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# Impacts of oxidative stress on acetylcholinesterase transcription, and activity in embryos of zebrafish (*Danio rerio*) following Chlorpyrifos exposure

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ABSTRACT

Organophosphate pesticides cause irreversible inhibition of AChE which leads to neuronal overstimulation and death. Thus, dogma indicates that the target of OP pesticides is AChE, but many authors postulate that these compounds also disturb cellular redox processes, and change the activities of antioxidant enzymes. Interestingly, it has also been reported that oxidative stress plays also a role in the regulation and activity of AChE. The aims of this study were to determine the effects of the antioxidant, vitamin C (VC), the oxidant, t-butyl hydroperoxide (tBOOH) and the organophosphate Chlorpyrifos (CPF), on AChE gene transcription and activity in zebrafish embryos after 72 h exposure. In addition, oxidative stress was evaluated by measuring antioxidant enzymes activities and transcription, and quantification of total glutathione. Apical effects on the development of zebrafish embryos were also measured. With the exception of AChE inhibition and enhanced gene expression, limited effects of CPF on oxidative stress and apical endpoints were found at this developmental stage. Addition of VC had little effect on oxidative stress or AChE, but increased pericardial area and heartbeat rate through an unknown mechanism. TBOOH diminished AChE gene expression and activity, and caused oxidative stress when administered alone. However, in combination with CPF, only reductions in AChE activity were observed with no significant changes in oxidative stress suggesting the adverse apical endpoints in the embryos may have been due to AChE inhibition by CPF rather than oxidative stress. These results give additional evidence to support the role of prooxidants in AChE activity and expression.



## 1. Introduction

Two cholinesterases (ChE) are present in vertebrates, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). Unlike many fish, zebrafish (*Danio rerio*) have a single gene for AChE but the gene or activity of BChE is not present (Bertrand et al., 2001). These characteristics make zebrafish an ideal organism for the study of AChE regulation. Zebrafish is a species convenient and cost-effective to work with from a technical and methodological point of view, and it provides conceptual insights into many aspects of vertebrate biology, genetics, toxicology and disease (Segner, 2009). The early life-stage test using the zebrafish embryo currently is one of the most widely used tools in environmental toxicology, especially for investigating the toxicity and teratogenicity of chemicals that could significantly affect environmental health (Schulte and Nagel, 1994).

ChE are inhibited by numerous pollutants that include organophosphate pesticides (OP), which are, among other pesticides the most toxic to vertebrates (Shadnia et al., 2005; Rahimi et al., 2006). OP causes the irreversible inhibition of AChE in the central and periferal nervous systems resulting in the accumulation of acetylcholine and excessive activation of muscarinic and nicotinic receptors, which may lead to death (Shih and McDonough, 1997). Thus, dogma indicates that the target of OP pesticides is AChE, but many authors postulate that these compounds also disturb cellular redox processes, and change the activities of antioxidant enzymes (Abdollahi et al., 2004; Possamai et al., 2007). OP metabolism produces reactive oxygen species (ROS) that are highly reactive molecules, which have one or more unpaired electrons (Altuntas et al., 2003). ROS cause lipid peroxidation resulting in the formation of highly reactive stable and unstable hydroperoxides of saturated and unsaturated lipids with eventual damage to DNA. Cells have mechanisms to

*Abbreviations:* AChE, acetylcholinesterase; CAT, catalase; CPF, chlorpiryfos; CPF-O, chlorpyrifos-oxon; GSH, glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; OP, organophosphate pesticide; ROS, reactive oxygen species; SOD, superoxide dismutase; tBOOH, tert butyl hydroperoxide, TCP, 3,5,6 trichloro-2-pyridinol.

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counteract damage caused by ROS. Many compounds have antioxidant capacity and can be categorized in two systems. One of them is enzymatic consisting superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). The other system is non-enzymatic and consists of a reduced form of glutathione and vitamins (C,E,beta-carotene) which directly inactivate ROS.

