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Long-term genotoxic effect of monocrotophos in different tissues of freshwater fish *Channa punctatus* (Bloch) using alkaline single cell gel electrophoresis

*Daoud Ali, Sudhir Kumar**

Molecular & Human Genetics Laboratory, Department of Zoology, University of Lucknow, Lucknow, 226007, India

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ABSTRACT

Monocrotophos, commonly known as azodrin, is one of the organophosphate pesticides extensively used in agricultural practices throughout the world. However, little information is available on its long-term genotoxic effects in different tissues of fish using genotoxicity biomarkers. The objective of the present study was to detect DNA damage, induced by monocrotophos in freshwater teleost fish *Channa punctatus* using the comet assay. The LC_{50}-96 h value of technical-grade monocrotophos was estimated for the fish species in a semi-static system. On the basis of the LC_{50} value, the sublethal and nonlethal concentrations were determined. The DNA damage was measured in the gill, kidney and lymphocytes as the percentage of DNA in comet tails of fish exposed to different sublethal and nonlethal concentrations of monocrotophos. In general, significant effects ($P<0.01$) from both concentration and time of exposure were observed in exposed fish. It was found that the tissues at all concentrations exhibited the highest DNA damage on day 4, after which there was a decline in percentage tail DNA. The comparison of DNA damage among tissues at different concentrations indicated that the gill cells were more sensitive to the pesticide than the kidney cells and lymphocytes. This study also explored the utility of the comet assay for *in vivo* laboratory studies using fish for screening the genotoxic potential of various agents.

1. Introduction

Organophosphate compounds are widely used as pesticide in agriculture and industry in recent years, due to their biodegradability and short environmental residence time. These exert their toxic effects on pests by phosphorylating the serine residues of the active site of AChE, leading to the accumulation of acetylcholine (Eto, 1974a). Monocrotophos [dimethyl (*E*)-1-methyl-2-(methylcarbomoyl) vinyl phosphate] is an important broad-spectrum systematic organophosphate pesticide, widely used in India. Laboratory data indicate that monocrotophos is a potent toxicant and controls a variety of pests (Corey et al., 1965).

The pesticides reach the aquatic environment through direct applications, spray drift, spraying, washing from the atmospheric precipitation and runoff from agricultural lands and ravage the biotic life (Nemcsok et al., 1987; Thangnipon et al., 1995). As a consequence of its widespread use, it has been detected in ground and surface rain-water (Waite et al., 1992). Fish are particularly sensitive to the environmental contamination of water and therefore, pollutants may significantly damage specific physiological and biochemical processes (Waite et al., 1992; Mahttiessen et al., 1995; Storm et al., 2000). Pesticides and heavy metals bioaccumulate in aquatic organisms and also exert harmful effects on humans through the food chain (Elia et al., 2006).

* *Corresponding author*. Tel.: +91 522 2740074.
E-mail address: panwarsk_p@yahoo.com (S. Kumar).

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Monocrotophos is classified as an extremely hazardous pesticide in India (ITRC, 1989). Its mutagenic potential has been extensively studied. It is reported to be genotoxic to aquatic organisms (Banu et al., 2001), *Salmonella typhimurium*, *E. coli*, *Saccharomyces cerevisiae*, and mouse lymphoma. It has also been found to induce sister chromatid exchange in Chinese hamster ovary cells. Joshi and Desai (1988) and Swami (1997) have reported that monocrotophos decreases the DNA content in *Tilapia mossambica*.

The genotoxic effects of environmental pollutants can be monitored using a broad range of both *in vitro* and *in vivo* biomarker assays but the comet assay is gaining popularity over others since it can detect low levels of DNA damage and requires shorter period to complete the study (Gedic et al., 1992).

Therefore, the present study was undertaken with a view to evaluate the effect of various concentrations of monocrotophos on the DNA damage of different tissues of *Channa punctatus* using the comet assay. The fish, *C. punctatus* was selected for the present study since it is edible, commercially valuable, breeds throughout the year and is distributed all over India.

2. Materials and methods

For the present study, technical-grade monocrotophos (36% effective concentration) was purchased from the market (manufactured by Hindustan Insecticides Ltd., New Delhi, India) since the monocrotophos of this grade is commonly used in field practices.

