Chronic exposure to quinalphos shows biochemical changes and genotoxicity in erythrocytes of silver barb, Barbonymus gonionotus

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ABSTRACT

An in vivo study was carried out on the freshwater fish Barbonymus gonionotus to evaluate the genotoxic effects of the organophosphate quinalphos. The fish were exposed to sub-lethal doses of quinalphos (0%, 10%, 25%, and 50% of LC50) for a period of 30 days. Analysis of biochemical characteristics (protein and lipid contents of different organs), nuclear abnormalities of erythrocytes (NAE) and morphological abnormalities of erythrocytes (MAE) were performed on peripheral erythrocytes sampled at post-treatment intervals of 0 and 30 days. The biochemical results revealed a significant dose-dependent decline in protein and lipid contents and increase in the frequencies of NAE as well as MAE. Our findings also confirmed that the morphological deformations of erythrocytes in addition to NAE on fish erythrocytes in vivo are effective tools in determining the potential genotoxicity of organophosphates.

KEY WORDS: pesticides; organophosphate; erythrocytes; nuclear abnormalities; micronucleus test; genotoxicity

Introduction

Due to their lower persistence in the environment, organophosphorus pesticides are used judiciously to control a wide variety of agricultural pests as well as ectoparasites of fish in aquaculture. However, uncontrolled use of these pesticides in agriculture and public health operations has increased the scope of ecological imbalance and thus many non-target organisms have become victims. The effect of pesticides on fish population may either be acute, resulting in death, or chronic, where the effects may be longer and more difficult to quantify. Sub-lethal effects of organophosphate insecticides on fish have been shown to include vertebral malformation, alterations of blood constituents (Mostakim et al., 2015; Sadiqul et al., 2016), impaired reproduction, inhibition of AchE activity (Keizer et al., 1991), reduced larval and adult growth (Seiki, 1992), reduction of liver DNA, RNA and protein content (Sadiqul et al., 2016), and structural changes of kidney and liver (Mostakim et al., 2015; Nannu et al., 2015). Quinalphos, a toxic organophosphorous pesticide for human systems, finds a wide applicability to control pests of various crops like paddy, sugarcane and potato (Suvardhan et al., 2005).

The biochemical changes occurring in the body of the organisms give first indication of stress. Exposure to environmental stressors can induce oxidative stress in cells and result in a decrease in reducing potential and metabolic transformation to reactive intermediates (Simmons et al., 2011). Reactive oxygen species (ROS) induce damage to proteins, nucleic acids, and lipids leading to various cellular dysfunctions including apoptosis and necrosis (Simmons et al., 2011). Alterations in the biochemical parameters show toxic stress in the treated animals, especially in blood and blood forming organs. Erythrocyte abnormality test (change in nucleus and morphology of erythrocytes) is one of the preeminent diagnostic tools to evaluate the genotoxicity caused by pollutants present in the aquatic ecosystem. Fish erythrocytes are distinct from mammalian erythrocytes because they possess a nucleus and their interpretation
in form of morphological changes became an important bioindicator of pollution. The formation of erythrocyte alterations, especially nuclear alterations, were first described by Carrasco et al. (1990) and various other abnormalities like blebbled, notched, binucleated and lobed nuclei have also been used as possible indicators of genotoxicity (Ayllón & García-Vázquez, 2000). Although the mechanisms responsible for nuclear abnormalities have not been fully explained, these abnormalities are considered to be indicators of genotoxic damage and they may thus complement the scoring of micronuclei through routine genotoxicity surveys. The present study was undertaken to examine the chronic effect of quinalphos on certain biochemical parameters, possible genetic damage on nuclear abnormalities of erythrocytes (NAE), and morphological abnormalities of erythrocytes (MAE) of silver barb (Barbonymus gonionotus), a representative fish which is available in our native water bodies and is variously affected by pesticides.

Materials and methods

Fish and chemicals

Silver barb (Barbonymus gonionotus), belonging to the family Cyprinidae, was chosen for this study because of its common availability in Bangladesh and also due to the proven sensitivity to genotoxic chemicals (Sadiqul et al., 2016). Specimens of juvenile silver barb (approximate age 30 days) with average weight and length of 5.23±1.05 g and 6.34±0.69 cm, respectively, were purchased from a local fish farm in the district of Mymensingh, Bangladesh. Before the experiments, without any selection, they were acclimated under laboratory conditions for 7 days at a population density of 10 specimens in 30 l aquaria, at 25 °C and 14:10 h dark-light modes. The fish were fed once a day with commercial fish pellets (Mega Fish Feed Co.). Feces and pellet residues were removed daily by suction. Animals were maintained and used in accordance with the guidelines of the Bangladesh Agricultural University.

