30 cm) of 40 L water capacity at laboratory conditions for two weeks by using water aerated with an aquarium air pump (Super-8300, made in China). The average values for the culture conditions in aquariums for zebrafish was temperature of 20±1 °C, pH 6.8 ±0.05, and dissolved oxygen of 8.15±0.06 mg/L. The natural photoperiod of 14:10 (Light: Dark) hours were maintained and the zebrafish were fed with dry flakes twice per day and add libitum with brine shrimp once a day. Prior to the acute toxicity tests, fish were starved for 2 days. Control texts were exposed to water only containing same volume of acetone. In all experiments, the exposure solutions were not changed.

2.3. Toxicity Test Method and Sample Collection

According to 203 for testing chemicals (OECD, 1992), a 1 L glass tank with 0.8 L of dechlorinated tap water was used in all experiments. Tests were set according to the literature [20], and the adding concentrations of CPF and EGCG were 0.010~ 1.0mg/L and 0.010~
10.0mg/L, respectively (seen in table 1 for the specific concentration), and the exposing time was 120h in the independent tests. For the combined effects of EGCG and CPF, the factorial analysis of CPF (3)*EGCG (7) was designed. Tests with three concentrations (0.5, 0.6, 0.7mg/L) of CPF and their solution with different ratios with EGCG were completed to determine lethal concentrations of the chemical in zebrafish. For each concentration, ten adult zebrafish were exposed to chemicals under static conditions. These experiments were performed in triplicate and fish were not fed during the test. Experimental conditions were maintained at 20 ± 1°C with a LD cycle of 14:10 h. The mortalities were observed.

At the end of the experimental period, the fish per group were sampled and anaesthetized on ice for 10 min and dissected, and liver and brain were used for the estimation of different parameters. 3 mL of pre-cooled homogenate buffer solution (0.05M PBS buffer pH 7.4) was added into the grinding sample of brain and homogenized in ice bath, centrifuged at 4000r /min for 10min at 4°C. The sample of liver was weighed and homogenized in an ice bath with 10 volumes of pre-cooled homogenate buffer (containing 0.075 M NaCl, 10% glycerol, 0.050 M PBS buffer pH 7.4), then, centrifuged at 12000 r/min for 20 min. The supernatant was for acetylcholinesterase and glutathione assay.

Samples for protein determination: Accurately weighed the test tissue, added by 9 times the volume of saline to tissues, processed homogenate in ice water bath, and centrifuged 2500 r/min for 10 minutes. The supernatant was tested after adding the saline (1:4, v/v).

2.4. Determination of Enzyme Activity and Protein
Glutathione (GSH) was determined using the reduced glutathione (GSH) assay kit (Spectrophotometric method). AChE activity was determined with a modified method from Ellman et al. (1961) [16]. The reaction mixture was composed of 0.284 mL of 0.1 M phosphate buffer (pH 8.0), 0.003mL of 3.3 mM DTNB, 0.003mL of 50 mM ATChI, and 0.01mL of crude enzyme extract. The total volume of the reaction mixture was 0.3mL and the reaction was initiated by the addition of crude enzyme extracts. The absorbance at 412 nm was read for 6 min. The samples of protein were balanced for 10 min after staining by coomassie brilliant blue and determined at 512nm by UV spectrophotometer [15].

3. Results
3.1. Independent and Combinational Exposing and Lethal Tests
In the independent tests, no zebrafish death was observed at the concentration of 0.10 ~ 1.0mg / L of EGCG during the exposing time of 120h and 2.0~10.0mg / L in 72 h. The LC50 of CPF was 0.6 mg / L after 72h, which was the similar concentration as that reported by Jeon & Lee (LC50 was 0.6mg/l after 71.2h) [20]. After 24 h exposure, the LD50 was 1.0 mg / L in current tests (Figure 1). The lower exposing concentration of 0.01 mg / L was also tested and the mortality was 50% after 120h exposing. The mortality of zebrafish stressed by the joint effect of EGCG-CPF with different proportions is shown in Figure 2(a-c). The data indicated that EGCG in a range from 0.125 mg/L to 2.8 mg/L significantly changed the mortality of zebrafish induced by Chlorpyrifos of 0.5, 0.6, 0.7 mg/L, respectively. In the three groups after 72h exposure, the ratio of EGCG to CPF between 100% and 200% was the most obvious for the lethal rate reduction of zebrafish in all joint effects tests.

The tests used the solution without change for 72h. We determined the concentrations of CPF in the exposing solution. The analysis data indicated that EGCG slightly decreased the concentration of CPF and the rate was no more than 5.0±0.02 %.
Figure 1. The mortality of zebrafish exposed in different concentrations of Chlorpyrifos (%)

Figure 2 (a). Combinational Effects of EGCG- Chlorpyrifos on zebrafish mortality (72h). [CPF]= 0.5mg/L.

