

Haemopoietic Function and Flowcytometry of Pronephric Kidney in *Clarias batrachus* L. under the Impact of Organophosphate- Sumidon 40

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Abstract

The impact of different doses of SUMIDON-40, an organophosphate pesticide on the haemopoiesis of *Clarias batrachus* L. (Clariidae) was tested. The small lymphoid haemoblast decreased and consequently the young erythrocytes increased significantly in both sublethal and LC50 dose treated fish. The percentage of mature reticulocyte increased significantly in LC50 dose treated group. The erythropoietic efficiency was increased significantly in fish exposed to sublethal dose and decreased in fish exposed to LC50 dose. Among leucocytes, the percentage of neutrophil increased significantly in both treatment groups and the percentage of macrophage increased only in LC50 dose treated group. The overall leukopoietic efficiency, however, was increased significantly in both treatment groups. Flowcytometric analysis of cell cycle in pronephric kidney cells confirmed that the cell death was increased and DNA synthesis was significantly decreased with increasing dose of pesticides. It is concluded that habitat deterioration caused by agrochemical impedes haemopoiesis in this species resulting to reduction of endurance levels to pollution.

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Introduction

Effective control of toxic substances is often the major factor in determining the success of fish culture or management of wild fish population. For this, haemopoiesis in fish is of considerable importance since any undesirable change in the environment or stress factors or ontogenetic, developmental and aging processes ought to have its influence on haemopoiesis. Knowledge of the physiological and toxicological responses of freshwater fish to stress has been generated from vast number of research. Comprehensive reviews are also available in recent literature (Sancho et al. 2000; Rajaguru et al. 2003; Sarmento et al. 2004). However, limited information on haemopoiesis in fish (Bihari et al. 2003; Wang et al. 2004; Gulden et al. 2005) has led to gaps in the understanding of fish response to agrochemicals, particularly in those, inhabiting shallow wetlands of tropics.

Clarias batrachus being obligatory air breathing fishes inhabit oxygen poor environment and are susceptible to erythropoietic stress to which metabolic adjustments are made. The dynamics of the progenitor erythropoietic cells in the haemopoietic tissues in fish have been found to be correlated with the environmental demand (Chudzik and Houston 1983; Alvarez et al. 1994; Lai et al. 2006). The impact of pesticide on the haematology of fish has been extensively studied and well documented (Larsson et al. 1980; Pandey et al. 1981; Venkateshwarlu et al. 1990) since pesticides are still being used extensively by farmers in many countries. Pesticides have been implicated in immunodepression in certain fishes as reported earlier (Ghosh and Banerjee 1992; Hart et al. 1997; Dautremepuits et al. 2004).

However, very few reports (Dheer et al. 1987; Pandey et al. 2005) are available on erythropoiesis and leucopoiesis under pesticidal impact. Therefore the present study on *Clarias batrachus* was aimed at understanding the impacts of an organophosphate pesticide on fish health using haemopoietic functions and flow cytometric analysis of cell cycle in the haemopoietic tissues as biomarker.

Materials and Methods

Clarias batrachus, an obligatory air breathing fish were collected from experimental fish farms at Kulia, Kalyani, Nadia, and West Bengal. Fish of 25-40 g weight and 15.5-18 cm length were selected from the catch and randomly divided into three groups of 10 and kept in large (570-1) glass aquaria where they were acclimatized to laboratory condition for two weeks. Fish were fed at the rate of 2 % of body weight daily at 11.30 h with commercial floating catfish pellets.

Stock Acclimation

All the experiments were conducted in 100-l glass aquaria, using dechlorinated well water source. The physico-chemical analyses of water were carried out following standard methods (APHA 1998). Average water temperature was 29.5 °C. Dissolved oxygen concentrations varied between 4.8 and 5.6 mg l⁻¹ and free CO₂ recorded was traced to 3.5 mg l⁻¹. Total alkalinity was recorded between 8.8 to 11 mg l⁻¹ and total hardness values varied between 150-260 mg l⁻¹. The pH of the water was between 7.2 -7.8.

