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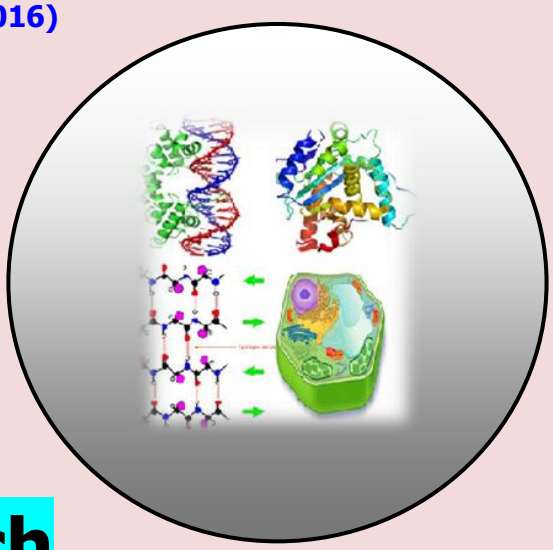

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RESEARCH PAPER

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Evaluation of Toxicity of Chlorpyrifos on the Haemocyte of *Pila globosa*

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ABSTRACT

Pila globosa (Mollusca: Gastropoda) is an important amphibian snail of Indian subcontinent capable of respiring both in land and water. This species constitute an important role in nutrition as it is the dietary source of protein for man, poultry and fish and potentially as aquacrop. Gastropods are important members of aquatic habitats and therefore present a high ecological relevance for freshwater ecosystem. Chlorpyrifos is an organophosphorous insecticide widely used in agricultural field against a broad range of insect pests. The freshwater bodies are particularly susceptible to contamination to pesticide by agricultural runoff during monsoon and from inadvertent overspray. Haemocytes are the circulating blood cells of mollusc and are capable of eliciting immunological response under the challenge of xenobiotics and infection. Toxicity of chlorpyrifos was investigated in the haemocyte of Pila globosa by exposing the animals to sublethal concentrations of toxin for 24h, 48h, 72h, 96h and 7 days in controlled laboratory conditions. The pesticide exposure inhibited the total haemocyte count in a dose and time dependent manner. Modulation in haemocyte density and neutral red retention time was observed in the toxin exposed animal. Depletion in intrahaemocyte cytotoxic molecule like nitric oxide (NO) suggests an immune-compromise in the animal. The pesticide exposure impaired the behavior and functions of haemocyte which is indicative of profound cellular stress and it also implies the nature of risk on the freshwater aquatic ecosystem under potential xenobiotic contamination.

Keywords: Pila globosa, Chlorpyrifos, Haemocyte, Neutral red and Nitric oxide.

INTRODUCTION

Agricultural field of India supports a wide variety of biodiversity including specific members of invertebrate phylum Mollusca. Few of the molluscan species inhabit in the regional environment of the subsoil

compartment shelter characteristics infaunas including *Pila globosa* (Mollusca: Gastropoda: Mesogastropoda), an edible amphibian snail (Athawale and Reddy, 2002). *P. globosa* is abundantly distributed both in the terrestial and freshwater ecosystem of Indian subcontinent (Ray et al. 2009). This species is suitably adapted for respiration for both in land and water. The foot of *P. globosa* is large, muscular and rich in protein, vitamin (A, B and D) and mineral and constitutes an important dietary item for lower income population (Roy and Prabhakar, 2009). Roy and Singh (2007) reported the ethno medical importance of meat of this species in curing diseases like ricket, nightblindness among tribal people of India. *P. globosa*, an important member of terrestrial and aquatic ecosystem is exposed to organophosphorous pesticide residues like chlorpyrifos for a considerable period of time as the pesticide is widely used by the farmer for protection of crop (Rittschof and Green, 2005). Chlorpyrifos is moderately lipophilic having water solubility of 1.4 mg/L and has a tendency to partition into soil, sediment and organic material (Poletika et al. 2002; Verma and Srivastava, 2001). The application of chlorpyrifos to temporary or flooded areas along with accidental spillage, spray drift, leaching and runoff from terrestrial applications have the potential to expose aquatic biota to residues. In this study toxicity of chlorpyrifos was examined in depth of relation to haemocyte function of *P. globosa* in controlled laboratory condition. Haemocytes, the circulating blood cells of molluscan haemolymph are capable of eliciting immunological response under the challenge of xenobiotics and pathogenic infection (Sauve et al. 2002). They exhibit diverse physiological functions including phagocytosis of non self particulates and generation of intracellular cytotoxic molecules. Total and differential haemocyte count is a marker of pollution which was determined both in control and pesticide exposed *P. globosa*. Damage to lysosomes, as a result of contaminant exposure has also been demonstrated in haemocyte. Capability of generation of intrahaemocyte cytotoxic molecule like nitric oxide (NO) is reported as potential strategy of immunological defense in mollusc which was estimated in the experimental model. The response of *P. globosa* in altered environmental condition validates the physiological and immunological parameters as biomarkers of aquatic toxicity in contaminated habitat.

