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Genotoxicity and Lipoperoxidation Produced by Paraquat and 2,4-Dichlorophenoxyacetic Acid in the Gills of Rainbow Trout (*Oncorhynchus mikiss*)

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Herbicides are compounds manufactured mainly to destroy weeds in an attempt to increase crop production. Their use has increased markedly in the past two decades; however, a solid amount of information has established that an excessive exposure to these chemicals may provoke a number of toxic effects in most organisms (Viveros 1990). This has raised concern about aquatic organisms exposed to herbicides, particularly in fishes, because of probable lower production, and the possible health damage to humans consuming contaminated organisms.

Paraquat (1,1'-dimethyl-4-4'-bipyridium dichloride) (PQ) is widely used as a broad spectrum herbicide. However, it is known that the compound modifies the level of several enzymatic activities in fishes and affects cardiac contraction and opercular ventilation, effects which may alter early development of the organism (Tortorelli et al. 1990).

PQ is readily converted by one electron-reduction to a free radical which reacts very rapidly with molecular oxygen. High concentrations of oxygen enhance its toxicity, an effect that has been related to various causes, one of which is the formation of superoxide (Tomita et al. 2001).

PQ genotoxic potential has been studied in different organisms since the 1970's. The studies have revealed genic alterations in bacteria, and chromosome damage in plants, Drosophila, mammalian cell lines, and mouse bone marrow; however, negative results have also been reported (Ortiz et al. 2000; Selipes et al. 1980). These include gene and chromosome effects measured in human lymphocytes, gene damage detected in bacteria, in mouse bone marrow and in spermatogonial cells, as well as dominant lethality in the mouse.

The systemic and selective herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is mainly used on broad-leaf species. In small amount, it mimics the action of the plant hormone indolacetic acid, but in large concentrations it is highly cytotoxic. Extensive genotoxic research has shown contradictory data; both positive and negative results have been reported in bacteria, plants, Drosophila, cultured mammalian cells, and in in vivo studies (Gollapudi et al. 1999; Madrigal-Bujaidar

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et al. 2001). An interesting characteristic of 2,4-D is its activity as a peroxisome proliferator reported in several mammals, which may be related with an increase in oxidative stress (Blair et al. 1990). In fish, the most common route of entry of 2,4-D is through the gills and, to a lesser extent, through the skin. In these organisms, the chemical may produce hemorrhages, enlarged kidney, impairment of renal functions, and liver degeneration (Gomez et al. 1998).

Considering the contradictory genotoxic reports on both pesticides, their relation with the production of free radicals and their contamination of the aquatic environment, the objectives of the present work were: a) to determine the level of DNA damage produced by the pesticides in the gills of the rainbow trout (*Oncorhynchus mykiss*), and b) to establish the potential of the pesticides to induce lipid peroxidation in the same organ.

MATERIAL AND METHODS

Paraquat (98% pure, Kow=-4.2) and 2,4-D (98% pure, Kow=45) were obtained from Sigma-Aldrich Chemicals (St Louis Mo. USA.). For the unicellular electrophoresis (comet) assay, the following compounds were purchased from the same company: low melting point agarose, dimethylsulfoxide, trizma base, triton X, EDTA, and ethidium bromide.

Normal melting point agarose, phosphate buffer saline (PBS), and the trypan blue stain were obtained from Gibco (Grand Island NY. USA). Sodium hydroxide and sodium chloride were purchased from Baker S.A. (Mexico City).

Concerning the lipid peroxidation assay, glutatione (reduced form, GSH), butyl hydroxytoluene (BHT), sodium dodecil sulphate (SDS), and 2-thiobarbituric acid were purchased from Sigma Chemical (St Louis Mo. USA). Sodium chloride, ferric chloride, sodium bicarbonate, potassium chloride, phosphoric acid, hexane, and isopropanol were obtained from Baker S. A. (Mexico City).

Rainbow trout (*Oncorhynchus mykiss*) weighing 3.6-4.6 g were obtained from the pisciculture center "El Zarco", located 30 Km west of Mexico City. The fishes were acclimated to laboratory conditions for 15 days prior to the experiment. They were maintained in an aquarium with reconstituted water at 9±1°C (hardness as $CaCO_3$=150 mg/L; alkalinity as $CaCO_3$=13 mg/L; dissolved oxygen=12 mg/L, and pH= 8.2). Water in the aquarium was air-saturated with air pumps.