Figure 2 (b). Combinational Effects of EGCG- Chlorpyrifos on zebrafish mortality (72h). [CPF]= 0.6mg/L.
3.2. Analysis of Acetylcholinesterase AChE Activity
Given the LC$_{50}$ at 0.5~0.7 mg / L after the exposure time of 72h were reported in most researches and the LC$_{50}$ at 0.6 mg / L detected in current test was corresponding with the results reported recently [20], we trialed the effects of 0.5, 0.6, 0.7 mg / L of CPF on AChE activity. As can be seen in Figure 3, EGCG greatly changed the concentration of AChE in the brain of adult zebrafish. The concentration of AChE was 3.8U / mg prot at the stress of 0.5mg / L of CPF, but with the additions of EGCG (from 25% to 400%, i.e. from 0.125 to 2.0 mg/L of EGCG), it reached to the maximum value of 14.00 U / mg prot at the ratio of 200%, i.e. [EGCG]: [CPF] = 1.0:0.5 (mg/L: mg/L). The concentration of AChE went down but still maintained at 12.00 U / mg prot at the ratio over the 200%. For 0.6mg / L of Chlorpyrifos, we noted that the maximum value of AChE was 12.7 U / mg prot with the addition of 0.3mg/L of EGCG corresponding to a mortality of 33.3%. More addition of EGCG did not increase the amount of AChE. But all the concentrations of AChE were higher than that pressed by CPF after adding EGCG. For the group of 0.7 mg / L of Chlorpyrifos, the increased values of the AChE concentration was lower than that of the group of 0.5mg / L of CPF, but a similar alteration trend was observed. These results indicated that EGCG increased the concentration of AChE in adult zebrafish brain which is an important target that influences the mortality of zebrafish and the significant effects was basically ranged from 100% to 200% of the ratio to CPF.

3.3. Changes of Glutathione Activity
Figure 4 showed that 0.5 mg / L of Chlorpyrifos inhibited the glutathione activity in zebrafish liver by the rate of 87.6%. Adding EGCG improved the inhibition and increased the activity of glutathione. When EGCG concentration was 0.25mg / L ([EGCG]: [CPF] = 0.25:0.5, mg/L: mg/L) and 1.0mg / L ([EGCG]: [CPF] = 1.0:0.5, mg/L: mg/L), the activity of glutathione increased 88.7% and 86.5%, respectively. During the tests at the 0.6mg / L and 0.7mg / L of CPF, we observed a similar phenomenon. For the 0.6mg / L of CPF, two peak value, 92.0% and 94.6%, of the increase rates appeared at 0.15 mg / L and 1.8 mg / L of EGCG, respectively. For the 0.7mg / L of CPF, one was at the concentration of 0.35 mg / L of EGCG, and the other peak appeared at 2.1 mg / L of EGCG with a rate of 23.5%. All the effects of EGCG were observed to be different at a varied concentration. These tests demonstrated that EGCG has a strong reparative effect on Chlorpyrifos-inhibited glutathione, but it is concentration-dependent and no concentration gradient correlation.
Figure 3. Combinational effects of EGCG and CPF on AChE. [CPF]=0.5mg/L

Figure 4. Combinational effects of EGCG and CPF on GSH. [CPF]=0.5mg/L

3.4. Protein Amount of Zebrafish
The protein in liver and brain of zebrafish under the Chlorpyrifos (CPF) stress and the combined action of CPF and EGCG were determination after 72h exposure (Fig 5, Fig 6). EGCG increased the liver protein of zebrafish exposed to 0.6 mg/L of CPF, improved partly that in the group of 0.5 mg/L, and had no significant effect found in group of 0.7 mg/L. For protein of brain, EGCG kept or decreased the concentration at the stress of 0.5 mg/l and 0.6 mg/l of CPF, and reduced it slightly at that of 0.7 mg/l. It was a reverse action for EGCG to the stress by CPF to the protein in liver and that in brain, which was difficult to illustrate in this tests.
Figure 5. Change of total protein under the combined effects of EGCG and CPF in liver. [CPF]=0.6mg/L

Figure 6. Change of total protein under the combined effects of EGCG and CPF in brain. [CPF]=0.5mg/L