MATERIAL AND METHODS

Collection and treatment of animal

The adult healthy *P. globosa* of uniform shell size (5-7 cm) were manually collected from selected wetlands of Coochbehar district of West Bengal. Animals were transported to the laboratory in rectangular plastic containers with dimension of 12'X18'X6' at a density of 4-6 individuals per box. Prior to experimentation, animals were acclimatized for 15 days in laboratory condition. During acclimatization, *P. globosa* were maintained in fresh supply of pond water with temperature of 29°C±3°C and the animals received uniform ration of illumination. During the course of acclimatization and experiment, the animals were fed with chopped *Hydrilla* (Ray et al. 2009). Routine replenishment of water was carried out in every 24 hours to avoid residual toxicity.

After a period of 15 days of acclimatization, animal were exposed to sublethal concentration of 0.05 ppm, 0.1 ppm and 0.15 ppm of aqueous formulations of chlorpyrifos dissolved in commercial preparation of Dursban (Chlorpyrifos E.C. 20%) for different spans of exposure, i.e. 24h, 48h, 72h, 96h and 7 days along with control. The pH of the solution was maintained at 7.2. Each experimental set consisted of 10 animals of same shell length. The experiment was carried out in static water environment.

Collection of haemolymph

Shells of the animals were cleaned by running tap water. From, control and post-treated animals haemolymph was collected by shell puncture method (Renwrantz et al. 1981) at a volume not exceeding 1ml per bleed per day. The bleeding and collection procedure was carried out at 4°C to prevent cell aggregation.

Haemocyte preparation

Haemocytes were sedimented by centrifugation of the haemolymph at 2000 rpm for 5-10 minutes. Cell density was adjusted with unit volume of sterile snail saline (SSS) (Adema et al. 1991) containing 4mM HEPES, 37mM NaOH, 36mM NaCl, 2mM KCl, 2mM $MgCl_2$, $2H_2O$, 4mM $CaCl_2$, $2H_2O_2$ with pH 7.8.

Cell viability

The viability of the haemocytes of *P. globosa* of all experimental variants was tested with 2% Trypan blue for 10 min following the dye exclusion principle.

Light microscopic study

A portion of the fresh haemolymph was placed on clean, sterilized glass slides in a moist chamber and the haemocytes were allowed to settle for 15-20 min at room temperature.

The adherent haemocytes were fixed with 1% glutaraldehyde for 5 min and rinsed with sterile snail saline. After fixation, the monolayer of haemocytes was stained with Giemsa's stain. The slides were then observed under a light microscope.

Total haemocyte count and Differential haemocyte count

Cell density was adjusted to unit volume with sterile snail saline (SSS) (Adema et al. 1991) and enumeration of cells was carried out under light microscope with Neubauer haemocytometer. Differential enumeration of diverse subpopulations was determined microscopically after Cain and Morado (2001).

Neutral red retention assay

The neutral red retention assay was done following the method of Lowe and Pipe (1994). Haemolymph was collected following shell puncture method of Renwarntz et al. (1981) in sterile snail saline so as to obtain a 1:1 v/v of cell per physiological saline suspension. The suspension obtained from both control and pesticide exposed *P. globosa* was spread on a slide (100µl/slide). It was then transferred to a light proof humidity chamber for 15 mins to allow the cells to adhere. The neutral red stock solution was made by dissolving 20 mg of dye in 1ml of dimethyl sulphoxide (DMSO) and the working solution prepared by diluting 10µl of the stock with 5ml of the sterile snail saline. 40µl of the neutral red (NR) working solution was added to both control and treated cell monolayer. After 15 mins incubation period, slides were examined systematically under a light microscope every 15 minutes. The time period between neutral red probe application and the appearance of the first evidence of dye loss from the lysosome to the cytosol or of other lysosomal abnormalities in at least 50% of the examined haemocytes, represent the neutral red retention (NRR) time.

Nitric oxide assay

The generation of the nitric oxide was measured as the amount of the nitrite released from the haemocytes with Griess reagent after Green et al. (1982). The concentration of the haemocyte suspension was incubated with equal volume of Griess reagent (1% sulphanilamide, 0.1% napthyl ethylenediamine dihydrochloride and 5% orthophosphoric acid) at 37ºC for 30 minutes in a humid chamber. The absorbance was recorded spectrophotometrically at 550 nm against a standard curve. A standard curve of sodium nitrite was used to determine generation of NO and was expressed as µM of NO generated per 10^6 cells.

RESULT

Cell viability

The average viability of haemocytes ranged from the highest value of 95.33± 3.01 under an exposure of 0.05ppm of chlorpyrifos for 24h to the lowest value of 88±1.32% under an exposure of 0.15ppm of chlorpyrifos for 96h (Table 1).