2.1. Procurement of animals

The healthy specimens of *C. punctatus* (Bloch) (Family Channidae; Order Channiformes) were procured from local outlets. The average wet weight and length of specimens were 30.0±2.0 g and 14.0±3.0 cm, respectively. The specimens were treated with 0.05% $KMnO_4$ solution for 2 min to minimize the dermal infection. These were acclimatized under laboratory conditions for 15 days in a semi-static system before exposure to monocrotophos. The fish were fed boiled eggs and goat liver. The faecal matter and other wastes were siphoned off daily to reduce the ammonia content in water. The fish were cultured in optimal conditions as suggested by Bennett and Dooley (1982) and no mortality was observed during the acclimatization.

2.2. Determination of sublethal concentrations

The acute toxicity bioassays to determine the LC_{50}-96 h value of monocrotophos were conducted in the semi-static system with the change of test water on every alternate day. Feeding was discontinued 24 h before exposure to test chemicals. A facility for oxygenation of the test solution was provided with the help of showers fixed above the test chambers. The acute bioassay procedure was based on standard methods (APHA et al., 2005). The stock solution of monocrotophos was prepared by dissolving it in acetone.

Median tolerance limit (LC_{50}) of monocrotophos to fish was estimated by exposing six groups of fish (10 fish per group) to different target concentrations of monocrotophos and the experiment was repeated twice to obtain the LC_{50}-96 h value of the pesticide for the selected fish, *C. punctatus*.

The LC_{50} value of monocrotophos was determined as 19.10 mg/l for *C. punctatus*, following the probit analysis as described by Finney (1971). Based on the LC_{50} value, the three test concentrations of monocrotophos viz., sublethal 1 (1/4th of LC_{50} = ~4.78 mg/l), sublethal 2 (1/8th of LC_{50} = ~2.39 mg/l) and nonlethal (1/12th of LC_{50} = ~1.59 mg/l) were estimated.

2.3. *In vivo* exposure experiment

The fish specimens were exposed to the three aforementioned test concentrations of monocrotophos in a semi-static system. The exposure was continued up to 21 days and tissue sampling was done at intervals of 1, 4, 7, 14 and 21 days at the rate of 5 fish per duration. The fish maintained in tap water and exposed to acetone (0.1%) were considered as control and solvent control groups, respectively.

On each sampling day, the whole blood, gills and kidney were collected and immediately processed for comet assay. The blood samples were collected from the fish by caudal vein puncture technique using heparinized syringe. The physico-chemical properties of test water, namely temperature, pH, dissolved oxygen, conductivity, chloride, total hardness and total alkalinity were analyzed by standard methods (APHA et al., 2005).

2.4. Alkaline single cell gel electrophoresis (SCGE)

The alkaline single cell gel electrophoresis (SCGE)/comet assay was performed as a three-layer procedure (Singh et al., 1988) with slight modification in which conventional microscopic slides were used (Klaude et al., 1996). The slides were cleaned with 100% ethanol and flame dried. Immediately after collection, the solid tissues (~50 mg) were washed three times with chilled phosphate buffer saline (Ca^{2+}–Mg^{2+} free) to remove blood cells and transferred to ice-cold homogenization buffer (1-X Hanks' balanced salt solution, 20 mM EDTA, 10% dimethyl sulphoxide (DMSO), pH 7.0–7.5). The tissue was cut into small pieces and homogenized to obtain a suspension which was centrifuged at 3000 rpm at 4 °C for 5 min. The cell pellet was finally suspended in chilled phosphate buffered saline for comet assay. The lymphocytes were separated from the whole blood using histopaque density gradient centrifugation and diluted 20 fold for the comet assay. Viability of the lymphocytes, gill and kidney cells was evaluated by trypan blue exclusion method (Anderson et al., 1994). The tissue samples showing cell viability higher than 84% were further processed for comet assay. In brief, about 15 μl of cell suspension (approx. 20,000 cells) was mixed with 85 μl of 0.5% low melting-point agarose and layered on one end of a frosted plain glass slide, pre-coated with a layer of 200 μl normal agarose (1%). Thereafter, it was covered with a third layer of 100 μl low melting-point agarose. After solidification of the gel, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris pH 10 with 10% DMSO and 1% Triton X-100 added fresh) overnight at 4 °C. The slides were then placed in a horizontal gel electrophoresis unit. Then fresh cold alkaline electrophoresis buffer (300 mM NaOH, 1 Mm Na_2EDTA and 0.2% DMSO, pH>13.5) was poured into the