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Research Article

BIOCHEMICAL EVALUATIONS OF QUINALPHOS EXPOSED ZEBRAFISH LIVER ORGANOTYPIC TISSUE CULTURE

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ABSTRACT

As being a broad-spectrum, organophosphorus insecticide and acaricide, quinalphos is widely used against a range of pests and has a great threat to aquatic systems. Although the methods for detecting and predicting the harmful effects of chemicals on non-target organisms are traditionally perfected by in vivo experiments, cell culture methods that were used widely in recent decades are also an important tool for these kinds of research. In order to evaluate the effects of quinalphos on liver organotypic culture system of zebrafish, tissue cubes (1-2 mm3) were prepared by dissection and slicing of the liver tissues, embedded in agarose and cultured. The cubes were exposed to three different concentrations of quinalphos (2, 4 and 8 mg/L) for 24 and 96 hours. By performing the comet assay as an emerging tool for cytotoxicity, it was detected that quinalphos causes DNA damage. Increased levels of catalase, superoxide dismutase and glutathione-s-transferase were also measured. All of these parameters were noted as concentration- and time-dependent. Our data suggest that organotypic liver tissue culture of zebrafish is a practical alternative to the whole fish..

Keywords: *Zebrafish liver organotypic culture, quinalphos, oxidative stress*

1. INTRODUCTION

Pesticides are widely used in agriculture to enhance food production by eradicating unwanted insects and controlling disease vectors (1). These chemicals have made great contributions to plant protection but at the same time, their unbounded and indiscriminate applications have resulted in serious health and environmental problems (2). It has been estimated that only 0.1 % of the applied pesticides reach the target pests and the remaining 99.9% find their way to different components of the environment (3). Pesticides are one of the major contaminants of our environment and many of them persist for longer durations (4), and pose great threats to the health of humans and non-target organisms (5).

Organophosphorus insecticides (OPIs) are the most usually applied pesticides, accounting for 50% of global insecticidal use due to their extensive insecticidal property, low mammalian toxicity, a lesser amount of persistence and rapid biodegradability in the environment (6,7). Unfortunately OPIs lack target specificity and cause severe, long-lasting population effects on terrestrial and aquatic nontarget species, particularly fish (8, 9).

Quinalphos (QP: O,O diethyl O-2 quinoxalinoxalinyl phospharothionate) is an OPI that extensively used in agriculture for pest eradication (10). It is effective against a wide range of pests of cotton, groundnuts, rice, tea, coffee, soybeans and so forth. QP has been classified as a moderately hazardous pesticide by WHO but has become a matter of concern because of its potentiality and hazardous effect on nontarget organisms. the primary target of QP action is the inhibition of AChE activity, the enzyme that degrades the neurotransmitter acetylcholine in cholinergic synapses. Experimental evidence showed that QP, besides its inhibitory effect on AChE, also induced oxidative stress.

Oxidative stress, defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses (11). It occurs when the critical balance between oxidants and antioxidants is disrupted owing to the depletion of antioxidants or excessive accumulation of the reactive oxygen species (ROS), or both, leading to damage

Pesticides are known to modulate antioxidant defense systems and to cause oxidative stress in aquatic organisms via ROS production. Pesticides are known to regulate antioxidant defense mechanisms and to cause oxidative stress in aquatic organisms via ROS Production (12, 13, 14). ROS such as hydrogen peroxide (H_2O_2) and the free radicals superoxide (O2 •) and hydroxyl radical (HO•) can react with biological macromolecules and produce enzyme inactivation, lipid peroxidation (LPO), DNA damage, and protein oxidation, resulting in oxidative stress (13, 15, 16). To reduce the adverse effects of ROS, fish possess an antioxidant defiance system similar to other vertebrates that use enzymatic and nonenzymatic mechanisms. The most important antioxidant enzymes are superoxide dismutase (SOD; EC 1.15.1.1), catalase, (CAT; EC 1.11.1.16), and glutathione-S-transferase (GST; EC.2.5.1.18, GST) (17; 18). One of the most important targets of ROS is the membrane lipids which undergo peroxidation (LPO). Thus, the estimation of LPO has also been successfully employed to signify oxidative stress and most frequently used as a biomarker in the evaluations of toxicological assays.