Subpopulation analysis

In the haemolymph, specific cells such as prohaemocyte (Figure 1A), agranulocyte (Figure1B), granulocyte (Figure 2B) and hyalinocyte (Figure 2A) were observed using light microscopes. Prohaemocytes were the smallest group of haemocytes bearing centrally placed round nuclei surrounded by rim of cytoplasm (Figure 1A). Agranulocytes exhibit the round or oval nuclei having basophilic cytoplasm (Figure 1B). This are lymphocyte like cells characterized by a relatively large and oval shaped nucleus, scanty cytoplasm with none or no or a few cytoplasmic granules and without pseudopodia. This cell do not spread, they retain their spherical shape upon adhesion to glass (Figure 1B). Granulocytes are almost round shaped cells having nucleus and cytoplasm is packed with granules (Figure 2B). In addition to granule, globular inclusions also occur in these cells. They are of various sizes and all size categorizes change their shape (pleomorphic), produce pseudopodia and form clumps of two to several hundred cells when spread on a glass surface. Granulocytes are reported to be phagocytic in nature. Hyalinocytes are spindle shaped cells and their cytoplasm is characterized with few pseudopodia (Figure 2A). Hyalinocytes generally do not involve in phagocytosis and agglutination of foreign material. Granulocyte population under the exposure of chlorpyrifos (0.15 ppm/7 days) resulted in apparent loss of cytoplasmic plasticity and degranulation (Figure 3A). Hyalinocyte subpopulation under chlorpyrifos exposure (0.15 ppm/7days) exhibit swollen appearance with appearance of discrete granules in the cytoplasm (Figure 3B).

Total and differential haemocyte count

Total haemocyte count was determined in *P. globosa* exposed to various concentrations chlorpyrifos for 24h, 48h, 72h, 96h and 7 days of duration along with control (Table 2).

Exposure to 0.15 ppm of pesticide for 96h and 7 days resulted a significant decrease in total haemocyte count against control value. Exposure to pesticide initiated to decrease of haemocyte density for varied span of exposure which is indicative of destabilization of haematological homeostasis.

Haemolymph of *P. globosa* contain diverse population of haemocytes i.e. blast like cell (prohaemocyte), agranulocytes, granulocytes and hyalinocytes. In this study, dynamics of haemocyte subpopulation was examined under the sublethal exposure of chlorpyrifos. Density of prohaemocyte and granulocyte increased against all concentrations of chlorpyrifos in all span of exposure (Figure 4,5,6,7 and 8). Chlorpyrifos induced decrease in percent of agranulocyte and hyalinocyte against control (Figure 4, 5, 6, 7and 8).

Neutral red retention assay

The haemocytes of animals exposed to sublethal concentrations of chlorpyrifos exhibited relatively less time of retention for the dye exhibiting the leaking of lysosomal components. The neutral red retention time of the haemocyte of *P. globosa* exposed to chlorpyrifos was recorded to be 30 ± 2 minutes. The haemocytes of animals exposed to sublethal concentration of pesticides exhibited relatively less time of retention for the dye. The decrease in neutral red retention time dependent is indicative of lysosomal constability under specific dose. Haemocyte of specimen exposed to 0.05, 0.1 and 0.15 ppm of chlorpyrifos for 7 days exhibit short mean dye retention time as 17 ± 1.6 minutes, 16 ± 1 minute and 15 ± 1.2 minutes against control value of 30 ± 2 minutes (Figure 9).

Nitric oxide generation

A decrease in intrahaemocyte NO production expressed a dose dependent pattern against 0.05, 0.1 and 0.15 ppm of chlorpyrifos exposure. Lowest concentrations of intrahaemocyte nitric oxide were recorded as 3 ± 0.01 µm/10^6 cells against 0.15 ppm of exposure for 7 days in respect to control value of 5 ± 0.16 µm/10^6 cells (Figure 10).Exposure to 0.05 and 0.1 ppm of pesticide varied span of days also show a dose dependent decrement in intrahaemocyte nitric oxide activity in compared to control (Figure 10).

Table 1. Viability (%) of haemocytes of *P. globosa* on exposure to 0.05, 0.1 and 0.15 ppm of chlorpyrifos for 24, 48, 72, 96 h and 7 days. Data are represented as mean± S.D. (n=5).

Concentration of chlorpyrifosexposure	Duration of exposure of *P. globosa*				
	24h	48h	72h	96h	7days
Control	96.18±2.37	96.01±2.05	95.92±1.33	96.02±3.01	95.23±1.24
0.05ppm	95.33±3.01	95.02±2.64	94.8±1.32	91.9±2.62	90.87±1.25
0.1ppm	94.39±2.05	93.24±2.91	91.3±3.25	90.3±2.62	89.45±1.43
0.15ppm	93.25±0.95	92.5±3.01	91.45±1.87	88.1±1.32	89.12±2.87

Table 2. Total haemocyte count (no. of cells x 10^6/mL) of *P. globosa* exposed to chlorpyrifos *in vivo.* Data is as Mean ± S.D. Statistical significance is shown at P<0.05*. (n=5).