An organophosphate pesticide, quinalphos 25% EC (Emulsifiable Concentration) [O,O-diethylO-quinoxaline-2-yl phosphorothioate, Square Pharmaceuticals Ltd. (Pesticide Unit) with 98.50% purity, Bangladesh] was collected from the authorized dealer of the pesticide in original sealed container from Mymensingh, Bangladesh. The expiration date of the test substance was checked prior to initiation of the treatment and found to be suitable for exposure.

Determination of LC50

An acute toxicity (LC50) test was conducted by the static renewal bioassay method to determine the toxicity of quinalphos on the freshwater fish B. gonionotus. The fish were exposed to various concentrations of quinalphos for 96 hours. The required quantity of quinalphos was drawn directly from this emulsified concentration using a variable micropipette. For determining the LC50 value, the data were subjected to Finney’s probit analysis (Finney, 1971). The concentration at which 50% survival/mortality occurred in quinalphos treated fish was taken as the median lethal concentration (LC50) for 96 h, which was 1.4 ppm.

Sub-lethal toxicity testing on protein and lipid contents

In the sub-lethal toxicity tests, 90 fish were exposed to 10%, 25% and 50% of the LC50 of quinalphos for a period of 30 days and the protein and lipid contents of different organs were assessed. The control (0% quinalphos) was run simultaneously. Changes in the levels of protein and lipid of the fish body were recorded on days 0 and 30.

Total protein determination

The protein concentration was determined by Lowry’s method according to the procedure described by Gerhardt et al. (1994). In this procedure, the whole body of the experimental fish was blotted dried, weighed, homogenized with 0.1 N NaOH, filtered and supernatant was taken to estimate total protein. Homogenate of 0.1 ml was taken and diluted to 1 ml with 0.1 N NaOH and 5 ml reagent “C” (2% of Na2CO3 in 0.1 N NaOH and 0.5% CuSO4 at the ratio of 50:1) was added to each tube and mixed thoroughly. The test tubes were kept for 10 minutes at room temperature. Then 0.5 ml of reagent “E” (Folin-Ciocalteu reagent) was added, mixed and allowed to stand for 30 minutes at room temperature. Optical density readings were taken at 620 nm. A blank was taken using 1 ml of 0.1 N NaOH. The percentage of protein recovery from the fish was defined as the ratio of protein yield obtained during the extraction process by spray drying to the amount of protein estimated Kjeldahl protein multiplied by 100 and was calculated as follows:

\[
\text{Percent protein recovery} = \frac{\text{recovered protein}}{\text{Kjeldahl protein}} \times 100
\]

The total protein yield from the fish was defined as the concentration of protein in the raw material multiplied by the average weight of part and was calculated as follows:

\[
\text{Total protein yield (mg)} = \frac{\text{recovered protein (mg)}}{\text{weight of raw material (mg)}} \times \text{average weight of fish part (mg)}
\]

Total lipid determination

Total lipid content was assessed by using the Folch method (Folch et al., 1957). Briefly, 500±0.1 mg of powdered oven dried tissue was mixed with 5ml of chloroform: methanol (2:1) mixture tightly covered with aluminum foil and kept at room temperature for 24 hours. It was then filtered by using Whatman no.1 filter paper and the filtered extract was taken in a pre-weighed petridish and oven-dried. The petridish was weighed with lipids and the difference in weight with pre-weighed petridish was taken as total lipid content and the percentage was calculated.

Abnormality test of erythrocytes

Silver barb were placed in the aquaria containing dechlorinated tap water (control) and three different concentrations of quinalphos corresponding to 10%, 25% and 50% of LC50, each having three replications. The experimental water was renewed twice a week and fish were fed twice
a day. For the nuclear abnormality and morphological abnormality tests of erythrocytes, blood samples were obtained on days 0 and 30 from the caudal vein of specimens following the exposure to quinalphos. A total of 90 silver barbs (at least five fish per concentration per duration group) were used for the experiments.