Fish are mainly used to evaluate the situation of aquatic systems and physiological changes in fishes serve as biomarkers of environmental pollution, and thus can ben used for the quality assessment of the aquatic system (19, 20, 21, 22). Zebrafish is one of the commonly used ecotoxicological models for understanding interactions of xenobiotics with living organisms. Especially in ecotoxicological studies, zebrafish is widely used for this purpose. From an ethical perspective, researchers need to minimize animal use in experiments. So that in vitro methods replace in vivo methods day by day. In vitro, toxicological methods are widely used for the assesment of chemicals effects. In this perspective, 2D and 3D culture methods

are used. Understanding the biochemical and metabolic characteristics of 3D models are important criteria for promoting their application in this field of ecotoxicology. A major promise in vitro systems is to obtain mechanism derived information that is considered pivotal for adequate risk assessment. Although two-dimensional (2D) cell culture enabled biologists to observe and manipulate cells and laid the foundation for cell and molecular biology, they do not completely recapitulate the three dimensional (3D) organization of cells and extracellular matrix (ECM) within tissues and organs. In the present study, we tried to explore genotoxic, biochemical effects of quinalphos with using in vitro organotypic liver 3D culture.

2. MATERIALS AND METHODS 22

2.1. Test Compound and Zebrafısh Maıntenance

Test compound, quinalphos was commercially obtained from local suppliers (Ekalux). Wild type zebrafish specimens were reared in glass aquaria (60 x 30 x 30 cm) of 40 L water capacity and carried out to standard husbandry procedures (23).

2.2. Liver Organotypıc Culture

Male healthy zebrafishes were selected randomly and anaesthetized by MS222 (0,5 mg/L, Sigma), liver samples were dissected and transferred into phosphate-buffered saline solution (PBS, Sigma). Tissue samples that were randomly pieced small cubes and embedded into %1 agarose dissolved in PBS; transferred into six-well plates. Leibovitz's 15 (L15) cell culture medium supplemented with %10 FBS were added to plates for incubation in 28 °C overnight.

2.3. Quınalphos Exposure

Quinalphos stock solution (10 mg/L) was prepared. Three different concentrations (2, 4, 8 mg/L) of test solution were diluted from stock with complete culture media. Organotypic culture of liver samples was exposed to different concentration of quinalphos with two different exposure duration. (24-96 hours).

2.4. Comet Assay

The alkaline comet procedure was performed. Briefly fully frosted slides were layered with normal melting point agarose (NMPA) and dried; then 100 µL of low melting point agarose (LMPA) were mixed with each disaggregated tissue sample and applied as a second layer onto the pre-coated slides. Finally the third layer of LMPA was added on top. The slides were placed in freshly prepared lysing solution (NaCl 2,5 M, EDTA 100 mM, Trizma base 10 mM (pH: 10), 1% Triton X-100 and 10% DMSO) for 24 h at 4 °C to leave the DNA uncovered; and were immersed in alkaline buffer (NaOH 300 mM and Edta 1 mM, pH > 13 for 30 min to allow the unwinding of DNA. Electrophoresis was conducted for 20 min at 25 V (0,66 V/cm) and 30 mA at the same pH. The slides were then placed in neutralizing buffer (pH: 7,5) three times each for 5 min. and dried at room temperature. They were stained with 50 µL of ethidium bromide for 1 min. The stained nucleoids were examined at 40x in an epifluorescent microscope (Leica LM200) with a digital camera (Leica) and images were evaluated with Image J. Tail lengths were also measured and statistical analyses were performed by SPSS v14.0 statistical program (IBM).

2.5. BIOCHEMICAL ANALYSES

2.5.1. Sample Preparatıon

The post mitochondrial fractions from the cultured tissues were used for biochemical assays. Cultured tissues were washed in ice-cold 1,15 % KCl solution, blotted and weighed. The tissues were homogenized with a homogenizing solution (50 mM phosphate buffer pH 7,4

containing 1 mM EDTA, 1 mM dithiothreitol (DTT), 0,15 M KCl and 0,01 % (w/v) PMSF). Homogenization was performed at 4 C by using homogenizer (sartorius), and centrifuged at 10,000 rpm for 20 min at 4 °C with a refrigerated centrifuge (Eppendorf 5810 R). The supernatants were stored at -20 °C until performing biochemical analysis.