Acute toxicity testing (for 72 hours)

Clarias batrachus specimens were exposed to a wide range of concentrations of Sumidon 40. The sublethal dose and LC 50 value for 72 hours were estimated following range finding test and short term definitive test (APHA 1998). In the present experiment, the 72 h LC 50 value was recorded as 0.1 ml l⁻¹ dose with adult *Clarias batrachus* on static bioassay. The concentration of 0.025 ml l⁻¹ was found to be a sublethal dose as the fishes were unaffected despite long periods of exposure and was able to recover from and acclimatize to such dose very easily.

Preparation of tissue imprint and staining

For the erythropoietic study, imprint or impression technique was employed following Mahajan and Dheer (1980). A small piece from the head kidney (pronephric kidney) was carefully dissected out and an impression or imprint of the tissue was made on clean glass slides. The preparation was air dried and first stained with Graham Knoll's benzidine method (Graham-Knoll 1918). The preparation was then counterstained with Giemsa, using the technique of Mahajan and Dheer (1979).

Imprint preparation of haemopoietic tissue was fixed in formal alcohol just for 30 seconds. The fixed slide of each tissue was rinsed with tap water and then with distilled water. The slide was air dried and stained with Benzidine stain for 7 minutes. After thorough washing with distilled water and air-drying, the slide was again stained with Giemsa stain for 3-4 hours. The stained slide was rinsed in a stream of water (pH 6.8). The film was dried and then mounted using DPX as a mounting medium and placed under a microscope for observation. The terminology for colour used for differentially staining is that of colour atlas of Dominguez and Villalobos (1947).

Cell differentiation criteria and nomenclature

Differential criteria and accompanying nomenclature were established after comparison of preliminary findings with data on humans (Hayhoe and Flemans 1992; Hoffman et al. 1995) as well as findings on channel catfish (Fijan 2002) and on *Oreochromis niloticus* (L.) and *Cirrhinus mrigala* (Ham.) (Maiti et al. 2000). The description of maturation stages within cell lineages was based on the following criteria; (i) earliest morphologically recognizable precursor

stages were distinguished by a large nucleus with loose chromatin and nucleoli as well as by a relatively sparse basophilic cytoplasm (ii) maturation was marked by loss of nucleoli, progressive chromatin condensation and reduction in nucleus size, shift of the cytoplasm to nucleus surface ratio in favour of the cytoplasm (except in maturation of some lymphoid cell), gradual shaping of morphology typical for mature blood cells (iii) the ageing process leading to cell death involved in reduction in cell and nucleus size, becoming increasingly condensed, pyknotic and eventually fragmented.

Haematopoietic efficiency determination

Evaluation of haematopoietic efficiency of *Clarias batrachus* was done using Nauber's double haemocytometer following the method described by Homechaudhuri and Jha (2001).

Flowcytometric analysis of cell cycle and DNA-synthesis of haemopoietic cells

To determine cell cycle phase distribution of nuclear DNA, head kidney cells were fixed with 3% p-formaldehyde, permeabilized with 0.5% Triton x 100 and nuclear DNA was labeled with propidium iodide (PI, 125 $\mu\text{g}\cdot\text{ml}^{-1}$) after RNase treatment using cycle TEST PLUS DNA reagent kit. Cell cycle phase distribution of nuclear DNA was determined by FACS (fluorescence activated cell sorter) calibur using cellquest software (Becton Dickinson), fluorescence detector equipped with 488 nm Argon laser light sources and 623 nm band pass filter (linear scale). Total 10,000 events were acquired for analysis. Leuco GATE was used to reduce debris, or other contamination, if any, and analysis of flowcytometric data was performed using Mod Fit software (Becton Dickinson). Histogram analysis of DNA content (X-axis, PI-fluorescence) versus counts (Y-axis) has been displayed. After performing t-test, treatments were taken to be significantly different if the *P* values were less than 0.05.

Results

The cell types enumerated from the pronephric kidney imprints were – small lymphoid haemoblast (SLH), basophilic erythroblast (BE), polychromatophilic erythroblast (PE), acidophilic erythroblast (AE), young reticulocyte (YR), mature reticulocyte (MR), young erythrocyte (YE), mature erythrocyte (ME), lymphocyte, neutrophil and macrophage.