To determine acute toxicity, three replicate groups with 12 organisms each, were placed in aquaria which contained 45 L of reconstituted water plus 2,4-D (316, 346, 389, 436, and 489 mg/L), or PQ (0.055, 0.066, 0.083, 0.116, and 0.133 mg/L). A control group with dechlorinated water was included in the assay. Water in the aquarium at 9±1°C was air-saturated with air pumps. The behavior and survival of the pesticide treated organisms were observed for 24 h and compared with the response of the control animals. The LC_{50} (24-h LC_{50}) was calculated by computerized log-probit analysis. For the comet assay, four groups with 12 each were put in aquaria containing 45 L of reconstituted water plus 2,4-D: 0.0, 5.0 (the

threshold limit concentration), 75.0, and 150.0 mg/L. The assay on PQ was carried out with concentrations of 0.0, 0.0005 (the threshold limit concentration), 0.005, and 0.05 mg/L. After 1, 4, and 8 days of chemical exposure, the fishes were decapitated, the gills were dissected and gently macerated in PBS to obtain a final cell suspension of approximately 10,000 cell/ml.

The comet assay was made according to the method described by Singh et al. (1988) with slight modifications. Normal melting agarose (110 μL) was pipetted on fully frosted slides which were covered with coverslips until the agarose solidified.

The cell suspension (10 μL) was mixed with 75 μL of 0.5 % low melting (37° C) agarose, placed on the normal agarose layer, and covered with coverslips for 5 min at 3° C; then, a third layer of low melting agarose was added and allowed to solidify at the same temperature. After the agarose polymerized, the slides were lowered into freshly prepared lysing solution at pH 10 (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, and 1% Na sarcosinate). The slides were incubated at room temperature in the dark for 2 h, after which they were placed on a horizontal electrophoresis tray with a freshly made buffer (0.3 NaOH/1 mM EDTA) that covered the slides for 15 min. For electrophoresis, the power supply (BioRad) was set at 25 v, 265-270 mA, pH<13, and the slides were exposed to this current in the dark at 3° C for 20 min. Afterwards, they were placed in another tray and coated with a neutralizing buffer (0.4 M Tris, pH 7.5) in the dark for 5 min. The slides were overlain with 90 μL of ethidium bromide (10 mg/mL), covered with coverslips and examined at 400X using a fluorescent microscope (Carl Zeiss) equipped with a B excitation filter and a 530-nm barrier filter.

The length and width of the DNA mass (tail length/nucleus width ratio, T/N) were determined using an ocular micrometer disk in 50 nuclei/dose/time. Cell viability corresponded to more than 90% for each dose/time by using the trypan blue exclusion technique.

The supernatant was transferred to another tube and evaporated with a nitrogen current in a water bath at 35° C. Next, the following substances were added to the lipid extract: SDS (0.28M) and GSH (130 mM dissolved in NaCl 0.15 M), ferric chloride (30.8 mM) dissolved in phosphoric acid (2.5 M), a methanolic solution of BHT (136 mM), and thiobarbituric acid (52.1 mM). After 30 min at 95° C, we added KOH (10.7 M) and cold ethyl acetate in agitation for 1 min, and then a centrifugation at 1,000 g was made for 10 min.

Finally, the color was spectrophotometrically assayed at 537 nm. For comparison, we established a curve type with tetramethoxypropane (TMP) using concentrations of 0.081, 0.162, 0.243, 0.324, 0.405, 0.486, and 0.567 uM. The results were expressed as nM of MDA/g of gill.

The experimental data obtained in the comet and the peroxidation assays were analysed by ANOVA, and the mean differences of each group were compared using the Dunnett test for multiple comparisons against a single control. Differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Rainbow trout from the control group displayed a typical behaviour with no signs of anomalies; however, the two high concentrations of 2,4-D and of PQ gave rise to some animals with signs of apnea (gasp) and white spots on the skin. The results of the LC_{50} showed that 2,4-D was less toxic (4,314 times) than PQ (Fig. 1). The LC_{50} for 2,4-D was 362.38 m/L (95% confidence limits: 326.76-401.89), and for PQ was 0.084 mg/L (95% of confidence limits: 0.075-0.094). We used the equation $Y= -28.86 + 13.23\ X$ to calculate the LC_0 of 2,4-D (which was equal to 150 mg/L), and the equation $Y= 26.65+ 20.10\ X$ for the LC_0 of PQ (which gave a result of 0.05 mg/L).