Concentration of chlorpyrifosexposure	Duration of exposure of *P. globosa*				
	24h	48h	72h	96h	7days
Control	5.5±0.92	5.75±1.01	5.59±0.67	5.56±0.32	5.43±0.95
0.05ppm	2.85±0.63*	1.98±0.57*	1.75±0.73*	1.83±0.43*	1.21±0.15*
0.1ppm	2.37±0.19*	1.87±0.55*	1.11±0.23*	1.45±0.77*	0.9±0.02*
0.15ppm	2.12±0.34*	1.23±0.43*	1.87±0.65*	1.46±0.29*	0.97±0.46*

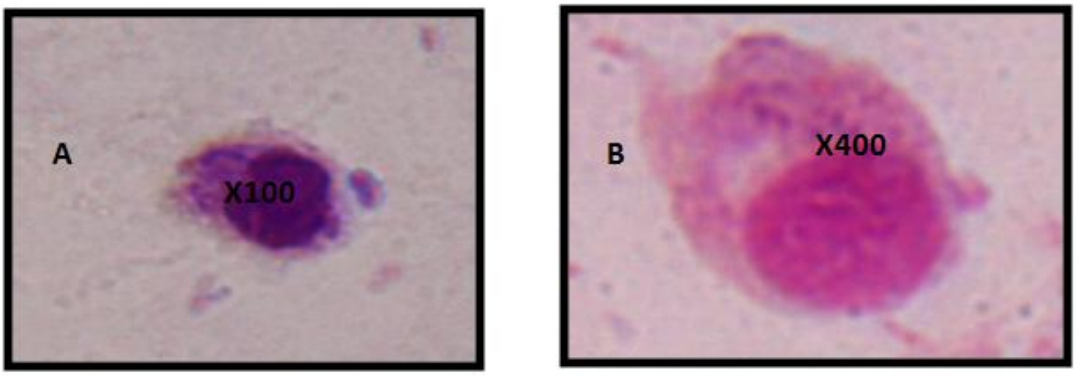

Figure 1. Light microscopic view of haemocyte of *P. globosa*. (A). Prohaemocyte having round nuclei. (B). Agranulocyte having oval nuclei.

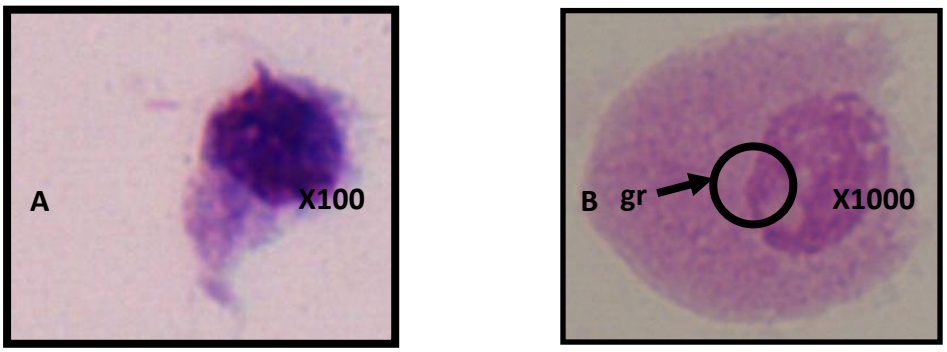

Figure 2. Light microscopic view of haemocyte of *P. globosa*. (A). Hyalinocyte. (B). Granulocyte exhibiting granules (gr).

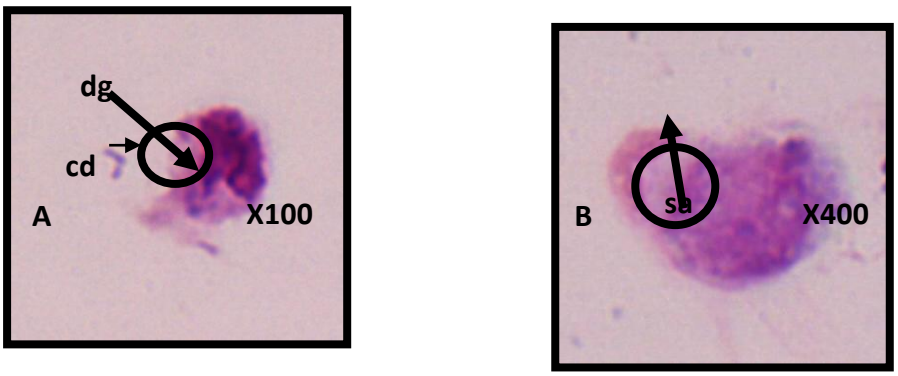

Figure 3. Light microscopic view of haemocyte of *P. globosa* exposede to chlorpyrifos *in vivo* (0.15 ppm/7days). (A). Granulocyte exhibiting cellular degradation (cd) and degranulation (dg). (B). Hyalinocyte showing swollen appearance (sa).