Sumidon 40 induced significant changes in the percentile distribution of haemopoietic cells and haemopoietic efficiency (Figs. 1, 2 and 3). A distinct decrease in the percentage of SLH was observed in the fish group exposed to both sub lethal (0.025 $\text{ml}\cdot\text{l}^{-1}$) and to LC 50 (0.1 $\text{ml}\cdot\text{l}^{-1}$) dose. The percentage of YE concurrently increased significantly in exposed fishes compared to control. The population of MR increased significantly in fishes exposed to LC 50 dose compared

to control (Table 1). Among the leucocytes, the percentage of lymphocyte decreased and the neutrophil cell population increased significantly in both treatment groups. The percentage of macrophage also increased significantly in fishes exposed to LC 50 dose.

Table 1. Effect of different doses of sumidon 40 on haemopoiesis in head kidney of *Clarias batrachus*.

Cell Types	% of cells \pm S.D. control	% of cells \pm S.D. exposed to sublethal dose (.025ml/l)	% of cells \pm S.D. exposed to LC 50 dose (.1ml/l)
SLH	30.60 \pm 1.80	12.96 \pm 00.62 *	10.01 \pm 2.23 *
BE	6.56 \pm 01.51	6.40 \pm 2.37	4.16 \pm 2.42
PE	4.79 \pm 01.11	3.61 \pm 00.89	3.63 \pm 00.88
AE	1.74 \pm 00.47	2.46 \pm 01.19	2.19 \pm 00.80
YR	8.50 \pm 3.98	11.98 \pm 1.55	8.63 \pm 00.95
MR	7.53 \pm 02.03	7.69 \pm 00.80	10.82 \pm 00.99 *
YE	6.53 \pm 00.70	9.71 \pm 00.50 *	11.14 \pm 01.43 *
ME	7.83 \pm 01.77	9.93 \pm 1.17	7.50 \pm 01.22
LYMPHOCYTE	16.03 \pm 6.91	12.44 \pm 2.20	12.63 \pm 02.04
NEUTROPHIL	7.28 \pm 1.17	16.96 \pm 2.96 *	27.17 \pm 00.98 *
MACROPHAGE	2.59 \pm 00.99	2.15 \pm 00.69	5.86 \pm 00.45 *

* Significant at 5%.

Figure 3 represents the comparative haemopoietic efficiency in treatment groups and control. The erythropoietic efficiency (EE) was significantly increased in fish group exposed to sub-lethal dose but decreased significantly in fishes exposed to LC 50 dose. The leukopoietic efficiency (LE) was, however, increased significantly in both treatment groups.

Flowcytometric analysis (Fig. 4)

Observation detecting percentage of cell death and DNA synthesis of cells in the head kidney revealed that cell death (M1) was augmented by the impact of pesticide Sumidon 40 at both sub lethal and LC 50 dose exposures. On the contrary DNA synthesis (M3) in the cells was affected by sub lethal dose and greatly inhibited on exposure to LC 50 dose.

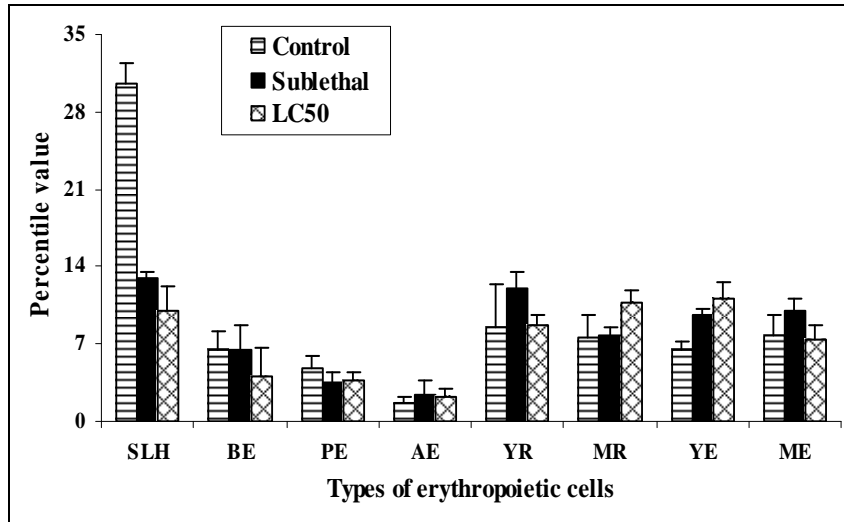


Fig. 1. Effect of different doses of Sumidon 40 on erythropoiesis in head kidney of *Clarias batrachus*.