With respect to the comet assay, both compounds were found to be strong inducers of DNA damage. Figure 2 shows that all tested concentrations of 2,4-D, at the three of timepoints study, caused a significant increase in the T/N ratio of the gill tissue with respect to the control value. With the two highest tested concentrations (75 and 150 mg/L), an approximate threefold increase was observed on day 4, an observation that was maintained in the last exposure time, where the strongest effect was produced. With respect to PQ, the main difference in regard to the effect described for 2,4-D was a reduction in DNA damage on day 8 with 0.05 mg/L of the chemical (Fig. 2).

The lipid peroxidation level in the control animals had a mean of 0.02 nmol MDA/g wet tissue with no statistical differences. With respect to the PQ peroxidation level, a significant increase was observed with all concentrations tested, also showing that the level was maintained along the three concentrations; however, the strongest effect was found on day 8 of the treatment. The highest increase (103 times with respect to the control value) was obtained with 0.05 mg/L (Fig. 3). Results concerning 2,4-D are shown in the same Figure; an increment in this parameter was observed throughout the experiment. The strongest effect was observed with 150 mg/L on day 8 of exposure, 220 times the control value. However, an increase was detected after a one day exposure.
Acute lethality tests have been useful in providing rapid estimates of the concentrations where xenobiotics cause direct, irreversible harm to the test organisms. In this study, fish mortality increased with respect to the concentrations of both herbicides; however, probit analysis indicated that PQ was clearly more toxic to *O. mykiss* than 2,4-D. This result agrees with acute toxicity data of the compounds in a variety of vertebrate and invertebrate organisms, which establish a higher mortality risk for PQ (Metelev et al. 1983).

The comet assay primarily measures DNA strand breakage in single cells, which can be a reflection of direct damage or of alkali-labile sites and of repair enzyme-mediated breakage. It is a sensitive and reliable method used in various toxicological areas, including genetic ecotoxicology (Cotelle and Férard 1999). In aquatic organisms, this assay has gained support due to the relationship of positive effects with carcinogenesis and reproductive damage related with pollution. In this effort, cultured fish cells have been used to test the genotoxicity of various xenobiotics, although most studies evaluate the effect of the compound by

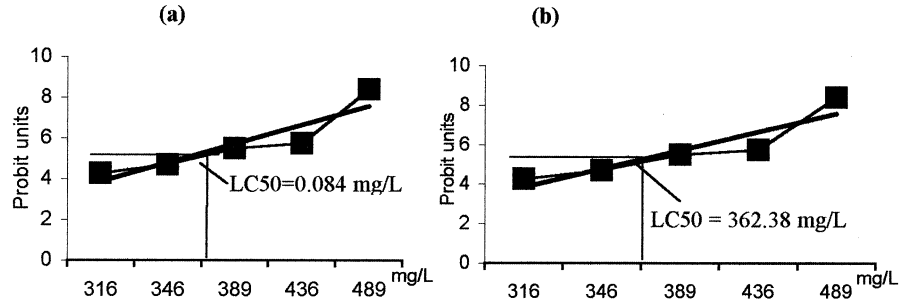

Figure 1. LC_{50} values obtained in rainbow trout treated for 24 h with paraquat (a) and 2,4- dichlorophenoxyacetic acid (b)

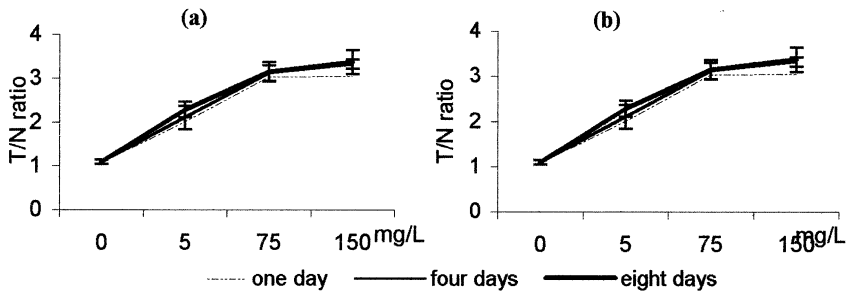

Figure 2. DNA damage determined in the gills of the rainbow trout exposed to paraquat (a) and 2,4-dichlorophenoxyacetic acid (b). The three tested concentrations of both herbicides were statistically significant along with treatment time versus the control. ANOVA and Dunnett tests, $P \geq 0.05$.