Examination of the frequency of the nuclear abnormality and morphological abnormality of erythrocytes

On each sampling, the fish were removed from the aquaria and immediately anesthetized with clove oil (5 mg/l). Peripheral blood of five fish from each group was collected from the caudal vein using a heparinized plastic syringe and smeared onto precleaned slides. The smear was fixed with methanol for 10 min and stained with 5% Giemsa and rinsed with distilled water. Then the slides were air dried overnight and mounted with DPX to be observed under an Olympus microscope (CX21) using 100× objective lens. All slides were coded and scored blind. Only cells with intact cellular and nuclear membrane were scored. For the scoring of micronuclei (MN), the following criteria were adopted from Fenech et al. (2003): the MN should be separated from or marginally overlap with the main nucleus as long as there is clear identification of the nuclear boundary; MN should have similar staining as the main nucleus. Nuclear abnormalities other than MN in erythrocytes were classified according to Carrasco et al., (1990). Briefly, blebbed (BL) had a relatively small evagination of the nuclear membrane and contained chromatin; pyknosis (PK) is characterized by the condensation of chromatin in the nucleus of the erythrocyte. The criteria for the identification of morphological abnormalities of erythrocytes (MAE) require them to be dissimilar from the regular erythrocyte cells with an oval shaped structure and condensed nucleus (Sadiqul et al., 2016).

Statistical analysis

Statistical analysis between experimental and control values for protein and lipid contents were performed by Fishers’ student t-test and the percentage decreased or increased over control was calculated for each value. In case of abnormality of erythrocytes, data were tested for normality via the Kolmogorov–Smirnov test and homogeneity of variance via Bartlett test before all statistical analyses. The data from nuclear abnormalities of erythrocytes (NAE) and MAE were used and analyses were performed by one-way analysis of variance (ANOVA), followed by the Tukey test. Statistical analysis was performed with the SPSS 16.0 computer program (SPSS, Chicago, IL, USA). All the data were expressed as means±SD. Differences were considered statistically significant at p<0.05 and p<0.01.

Results and discussion

Chronic effect of quinalphos on protein contents of different organs

The changes in protein levels in different tissues of fish after 30 days of treatment with sub-lethal concentrations of quinalphos are presented in Table 1. Protein content in the control group in the liver, muscle and intestine are normally distributed and the values are approximately 22.58, 18.60 and 21.10 mg/g, respectively. Bogard et al. (2015) found almost similar proximate compositions of protein in B. gonionotus with values of 17.5%, 15.74% and 18.4%, respectively. While analyzing the changes in protein, it became clear that they fluctuated in different sub-lethal concentrations. The level of protein was found to be decreased significantly (p<0.05) in all the tissues in comparison to control. The extent of decrease in protein was greater in the high concentration upon 30 days exposure. In the tissues, the trend of decrease in protein contents was liver>muscle>intestine. Exposure of silver barb to sub-lethal concentrations of quinalphos produced changes in the protein contents of the liver, muscle and intestine. The reductions in tissue protein of silver barb indicated rapid utilization of energy stores to meet the energy demands required by the environment. The observed reductions in tissue protein on treatment with sub-lethal doses of quinalphos were suggestive of proteolytic activity, possibly to meet the excess energy demands under toxic conditions. Similarly to our findings, Sastry and Siddique (1984) reported decreased protein content in liver, muscle, kidney, intestine, brain and gill when Channa punctatus had been treated with quinalphos. In this study, the highest rate of decrease in protein content was found in the liver as the metabolic center for detoxification and also the host in absorbing greater organophosphate pesticide residues. Increasing protease activity under stress condition clearly suggests that quinalphos induces high protease activity which leads to the formation of higher free amino acid content causing hepatotoxicity (Dwivedi et al., 1998). For instance, it has

Table 1. Total protein content (mg/g) in the liver, intestine and muscle, and lipid content (mg/g) in the gonad (both male and female) of silver barb exposed to zero (control) and sub-lethal concentrations (0.14, 0.35, and 0.70 ppm are 10, 25, and 50% of LC50, respectively) of quinalphos for a period of 30 days. The changes (%) with respect to the control are shown in parentheses.

| Biochemical components | Organ   | 0% (control) | 10% | 25%     | 50%     |
|------------------------|---------|--------------|-----|---------|---------|
|                        | Liver   | 22.58±1.62   | 20.03±0.96 (−11.29%) | 18.28±0.8 (−19.04%) | 16.38±2.23 (−27.45%) |
|                        | Muscle  | 18.6±0.76    | 17.2±0.35 (−7.53%)  | 15.12±0.42 (−18.70%) | 14.62±1.25 (−21.40%) |
|                        | Intestine | 21.1±0.42   | 19.03±0.41 (−9.81%) | 17.51±0.49 (−17.01%) | 17.12±0.38 (−18.86%) |
|                        | Gonad   | 70.94±1.15   | 27.59±0.87 (−61.11%)** | 21.5±1.54 (−69.69%)** | 19.28±1.94 (−72.83%)** |