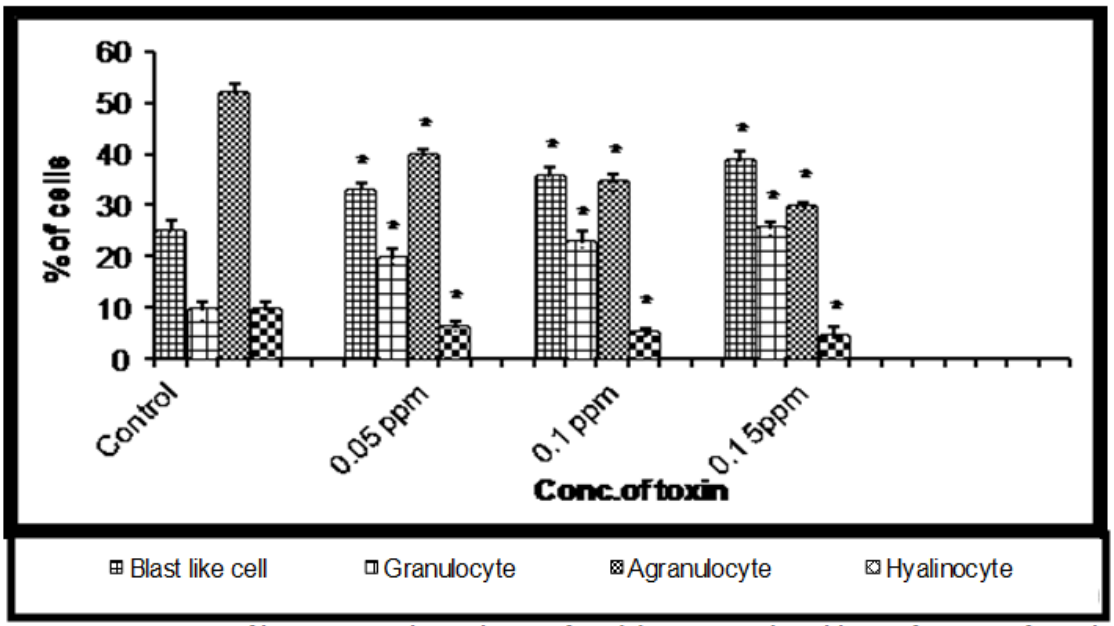

Figure 5. Variation of haemocyte subpopulation of *P. globosa* exposed to chlorpyrifos *in vivo* for 48 hrs. Values reported as Mean± S.D. Statistical significance is shown at P<0.05*. (n=5).

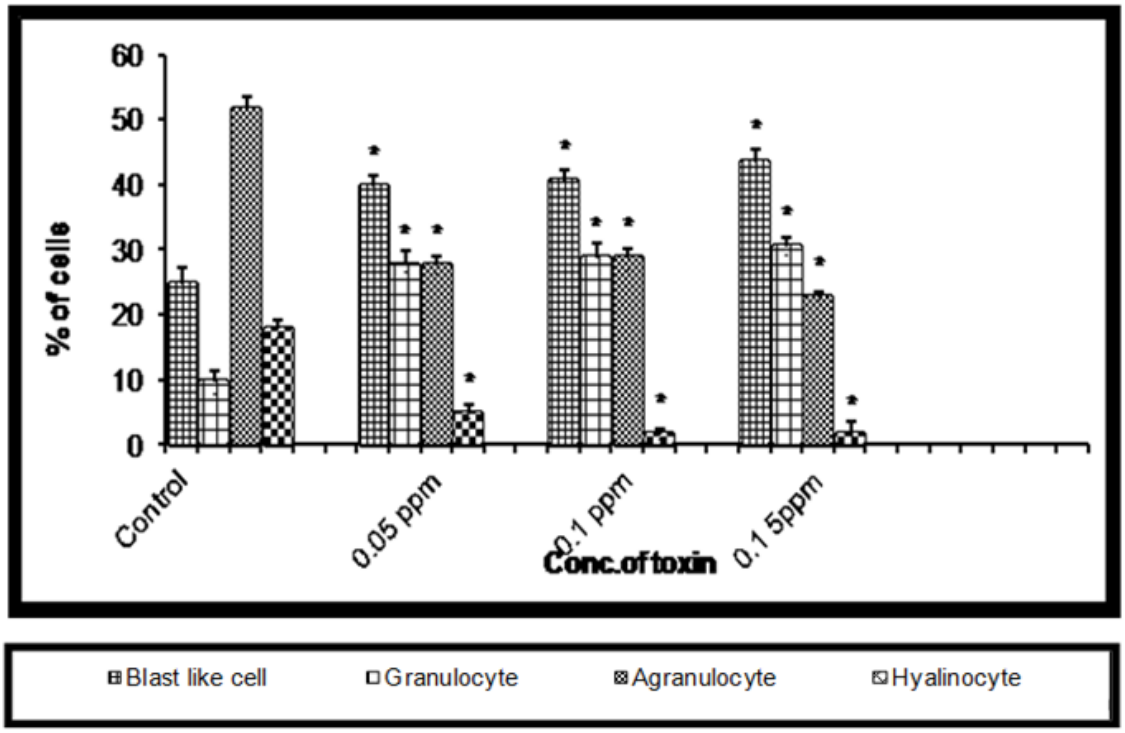

Figure 6. Variation of haemocyte subpopulation of *P. globosa* exposed to chlorpyrifos *in vivo* for 72hrs. Values reported as Mean± S.D. Statistical significance is shown at P<0.05*. (n=5).