2.5.2. Assay Of Lıpıd Peroxıdatıon

Lipid peroxidation was determined by thiobarbituric acid (TBA) reaction (24). The absorbances were read at 532 nm after the removal of any flocculated material by centrifugation. The amounts of thiobarbituric acid reactive substances (TBARS) were then calculated by using an extinction coefficient of 1.56 x105 M^{-1} cm^{-1} and expressed as nmol TBARS formed/mg protein.

2.5.3. Assay Of Antıoxıdant Enzymes

Superoxide dismutase (SOD) activities were measured based on the inhibition of oxidation of NADH by SOD (25). One unit of enzyme activity was defined as 50% inhibition of oxidation of NADH in the reaction. The reaction rate was recorded at 340 nm and expresses as units/mg protein. Catalase (CAT) activities were determined based on the decomposition rate of H_2O_2 by the enzyme. Absorbance was measured at 240 nm and enzyme activity was expressed as units/mg protein. One unit of catalase activities were defined as decomposition of 1.0 nm of H_2O_2 to oxygen and water per minute at pH: 7.4 and 25 C. Glutathione –S-Transferase (GST) activities were measured by using 1-chloro-2, 4-dinitrobenzene as a substrate (26). The reaction rate was recorded at 340 nm and the activities were expressed as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 Mm-1CM-1.

3. RESULTS AND DISCUSSION

Comet assay or single nuclear electrophoresis are commonly used methods to observe DNA damage and genotoxicity can be identified by measuring tail lengths. As expected, highly genotoxic effects of quinalphos are seemed to be depended on concentration and exposure duration (Figure 1). In rats genotoxicity of quinalphos was reported with similar findings obtained by micronucleus and comet assay (27).

Figure 1. Tail lengths of DNA damaged cells were depended on concentration and exposition time.

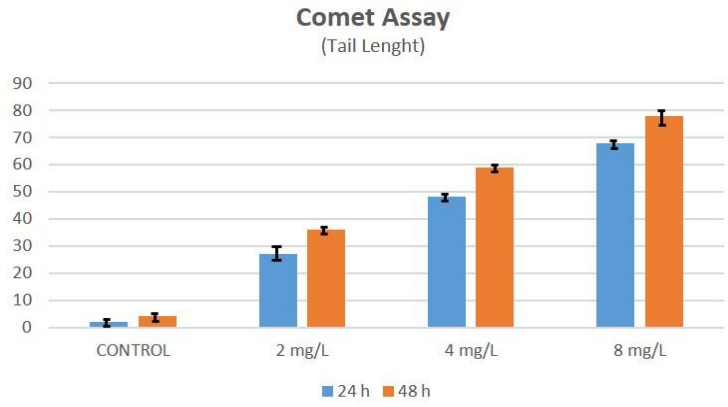
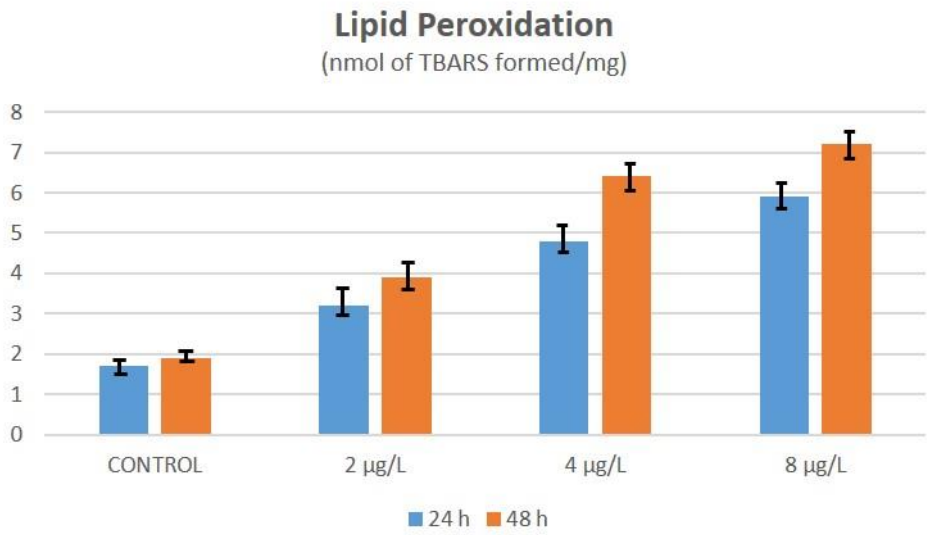