Values are mean±SD of five replicates. Asterisks indicate significantly different (*p<0.05, **p<0.01)
thus been shown that large doses of acetaminophen can result in increased degradation of the hepatic cytochrome P450 (CYP) enzymes in vivo. The lysosomal pathway, mainly mediated by cathepsin D, appears to be the major proteolytic pathway involved in the degradation of the P450 enzymes induced by toxic doses of acetaminophen (Zhang et al., 2004). Decreasing protein content might also be attributed to the destruction or necrosis of hepatocytes and consequent impairment in the protein synthesis machinery. The amount of protein depends on the rate of protein synthesis or on the rate of its degradation. The amount of protein may also be affected due to impaired incorporation of amino acids into polypeptide chains (Ram et al., 2003). However, depletion of protein content in the studied organs may be caused by increased utilization of protein to meet the energy demand when the fish is under stress conditions. It is thus inferred that exposure to quinalphos in sub-lethal doses affects protein metabolism.

**Chronic effect of quinalphos on lipid contents of gonads**

Present observations indicated that the rate of decreasing lipid in gonads was higher in high concentration of quinalphos as compared to control upon 30-day chronic exposure (Table 1). The change in lipid contents in the organ studied was due to pesticidal stress, indicating the change in activity of an organism. A declining trend in percentage change of lipid contents in gonads was observed as −61.11%, −69.69%, and −72.83% after exposure of 30 days at 10%, 25% and 50% concentrations of LC50, respectively (Table 1). The toxicity of pesticides may be accumulated in the reproduction tissue and causes disturbance in reproduction activity and thus decrease in the fish population, leading to a decrease in economic fortune. In the present work, the considerable decrease in total lipids in gonads might be due to drastic decrease in glycogen content in the tissue, which is an immediate source of energy during toxic stress conditions. For such a type of mechanism Riediger et al. (2007) have suggested that quinalphos do not dispose of reactive groups capable of inducing oxidative stress directly, inducing rather a secondary oxidotoxicity by the main metabolite 2-hydroxyquinoxaline (HQO), which should easily undergo redox reactions. Extra clues for such a secondary toxicity by a quinalphos metabolite came from studies on phototoxicity (Behrends et al., 2004). When directly applied to the depilated guinea pig skin, quinalphos did not elicit any photosensitization, yet phototoxicity became apparent when the pesticide was administered orally to mice, so that the effect would have resulted from a metabolite formed in the organism (Behrends et al., 2004, 2007). Recently Riediger et al. (2007) showed that HQO was cytotoxic to ciliates, dinoflagellates and rotifers, to increase hydroperoxides and protein carbonyl, and to be genotoxic in an Ames test. In addition, previous researchers like Shanthi et al. (2009) have suggested that the decrease in lipid content in the tissues of fish could be due to the utilization of lipid for energy demand under the condition of stress or due to increased lipolysis or mitochondrial injury, which affect the fatty acid oxidation mechanism. Thus any changes in lipid metabolism affect the ability of fish to store energy obtained from nutrients and in long term the stability to survive (Sancho et al., 1997).% 0.975 2.505 42.2775 13.8425

**Figure 1.** Frequency of individual nuclear abnormalities of erythrocytes (NAE) of silver barb exposed to quinalphos as well as control is shown in Figure 1. The most commonly detected nuclear abnormality was micronucleus (MN). After chronic exposure to quinalphos there was a significant increase (p<0.01) in the frequency of MN in relation to control. Also, a significant increase (p<0.01) in the frequency of pyknosis (PK) and blebbed (BL) were observed. In general, frequencies of NAE were found in the following order: MN>PK>BL. Examples of NAE are presented in Figure 2. The morphological and different nuclear abnormalities in erythrocytes are considered the main biomarkers to assess the pollution-related toxic effects and demonstrate the associations between mutagenicity and chronic health effects. Micronucleus (MN) assays with fish have shown their potential as an in situ biomonitoring tool for detecting genotoxic agents in the aquatic environment (Bolognesi & Hayashi, 2011). An increment of the number of MN is an indirect marker of structural and numeric chromosomal abnormalities caused in the cells by many agents. As chromosome aberrations and MN formation are adjacent cytogenetic indicators of genotoxicity, it was concluded that quinalphos might be capable of inducing significant chromosome damage (Chauhan et al., 2015). Rupa et al. (1991) also demonstrated that quinalphos can induce chromosome breaks/fragments in mice and human lymphocytes. In the present study, quinalphos was found to be capable of inducing significant genotoxic damage. Fish species
could be model organisms for determining the extent and formation of MN in response to aquatic genotoxins. Thus MN and other NAE tests in our study revealed that extensive use of pesticide can cause genetic damage in the silver barb.