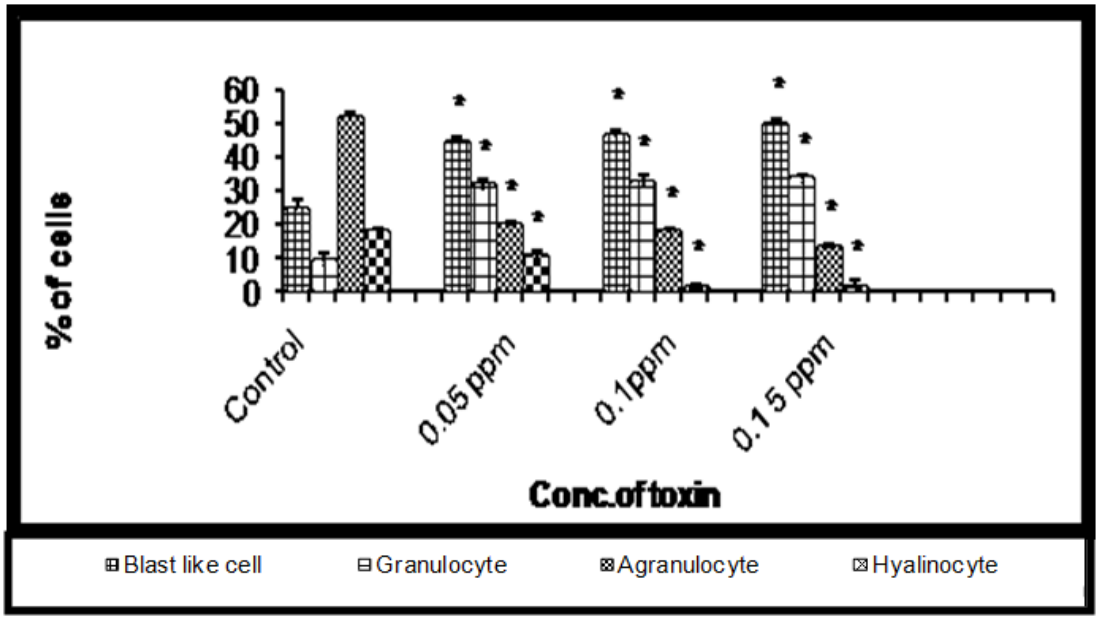

Figure 7. Variation of haemocyte subpopulation of *P. globosa* exposed to chlorpyrifos *in vivo* for 96 hrs. Values reported as Mean± S.D. Statistical significance is shown at P<0.05*. (n=5).

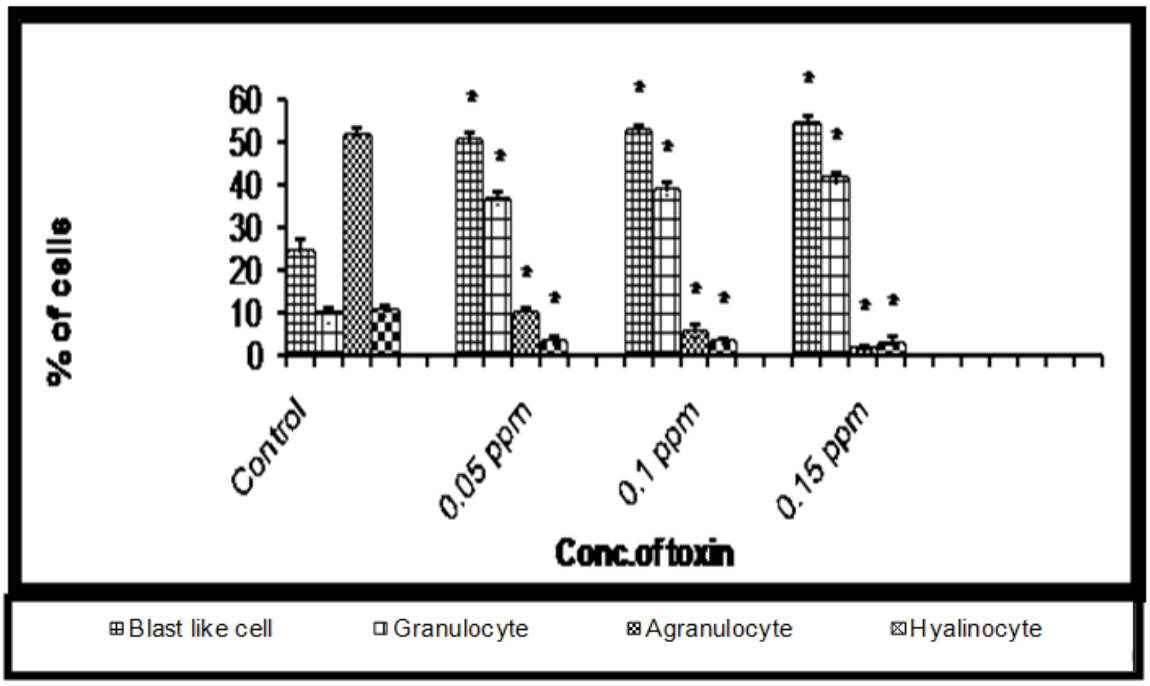

Figure 8. Variation of haemocyte subpopulation of *P. globosa* exposed to chlorpyrifos *in vivo* for 7days. Values reported as Mean± S.D. Statistical significance is shown at P<0.05* (n=5).