Interestingly, it has also been reported that oxidative stress plays also a role in the regulation and activity of AChE. For example Schallreuter et al. (2004) identified activation/deactivation of human AChE by hydrogen peroxide; De Carvalho Corrêa et al., 2008 found that oxidative stress during hypertension changes AChE activity in vivo, and it has also been reported that ethanol, which produces ROS, alters genetic expression and activity of AChE (Rico et al., 2007).

Considering that changes in antioxidant levels contribute to many diseases and developmental abnormalities, the importance of AChE in nervous system, and the extensive use of OPs, further research into the effect of OP on oxidative stress and how it interacts with AChE inhibition and regulation is necessary. The aims of this study were to determine the effects of Chlorpyrifos (CPF), the antioxidant vitamin C (VC), the pro-oxidant t-butyl hydroperoxide (tBOOH) and their co-exposures on AChE gene transcription and activity as well as their effects on oxidative stress and the development of zebrafish embryos.

## 2. Materials and methods

This study was conducted in accordance with institutional guidelines for the protection of animal welfare. Adult wild-type zebrafish were obtained from Enmanuel fish farm (Hunucma, Yucatan). Organisms were separated by gender and acclimated for 1 month at the Unit of Chemistry of the National Autonomous University of Mexico in Sisal, Yucatan, using 40-L glass aquariums (density of 1 fish/liter) with constant aeration at 26 ± 2 °C, pH 8.0 ± 0.5, conductivity 600 ± 50 μS/cm. Fish were fed twice daily with commercial dry food, once per day with *Artemia* sp. nauplii and once with *Daphnia pulex* ad libitum. After acclimation period, mating was done in 10 L glass aquariums with a density of 5 fish per liter and a female to male ratio 2:1, water temperature was elevated to 28 ± 2 °C (Westerfield, 2000). Spawning was induced by the onset of light. Eggs were collected and rinsed with clean water before exposures. Fertilized eggs were used for exposures 2 h post fertilization.

Zebrafish embryos were transferred to 6 well plates, 50 embryos per well and 3 replicates per concentration for each of the evaluated endpoints were used in all the exposures. Zebrafish embryos were exposed to 200 and 400 μg/L CPF; 200 and 400 μg/L VC and 200 and 400 μg/L tBOOH. Additional bioassays consisted in the co-exposure with 400 μg/L of CPF and either 200 or 400 μg/L VC or tBOOH. All bioassays included a negative control and a 0.01% ethanol control (if needed).

After 72 h, larvae were placed in clean 1.5 mL microcentrifuge tubes and snap frozen in liquid nitrogen. Samples used for qRT-PCR were treated with 200 μL of RNA later® (Sigma, USA). Samples were kept at −80 °C for later analysis.

Concentrations of CPF were verified by solid-phase microextraction and gas chromatography-mass spectrometry (SPME/GC-MS), based on the methods reported by Tomkins and Ilgner (2002). A sample aliquot of 10 mL was placed in a 20 mL-SPME vial (Supelco, USA) with a screw cap with a PTFE/silicone septum (Supelco, USA). Extraction of CPF was performed by direct immersion for 30 min at 55 °C with a 65 μm PDMS/DVB SPME fiber (Supelco, USA) and 700–900 rpm magnetic stirring. After the extraction, CPF was quantified using an Agilent Technologies 6850 Gas Chromatography System equipped with a 5975B mass detector and a Zebron ZB-5MSi capillary column (30 m long, 0.25 mm i.d. and 0.25 μm film thickness, Phenomenex, USA). The Inlet temperature was 250 °C and samples were injected in a splitless mode (1 min purge time) with 10 min of desorption time. The oven temperature program started at 50 °C for 1 min, ramp 1 was 10 °C/min until 180 °C, ramp 2 was 1.5 °C/min until 200 °C and held for 2 min, and ramp 3 was 30 °C/min until 290 °C and held for 2.67 min; the carrier gas was helium. CPF was determined in selective ion monitoring (SIM) mode (target ions: 314, 316 *m/z*). The spectra generation frequency was 20 Hz, and the interface and ion source temperatures were 290 and 230 °C, respectively. The MS ionization mode was electron ionization (EI). Calibration solutions were used to quantify the CPF in the samples. The method detection limit was 10 ng/L.