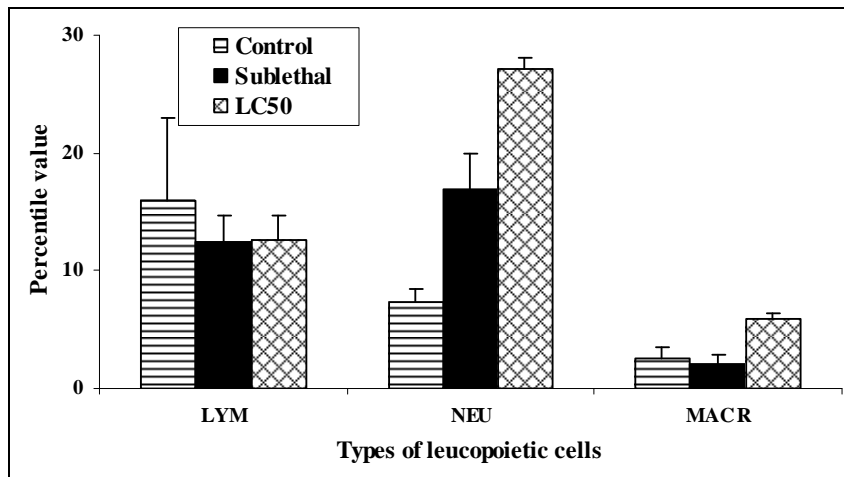


Fig. 2. Effects of various doses of Sumidon 40 on leucopoiesis in head kidney of *Clarias batrachus*.

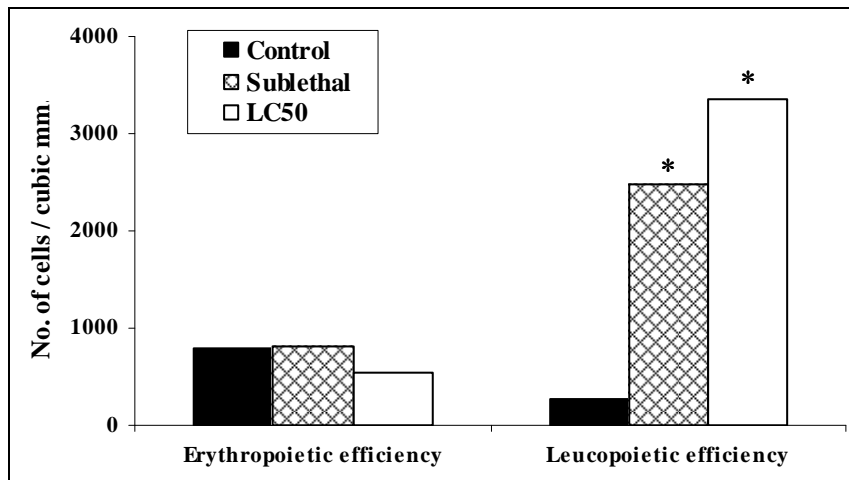


Fig. 3. Effect of different doses of Sumidon 40 on haemopoietic efficiency in *Clarias batrachus*.

* Significant at 5%.

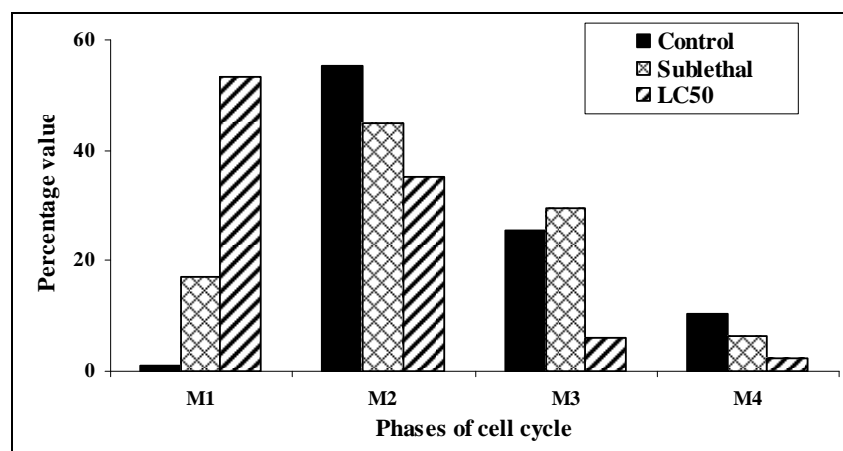


Fig. 4. Effects of various doses of Sumidon 40 on the head kidney cell cycles phase distribution in *Clarias batrachus*.