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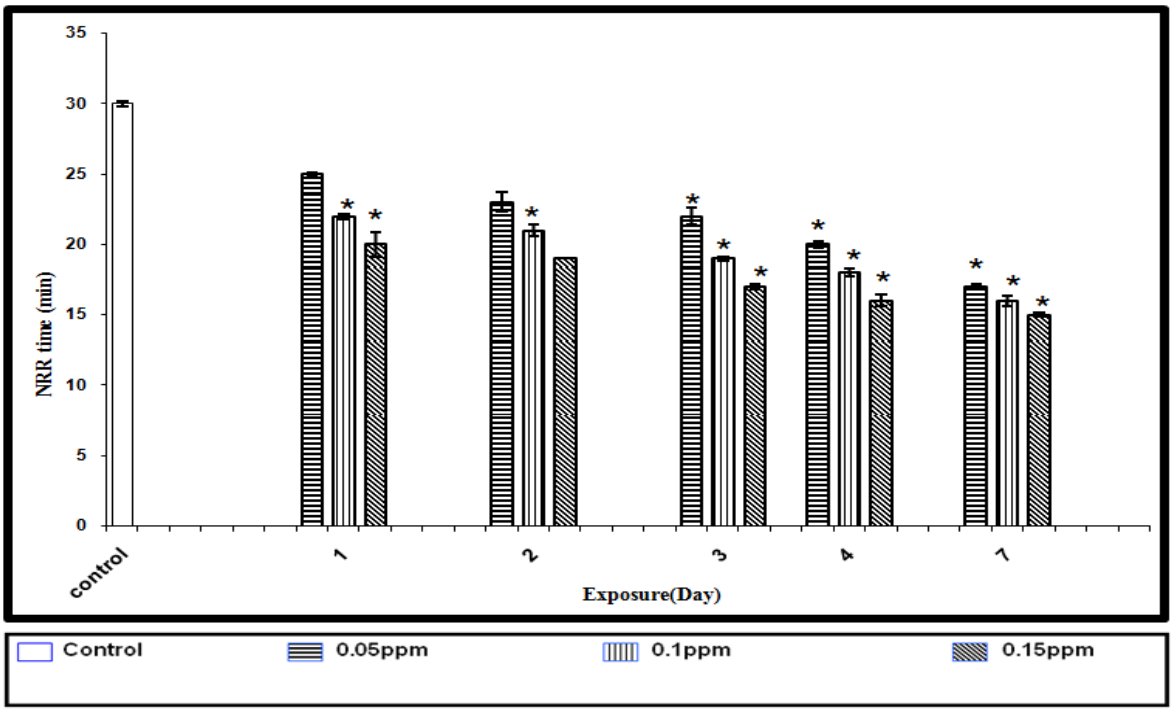

Figure 9. Neutral red retention (NRR) time of the haemocyte of *P. globosa* exposed to chlorpyrifos *in vivo*. Values reported as Mean ± S.D. Statistical significance is shown at P<0.05*. (n=5).

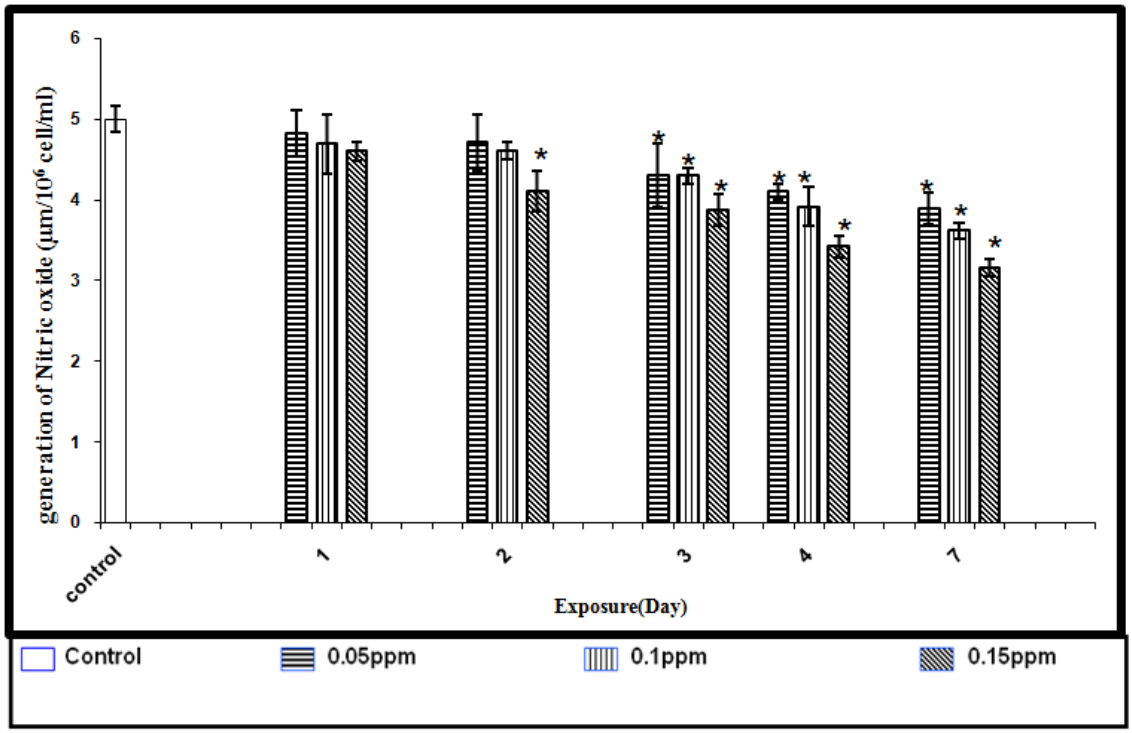

Figure 10. Generation of nitric oxide in haemocyte of *P. globosa* exposed to chlorpyrifos *in vivo*. Data is represented as Mean ± S.D. Statistical significance is shown at P<0.05*. (n=5).