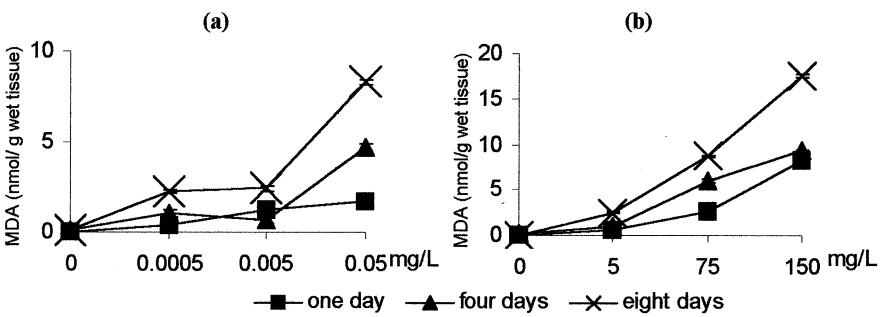

Figure 3. Lipid peroxidation level in the gills of rainbow trout exposed to paraquat (a) and 2,4-dichlorophenoxyacetic acid (b) 1, 4, and 8 days of exposure. The three tested concentrations of both herbicides were statistically significant along with treatment time versus control. ANOVA and Dunnett tests, $P \geq 0.05$.

into the organism, or mixed to water or food (Cotelle and Férard 1999). Our results on the genotoxic effect of the two pesticides were obtained by the sensitivity of the comet assay. Significant DNA damage was noted with both

compounds starting from the first concentration tested. The response was concentration and time dependent; however, a decrease appeared in the last period of PQ treatment, probably as an outcome of DNA repair stimulated by the mutagen. Our data established clearly that the studied pesticides are DNA damaging agents in *O. mykiss*. This damage may be induced directly in the DNA molecule or through the production of free radicals; the latter possibility is supported by the strong peroxidation induced by the pesticides, and by the parallelism observed between this effect and the rate of DNA breaks. The peroxidation of polyunsaturated lipids generates a range of substances that possess DNA damaging potential. This includes lipid peroxydes and various species that contain unpaired electrons, such as the alkoxyl and peroxil radicals. In addition, a range of genotoxic carbonyl-containing compounds are formed, such as malondialdehyde, various 4-hydroxy-2-alkenals and a number of 2-alkenals (Burcham 1998).

Under our experimental conditions, lipid peroxidation was higher with 2,4-D, an event probably related with the mechanism of action of the chemicals. While PQ needs a reduction to produce free radicals, 2,4-D alters Ca2+ homeostasis and activates proteases and lipases accelerating phospholipid hydrolysis (Arguello et al. 1990). Oxidative stress by PQ has been verified in several models, including oxidative stress-inducible specific genes and modifications on a number of mitochondrial enzyme activities (Konstantinova and Russanov 1999). It is also well documented that free radicals are related with DNA strand breakage and deoxyribose degradation, and that PQ is involved in these kind of damage (Salam et al. 1993). Moreover, PQ has shown positive results in human lymphocytes evaluated with the comet assay (Ribas et al. 1995). In this matter, research on 2,4-D is less conclusive; for example, a study with the comet assay in *Rana catesbeiana* showed a negative result (Clemens et al. 1997), a difference with our result probably related with the herbicide presentation (2,4-D amine), and with the exposure time in a different organism (24 h). However the chemical is a peroxisome proliferator in fish; since peroxisomes contain enzymes to catalyze hydrogen peroxide, the activity may be related with an excess of free radicals. Besides, 2,4-D may interact with the cell membrane releasing a chemical (probably a second messenger) which reaches the nucleus and modifies the transcription process (Hassal 1990). Various studies indicate there is no 2,4-D genotoxicity (Gollapudi et al. 1999). Contrary findings by other authors, however, suggest it is pertinent to continue evaluating it in two ways; first, establishing the genotoxic potential in different organisms, which coincides with our purpose; then, risk assessment. Finally, our study also agree with previous research which suggest the effectiveness of rainbow trout to monitor toxic effects of xenobiotics.

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