Discussion

Previous experiments indicated that organophosphate insecticides mostly caused increases in hematological variables (Natarajan 1984; Lal et al. 1986). Evidence also supports the idea that the effect is primarily due to histological damage to gill tissue that produces an internal hypoxia and subsequent stimulation of erythropoiesis (Areechon and Plumb 1990). The comparison of cytotoxic potencies also shows that in general the fish cell lines and the mammalian cell lines are almost equally sensitive towards the cytotoxic action of chemicals (Gulden et al. 2005). Furthermore, investigations revealing an increase in fish disease prevalence in polluted sites have suggested that these environments evoke stress responses which enhance susceptibility to disease (Brown et al. 1979).

Significant decrease of SLH cells in the exposed fishes in the present study is indicative of certain physiological stress response. Pesticide induced internal hypoxia due to gill damage invariably generates erythropoiesis to release more red blood cells in circulation. Therefore, due to rapid turnover of SLH cells to matured red blood cells through transient stages, the percentage of SLH declines. In case of emergency an increase in the number of erythroid precursor cells may occur, rapidly differentiating into young red blood cells. Otherwise, an increased release of stored younger erythroid cells into circulation from various marginal and defined storage sites is a possibility. The results of the present experiment clearly reflect both the above situations. Agarwal and Mahajan (1983) reported significant increase in the earlier stages of erythrocytic development (BE and PE) with a simultaneous decrease in the later stages (YR and MR) in pyridoxine deficient fish, *Channa punctatus*. The present result differs having significant increase in YR and MR in fish exposed to pesticide. Increase of Erythropoetic Efficiency (E E) (Fig. 3) upon exposure to sub lethal dose is indicative of the above facts.

Moreover agricultural pesticides could affect one or more of the immunological functions in aquatic organisms. Christin et al. (2004) documented altered immune response in frogs due to agricultural pesticide which contribute to their global decline by exposing them to certain threats from infections. In fish lymphocytopenia was observed following pesticidal stress (Johansson-Sjoberg et al. 1978). It has been presumed such stresses affecting immunological functions in fish make them more susceptible to various diseases (Snieszko 1974). From the present work, it seems that organophosphorus insecticides might have induced immunotoxicity in *Clarias* (Fig. 2) as significant increase of neutrophil cell population in both treatment groups has been observed. This fact is in agreement with previous works in lake trout, *Salvelinus namaycush* and coho salmon, *Oncorhynchus kisutch* (Walsh and Ribelin 1975).

Interestingly different classes of DNA cell cycle alteration found in the study of mussels (*Mytilus galloprovincialis*) mirror either acute or cumulative genotoxic effects of the surrounding environment of mussel hemocyte DNA (Bihari et al. 2003). Arsenic induced toxicity was studied in two fish cell lines JF (fin cells of *Therapon jarbua*) and TO-2 cells (ovary cells of Tilapia) in two ways to mimic acute and sub acute exposure. The results indicate that sodium arsenite induces apoptosis in JF cells probably by causing oxidative stress and disturbs the cell cycle of TO-2 cells (Wang et al. 2004). In his study with *Clarias batrachus* cell death associated with reduced DNA synthesis in head kidney was found to be augmented by the impact of sublethal and LC 50 dose of pesticide (Sumidon-40). Kaminsky et al. (2003) found osteosarcoma 143B cells with typical apoptotic and necrotic features on menadione treatment. Therefore, the mass decline of air breathing fish population in diverse geographic location can be explained from the stress related agrochemicals as evident from the present study of haemopoiesis.

Conclusion

The air breathing *Clarias batrachus*, normally inhabiting oxygen deficient shallow environment might be affected by even subtle changes in pesticidal concentrations. The study indicates that the haemopoietic impediments reduce the fish endurance to pollution resulting in population decline to arise concern.

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