Figure 2. shows the results of MDA analysis in the organotypic liver culture of zebrafish after 24 h and 96 h of exposure. There was a significant increase in MDA levels that showed the level of lipid peroxidation in liver tissue in all test concentrations when compared with control groups. Lipid peroxidation (LPO) has been widely used for a biomarker of oxidative damage in fish exposed to xenobiotics (28). LPO is the process of oxidative degeneration of

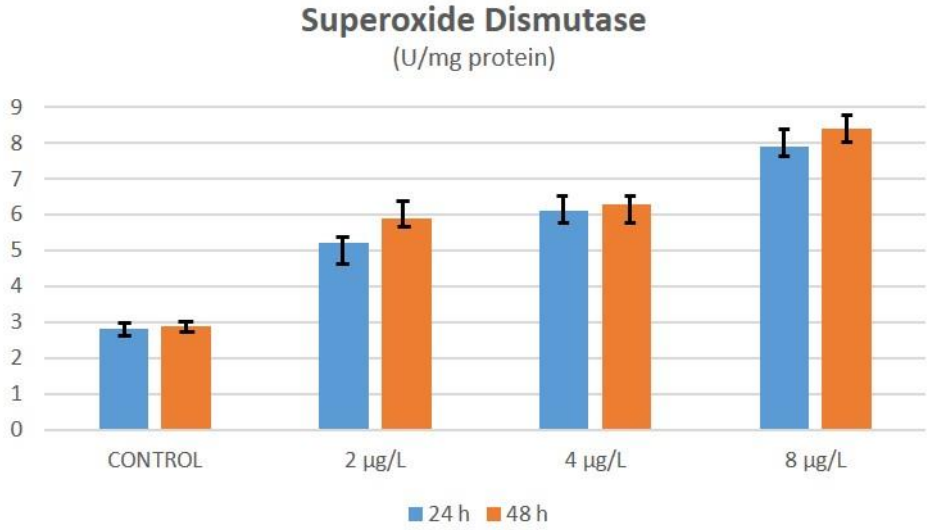
polyunsaturated fatty acids (PUFAs) that is essential for membrane function, structural integrity and inactivation of several membranes bound enzymes (29). MDA is a major oxidation product and the measurement of MDA provides a convenient index of LPO (30). A similar increase in MDA level in the fish liver was reported chronic exposure to lindane, an organochlorine pesticide in an in vivo study (31). Also another study showed that MDA levels in the liver of O. Mykiss were increased by exposure to methyl parathion and diazinon (32). However, there is no data in the literature to combine about in vitro organotypic liver culture of zebrafish. Our observations led us to conclude that the administration of different concentration of quinalphos promotes the concentration of MDA levels in in-vitro 3D tissue culture system.

Figure 2. Lipid peroxidation of QP exposure organotypic tissue culture. Compared to controls lipid peroxidation was increased depending on the exposure time and concentration.