DISCUSSION

Gastropods are distributed in varied ecosystems which often bear the risk of contamination by xenobiotics of diverse chemistry and toxicity (Ottaviani, 1992). Amphibian molluscs like *P. globosa* are often distributed in the land and water interface. This unique habitat of the species is a less studied area which demands a special attention to toxicologists and ecologists. *P. globosa* bears a wide range of humidity tolerance as evident from its adaptabilities in both dry and wet environment. They are capable of performing gaseous exchange both by pulmonary sac and ctenidium in terrestrial and aquatic habitat respectively. During monsoon, agricultural run off contaminates the aquatic waterbodies including the habitat of *P. globosa*. The density of circulating haemocyte is considered as an effective tool in monitoring the health status of *P. globosa* distributed in biounsafe environment.

Total haemocyte count (THC) and Differential haemocyte count (DHC) are considered as an important biological marker which predicts the immunological efficiency of the animal during the period of stress or in disease condition. Total haemocyte count in the blood of *P. globosa* was recorded as$5.5\pm0.75\times10^6$ cells/ml. A significant decrease in haemocyte density was recorded against 0.05, 0.1 and 0.15 ppm of chlorpyrifos exposure for varied span of days. Data was suggestive of drastic alteration of total count of haemocyte of *P. globosa* exposed to pesticide which is indicative of onset of cellular stress of the animal (Chakroborty et al. 2008). Prohaemocyte are blast like cells which differentiate into other cell types of varied function. Density of prohaemocyte increased in respect to control under sublethal exposure of toxin for varied span of exposure that may be indicative of impairment in haematopoisis (Adamowicz and Bolaczek, 2003). Percentage of granulocyte subpopulation was increased against 0.05, 0.1 and 0.15 ppm of chlorpyrifos in all span of exposures specified. Increase in granulocyte subpopulation and appearance of dense packed cytoplasmic granules is suggestive to the toxic response of chlorpyrifos on haemocyte of *P. globosa*. Agranulocyte and hyalinocyte subpopulation percentage decreased in a dose dependent manner that may be due to cellular degradation, vacuolization of the cytoplasm (Lopez et al. 1997) under the toxic influence of cholrpyrifos.

The neutral red retention assay (NRR) is a useful technique applied for monitoring alterations in the permeability of the lysosomal membrane caused by xenobiotic (Koukouzika and Dimitriadis, 2005). It has been reported that lysosomal membrane damage caused by the impact of natural stressors and cytotoxic agents decrease the NRR times, by inducing the leakage of lysosomal components (Maleri et al. 2008). Such leakage of lysosomal vesicles might prove to be detrimental for the health of haemocytes as the lysozymal enzymes bear the potential to damage the self cellular components. A depression in the NRR time was recorded in gastropod following exposure to xenobiotics (Lowe et al. 1995). By reducing the NRR time of the haemocytes, chlorpyrifos has been shown to induce cytotoxicity in *P. globosa*. Such toxic effects of pesticide appear to be detrimental to the other aquatic invertebrates of freshwater ecosystems in India.

Haemocytes are reported chief phagocytes capable of generating nitric oxides- a potential cytotoxic agent. Exposure to chlorpyrifos affected the phagocytic efficiency and generation of nitric oxide in haemocytes. Sublethal concentrations of chlorpyrifos had suppressed this primary defence response in the gastropod leading to a state of immune compromise. In the past two decades, NO mediated pathogen killing and superoxide remediation has earned recognization as an effective immune strategy in the animal world (Arumugan et al. 2000). As NO plays the dual role of scavenging O_2^- radicals and production of the immune molecule affects the animals dually: unscavenged naturally produced O_2^- will pose damage to the conformity of the internal cell and tissue structures of the animal and will make the animal vulnerable to opportunistic microbial attack. Primary defense reaction in invertebrates is defined as “cellular response” (Ray et al. 2009). Cellular response plays a pivotal role in molluscan immunity under the challenge of parasites and xenobiotics. Molluscs lack immunoglobulins and mostly depend on circulating haemocytes to elicit immunological reactions towards xenobiotics and invading pathogens. Impairment in total haemocyte count and differential haemocyte count is indicative of irreversible breakdown of blood cell homeostasis (Lesser, 2006). In the biological unsafe environment, the molluscs are capable of eliciting phagocytic response against invading pathogens (Adamowick and Wojtazek, 2001) and can generate nitric oxide as principal defence molecule. This innate immune response of mollucs help the animals to combat with toxic pathogens abundantly distributed in their natural habitat. Pesticide induced decline in activity of cytotoxic molecules that may lead to opportunistic growth of microorganisms in the blood and other tissues of *P. globosa*. This situation may lead to rapid loss of this important edible species from freshwater ecosystem of India.

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