VC in exposure solutions was measured using Ascorbic Acid Assay Kit (MAK074, Sigma-Aldrich, USA) and VC concentration was determined by a coupled enzyme reaction, which results in a colorimetric (570 nm) product proportional to the ascorbic acid present in the sample. Analyses were made following the instructions indicated in the technical bulletin provided by the manufacturer.

TBOOH in exposure solutions was measured using PeroxiDetect Kit (PD1; Sigma-Aldrich, USA) for the determination of aqueous peroxide solutions. Color formation was measured colorimetrically at 560 nm using a Lambda 25 UV/Vis spectrometer (Perkin Elmer, USA).

AChE activity was measured using a modification of the method of Ellman et al. (1961) adapted to a microplate reader (Rodríguez-Fuentes et al., 2008). In summary, each well contained 10 μL of the enzyme supernatant and 180 μL of 5,5′-dithiobis(2 nitrobenzoic acid) (DTNB) 0.5 mM in 0.05 M Tris Buffer pH 7.4. The reaction started by addition of 10 μL of acetylthiocholine iodide. The rate of change in the absorbance at 405 nm was measured for 120 s.

Activities of CAT, SOD and total glutathione concentration were determined colorimetrically using Cayman Chemical kits (Cayman Chemical, USA) following the manufacturer's instructions. CAT assay (Johansson and Borg, 1988) utilizes the peroxidatic function of the enzyme for determination of activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of $H_2O_2$. The formaldehyde produced is measured colorimetrically with 4-amino-3-hydrazini-5-mercapto-1,2,4 triazole (Purpald) as the chromogen. Purpald forms a compound with aldehydes which upon oxidation changes from colorless to a purple color. SOD assay utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. Glutathione assay (Baker et al., 1990) utilizes an enzymatic recycling method, using GR. The sulfhydryl group of GSH reacts with Ellman's reagent and produces a yellow colored compound that is read at 405 nm. Concentrations of protein in samples were measured as described by Bradford (1976). All enzyme activities were normalized respect to its protein content.

Quantification primers for AChE, CAT, and β-actin were designed from the reported GenBank sequences with accession numbers **NM_131846.1**, **NM_130912** and **BC165823.1**, respectively, to obtain amplicons of 100–200 base pairs. SOD quantification primer sequences were taken from Gonzalez et al. (2006). Primer sequences are reported in Table 1. Before qPCR, all amplicons were purified and sequenced to verify primer specificity, and efficiency curves were also run to validate the method.

Total RNA was extracted using Gene Elute® Mammalian Total RNA Mike Prep Kit (Sigma, USA). Total RNA concentration was determined by evaluating fluorescence with the Quant-it® RNA Assay Kit (Invitrogen, USA). Two hundred nanograms of total RNA was used for the synthesis of cDNA with the iScript® Kit (Biorad, USA); 1 μL of cDNA was used for qPCR

**Table 1**
Primer sequences for qPCR for acetylcholinesterase (ACHE), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and β-actin (BAC) genes in zebrafish larvae.

| Gene | Forward | Reverse | Tm° | |
|---|---|---|---|---|
| | | | F | R |
| AChE | CAAGTTCTTCCCTGGAGCAG | TCCCTCATCCTGATTTACGC | 63.9 °C | 63.9 °C |
| GPx | GAAATACGTCCGTCCTGGAA | TCTCCCATAAGGGACACAGG | 63.8 °C | 63.9 °C |
| $SOD_{CU/ZN}$ | TGAGACACGTCGGAGACC | TGCCGATCACTCCACAGG | 62.8 °C | 66.4 °C |
| CAT | CAGGAGCGTTTGGCTACTTC | ATCTGATGACCCAGCCTCAC | 63.8 °C | 64.2 °C |
| BAX | GTGCCATCTACGAGGCTTA | TCTCAGCTGTGGTGGTGAAG | 63.8 °C | 64.3 °C |