Except MN, other NAE observed in the study were blebbed and pyknotic cells, designating genotoxicity of the existing contaminants in the silver barb. The increased frequency of erythrocytes with blebbed, pyknosis and cells with nuclear micronuclei indicating mutagenic and genotoxic effects in the present study could be due to higher production of caspase activated DNase, which results in cleavage of nuclear and cytoskeletal proteins and aneuploidy (Hussain et al., 2012). According to many authors (Barsiene et al., 2006; Ergene et al., 2007), observation of variations in the nucleus shape could represent an alternative approach to detect cytogenotoxicity. These changes in nuclear morphology have been found to originate from a genotoxic event as a result of exposure to xenobiotic contaminants. In our study the findings of nuclear abnormalities in silver barb exposed to quinalphos showed a significant increase after chronic exposure, indicating that nuclear abnormalities are a better biomarker for exposure to an organophosphate like quinalphos than MN alone. Steenken (1989) also hypothesized that abnormalities arise due to damage caused to the genetic material by free radicals produced under oxidative stress. Previously, various studies have shown that these nuclear alterations could be due to cytotoxic impacts of a variety of toxic compounds which induce chromosomal abnormalities (Lim et al., 2009; Sharaf et al., 2010).

**Frequency of morphological abnormalities of erythrocytes (MAE)**

The frequency of morphological abnormalities of erythrocytes (MAE) in silver barb exposed to quinalphos is shown in Figure 3. The most commonly detected morphological alteration of erythrocytes was ellipsoid.

It was observed that the frequencies of abnormalities in all treatments were in the order: ellipsoid > twin > spindle > vacuolated cytoplasm > fusion > enucleated > schistocyte. Examples of MAE are presented in Figure 4. In the control group erythrocytes were oval shaped with regular condensed nucleus (Figure 4a), whereas in the quinalphos treated groups erythrocyte morphology showed enucleated (Figure 4b), schistocytic (Figure 4c), fusion and twin (Figure 4d), ellipsoid and spindle shaped (Figure 4e), vacuolated cytoplasm (Figure 4g), and others (including normal shaped cells and a few other abnormal cells). In the present study, various morphological changes including ellipsoid, enucleated, schistocyte, fusion, spindle...
shaped, twin, and vacuolated cytoplasm of erythrocytes were significantly increased in the treated groups. It can be hypothesized that such types of MAE may undergo morphological alterations in their plasma membrane affecting surface deformability and make erythrocytes more susceptible to burst when crossing small capillaries. The various morphological alterations might be due to concentration dependent increase of lipid peroxidation products in erythrocytes of fish exposed to quinalphos. It is well established that many toxicants with oxidative stress potential may assault DNA, resulting in clastogenic, molecular and morphological damage (Jha, 2008). Nuclear and morphological changes might also happen due to increase in production of reactive nitrogenous and oxygen species as results of oxidative stress to the mitochondrion (Hussain et al., 2012). In mitochondria, toxicants can bind to crucial enzymes and respiratory protein complexes uncoupling oxidative phosphorylation, leading to the generation of reactive oxygen species (ROS), and acting on the plasma membrane causing lipid peroxidation, or directly on the DNA molecule, causing damage (Ahmad et al., 2006). Although organisms have antioxidant defenses to protect tissues against oxidative damage, if the rate of ROS production exceeds the capacity of the defense mechanisms, injury can occur in the cells and DNA (Cadet et al., 2003), inducing damage to their bases, causing breaks in the DNA strand (Reinecke & Reinecke, 2004). Our results also support the findings of extensively studied quinalphos exposed to different biological systems. For example, sub-lethal concentration of quinalphos promoted the concentration of malondialdehyde and the antioxidative systems in the liver and kidney of Cyprinus carpio (Hemalatha et al., 2016; Padmanabha et al., 2015), alteration of the pro-oxidant/antioxidant balance in subcellular fractions of Mozambique tilapia gill (Ramya et al., 2015). Further, Ateeq et al. (2002) elaborated the sequence of cellular degradation under the impact of toxicants and suggested that toxicants could cause hypoxic conditions which result in depression of ATP, leading to abnormal shape of erythrocytes. Thus, the presence of quinalphos induced such changes in silver barb causing cytotoxic damage resulting in death of the fish.

Conclusion

The present study confirms that quinalphos poses changes in the biochemical profiles indicating their rapid utilization for providing excess energy to cellular biochemical processes in order to cope with stressful conditions.
Further, hematological and mutagenic effects in fish may be hazardous to the other species when exposed to this organophosphate.

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Conflict of interest

The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of the paper.

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