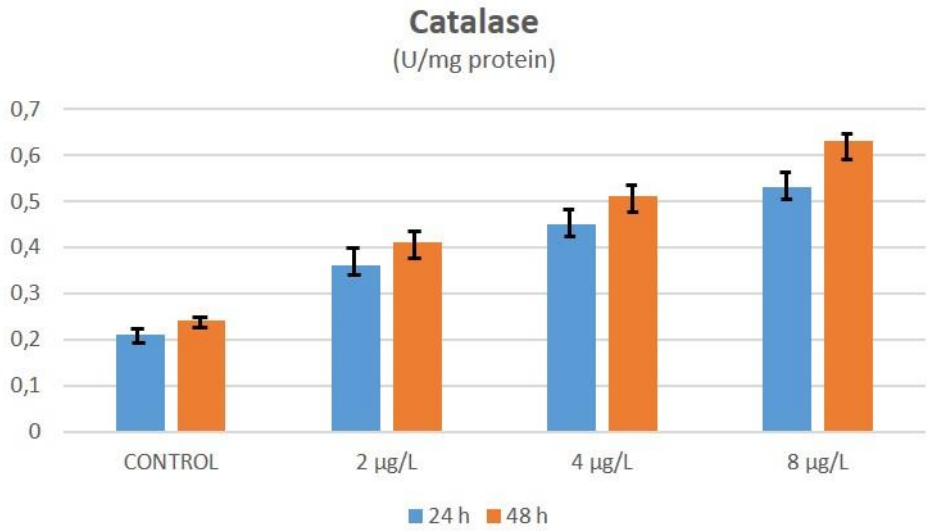
SOD levels of organotypic 3D liver cultures of zebrafish exposed to quinalphos were shown in figure 3. SOD is a group metalloenzymes that plays an important role in cellular defiance against free radical induced damage by catalyzing the dismutation of superoxide and produced in peroxisomes and mitochondria to H_2O and H_2O_2 (33; 34). In our study SOD levels were increased significantly in time and concentration depended. In other studies, performed in vivo techniques, it was observed that SOD activity in the liver of *D. labrax* after 96 h exposure to fenitrothion which is an OP pesticide, was increased (35). Different studies performed with different teleost species and different OP pesticides, it was clear that SOD is a common biomarker for exposure of OP pesticides in vivo. Our study also has shown that in vitro methods such as the organotypic culture of tissues like the liver could be used for assessment of xenobiotics, instead of whole organisms.

Figure 3. Superoxide Dismutase (SOD) activities of QP exposed liver organotypic tissue culture. Enzyme Activities increased significantly depending on increased concentration and exposure time.



CAT has been implicated as essential defiance against the potential toxicity of superoxide anions (36). It is an antioxidant enzyme that acts specifically on H_2O_2, forming oxygen and water. CAT is mainly located in the peroxisomes and it is responsible for the reduction of hydrogen peroxide produced from the metabolism of long-chain fatty acids in peroxisomes (37). In our study exposure of quinalphos in different concentrations and exposure time, CAT activity levels increased significantly depending time and concentration (Figure 4). In related studies hepatic CAT activity was determined to be increased after different OP pesticides such as cypermethrin and malathion in different teleost species such as *O. niloticus* and *L. rohita* (38).

Figure 4. Catalase (CAT) enzyme activities of organotypic liver tissue culture exposed to QP. CAT levels increased meaningfully depending on increased concentration and exposure duration.



The same increase level was observed in GST activities (Fig 5). The enzyme GST is involved in the detoxification of many xenobiotics and this plays an important role in protecting tissues from oxidative stress (38). An increase in GST activity has also been observed in the studies with *C. carpio* after 2,4-D and azinphos-methyl exposure (39). Studies have shown that GST is the one of the biomarker for exposure of OP pesticides and our study conclude that in vitro experimental designs could be used for analysis of GST activity for evaluating the toxicity of OPs.

Figure 5. Glutathione-s-Transferase (GST) enzyme activities of organotypic liver tissue culture exposed to QP. GST levels increased meaningfully depending on increased concentration and exposure duration.

It is clear that quinalphos is not only genotoxic, but also cause lipid peroxidation and oxidative stress. Levels of lipid peroxidation and oxidative stress were elevated by increased concentration and longer exposure duration. These findings are in accordance with the report of Padmanabha et al. (40) who examined acute and chronic exposure of quinalphos on *C. carpio*. As a conclusion, it is clear that 3D tissue culture gives more efficient results when compared with 2D; and organotypic tissue culture is a more efficient and simple method to observe the effects of chemicals when compared to in vivo methods. We have to inform that only seven zebrafishes were sacrificed for his investigation.

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