4. Discussion
The toxic of CPF to zebrafish was reported differently in extensively researches. In the current exposure, the toxic of CPF was positively correlated with the concentration and the exposing time, i.e. the higher of the concentration and the longer of the exposing time, the higher of mortality was observed. We tested the LC50 of CPF to the adult zebrafish in a large rang of concentration and time. It was 0.6 mg/L after 72h exposure in static water adding CPF once at the beginning, which was the similar concentration as that reported by Jeon & Lee (LC50 was 0.6mg/l after 71.2h) [20]. For the exposure mortality experiment results, we noted there was a concentration dependent of EGCG combined with CPF. Most researches demonstrated that the antioxidation of EGCG was dependent on concentration. Zebrafish are not sensitive to EGCG from 0.01 to 10 mg/L, a wide range of concentrations, in our experiments. However, when CPF and EGCG worked together, it was observed there was a lethal antagonistic effect on zebrafish mortality, which depending on the concentration of EGCG ranged from 0.125 to 2.8 mg/L.

CPF is not only the nerve poison but also involved in the oxygen stress to zebrafish. Catechin [22]. The extract from green tea (GTE) [23] have been reported the protective effect on rat from Chlorpyrifos toxic by restoring antioxidant enzyme activities and improving histopathological changes as well as alleviating lipid peroxidation [22, 23]. That GTE could inhibit hepatotoxicity induced by exposure to Chlorpyrifos [24] is considered to be possibly due to the antioxidant effect of Catechin [22]. EGCG is one of the strongest antioxidative and
biological activate substances in green tea and belongs to Catechin [17]. Biasibetti and Tramontina [18] investigated the effects of EGCG on GSH of rat in their evaluation on the effects of sub-chronic EGCG treatment in rats that were submitted to ICV infusion of STZ. Even though their results show that EGCG single was not able to modify glutathione content but the glutathione peroxidase activity and reactive oxygen species content were completely reversed by EGCG administration. CPF was demonstrated the oxidative stress to liver [11]. We investigated the effects of EGCG on glutathione activity in the liver under oxidative stress of Chlorpyrifos using the adult zebrafish and observed that when acted individually, CPF reduced GSH concentrations while EGCG did not change the amount of GSH, which was accord to the results of Biasibetti and Tramontina [18]. However, when EGCG was co-exposed with CPF, an increase in total GSH concentration prohibited by CPF was detected. The results in the current study confirmed our hypothesis that EGCG can reduce the oxidation stress induced by CPF in the liver of zebrafish. Besides, the effect of EGCG on the protein quality can also be explained by the existence of multicomponent repair of EGCG to the Chlorpyrifos-induced hepatotoxicity. But, the role of the repair is complex corresponding to the concentration of EGCG, the properties of EGCG with CPF, and the exposure level of CPF. It is also no gradient correlation with the concentration of EGCG. Thus, it is suggested to experiment and acquire the data on a specific case.

In the trial of Biasibetti and Tramontina [18], EGCG was reported to have no effect on AChE but was neuroprotective and change the amount of AChE when AChE was decreased by exposed to CPF in the rat. We reproduced the Chlorpyrifos’ inhibitory on the AChE of zebrafish in the experiments and also demonstrated that EGCG alone did not alter the amount of AChE. What’s different is that, when AChE is reduced by CPF, the co-exposed EGCG repairs the AChE by increasing the amount of AChE, even up to that of non-CPF exposure. This indicated that EGCG affects the amount of AChE in zebrafish as well as that in mice exposed to Chlorpyrifos [18]. AChE is the main targets by CPF in brain. EGCG partly restored the impairment of CPF on AChE and decreased the lethal rate stressed by CPF in the exposure tests. Even though we did not acquire the exact match of data on the change of the AChE and the mortality, we still speculated that the presence of EGCG was an important factor on the mortality of zebrafish stressed by CPF.

Contrary to results reported that antioxidant, i.e. VC, protect AChE by decrease its amount in embryos of zebrafish [16], our experiments showed that EGCG protected zebrafish nerves by increasing the amount of AChE repressed by Chlorpyrifos. What we are interested in is why the two antioxidants produced the positive effects on Zebrafish's neurons by acting on AChE in reverse ways. If, as some investigators believe, the inhibition of AChE may also involve redox, the two reverse actions might be understood from the viewpoint of buffer potential.

5. Conclusion
We investigated the effects of EGCG on the Chlorpyrifos impairments to Zebrafish and demonstrated our expected results. The mortality of zebrafish induced by Chlorpyrifos was decreased when EGCG was present at a certain concentration. By analysis the activity of AChE and glutathione (GSH), we concluded that EGCG protected Zebrafish's neurons from Chlorpyrifos toxic and resisted to the oxidative stress from Chlorpyrifos. The concentration dependence without the gradient correlation complicated the situation. These data may help us understand the functions of EGCG on the adult zebrafish’s response to Chlorpyrifos exposure.

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7. References
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