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Research Journal of Agricultural Sciences
An International Journal

P- ISSN: 0976-1675
E- ISSN: 2249-4538

Volume: 12
Issue: 04

Res Jr of Agril Sci (2021) 12: 1262–1266

CARAS

Res. Jr. of Agril. Sci. (July-Aug 2021) 12(4): 1262–1266
www.rjas.org

Full Length Research Article

Evaluation of Phagocytosis and Cytotoxicity Response in Fresh Water Snail, *Bellamya bengalensis*, Following Exposure to Chlorpyrifos

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Received: 20 Apr 2021 | Revised accepted: 27 Jun 2021 | Published online: 26 July 2021

A B S T R A C T

Freshwater edible molluscs *Bellamya bengalensis* (Molluscs: Gastropoda) is an economically important species. Chlorpyrifos is a crystalline organophosphate insecticide and is used in the agricultural field for control insect pests. Mollusc mostly elucidates effective immunological responses by producing cytotoxic molecules like generation of superoxide anion and nitric oxide against environmental xenobiotics. Haemocytes of *B. bengalensis* are an immune effector cell of haemolymph and are capable of discrimination self and nonself surface, phagocytosis of foreign particles and production of cytotoxic molecules as an antimicrobial agent. The cells lining the digestive tubule participate in moving the food, secreting substances in the lumina, phagocytosis and generation of cytotoxic molecules. Fresh water *Bellamya bengalensis* were exposed to sublethal concentrations of chlorpyrifos for varied span of time in controlled laboratory condition to examine phagocytic response in haemocyte, histopathology and cytotoxic activity in digestive tubule. The tissue pathology demonstrates a state of inflammation which is related to possible disruption of cellular homeostasis Alteration in phagocytic response of haemocyte challenged with yeast (*Saccharomyces cerevisiae*) and increment of activity of superoxide anion (SOA) along with parallel decrease in the activities of nitric oxide (NO) in digestive tubule appeared to be detrimental for survival of *Bellamya bengalensis* in the chlorpyrifos contaminated environment. Data is indicative of cellular metabolic stress in the edible gastropod that may lead to decline of population size in freshwater aquatic system of West Bengal.

***Key words*:** Chlorpyrifos, *Bellamya bengalensis*, Haemocyte, Digestive tubule

Bellamya bengalensis is a freshwater mollusc widely distributed in the wetland of different states of India. Animal is regularly consumed by human population and serves as a source of dietary protein to human, poultry and fish. Chlorpyrifos is a crystalline organophosphate insecticide used for control insect pest of various agricultural crops [1]. Freshwater natural habitat of the animal faces the risk of pesticide contamination by agricultural runoff during monsoon [2]. Haemocytes, the circulating blood cells of gastropod, function as the immunological effector cells under exposures of toxin and parasites [3]. They are involved in various types of physiological functions such as cell aggregation, self-nonself discrimination; wound repairing and phagocytic responses [4].

Digestive tubule is the principal site of detoxification and multiple metabolic activities of molluscs [5]. The digestive diverticula of gastropod are closely packed together containing secretory basophilic cells. Superoxide anion is reported as a defence molecule against intruding pathogenic microorganisms [6]. Nitric oxide is a vital cytotoxic molecule generated in response to oxidative stress and provides immunological defence to the host by deactivating foreign microorganisms [7]. Information of toxicity of chlorpyrifos in freshwater gastropod of India is scanty. In this present study, cellular modulation of haemocyte, histopathology and cytotoxic activity of digestive tubule were examined under the sub-lethal exposure of chlorpyrifos in controlled laboratory condition. Information will provide a data in understanding the degree of cellular modulation in haemocyte and impairment of digestive tubule function of gastropod in presence of sublethal concentrations of chlorpyrifos and to establish its suitability as a biomarker of aquatic toxicity in contaminated habitat.

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MATERIALS AND METHODS

Collection and acclimatization of animal

The adult healthy *B. bengalensis* weighing 2 gms ± 1.66 with an average shell size of 3.77 cm ± 0.26 were manually collected from the selected wetlands of the district of Cooch Behar of West Bengal. Animals were transported to the laboratory in rectangular plastic containers with a dimension of 12' × 18' × 6' at a density of 8-10 individuals per box in moist condition. Prior to experimentation, animals were acclimatized for 10 days in the laboratory. During acclimatization, *B. bengalensis* were maintained in aquaria with 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 sp*. and some common aquatic weeds [8]. Routine replenishment of water was carried out in every 12 hours to avoid residual toxicity.

Determination of LC_{50}

Aqueous solutions of Dursban (Dow Agro Sciences, India, Chlorpyrifos (E.C. 20%) formulations were prepared in Borosilicate glass containers with chlorpyrifos concentrations of 1, 2, 3 and 5 ppm. The pH of the solution was maintained at 7.2. Each experimental set consisted of 10 animals of same shell length. Animals were exposed to a volume of 1litre of pesticide solution. For control, a set of animals were kept in identical volume of pesticide free analytical grade water. The LC_{50} study was carried out in static water environment and fresh solutions of pesticide were replenished in every 12 hour. The mortality of the animals was recorded after every 24 hour for all the concentration of exposures. The LC_{50} values of chlorpyrifos of *B. bengalensis* were evaluated by arithmetical method [9].

Treatment of animal

Aqueous solutions of pesticide (Chlorpyrifos, E.C. 20%) formulations were prepared in Borosilicate glass containers with sublethal chlorpyrifos concentrations of 0.05, 0.1, 0.5 ppm. The pH of the solution was maintained at 7.2. Each experimental set consisted of 10 animals of uniform shell length. Animals were exposed to a volume of 1 litre of pesticide solution for varied span of exposure i.e., 1,2,3,4 and 7days. For control, a set of animals were kept in identical volume of pesticide free analytical grade water. The experiments were carried out in static water environment and fresh solutions of pesticide were replenished in every 12 hour.

Phagocytic response assay

Haemolymph was collected following shell puncture method of Brousseau *et al*. [10] and was stored in prechilled glass vials. A portion of the fresh haemolymph was smeared on clean, sterilized glass slides in a moist chamber so as to get a haemocyte monolayer on the glass surface. The haemocytes were allowed to settle for 15-20 minutes at room temperature. The phagocytic efficiency of the haemocytes was examined by challenging them with freshly cultured yeast at an optimal phagocytic ratio of 1:10. Both cell types were maintained in short term culture system for 6 hours to complete the phagocytosis *in vitro*. Cells were subsequently processed fixed and stained for microscopic observation. Percentage of phagocytic haemocyte was determined microscopically following the method of Adamowicz and Wojtaszek [11].

$$\text{Phagocytic haemocytes (\%)} = \frac{\text{Total number of phagocytosed cells}}{\text{Total number of cells}}$$

Histopathology

Tissues like digestive tubule were isolated from normal and experimental mollusc. Sterile Snail Saline was used to rinse and clean the tissue. They were fixed in aqueous Bouin's solution for 48 hour processed through graded series of alcohols, cleared in xylene and embedded in paraffin wax. Sections were cut at 6 µm thickness with the help of rotatory microtome, stained with hematoxylin-eosin and were mounted in Canada Balsam following the method of [12].

Superoxide anion assay

Superoxide anion productions by tissue suspension were determined by a modified method of Bell and Smith [13]. Assay consisted of 1ml of freshly collected tissue suspension of digestive tubule (1×10^6cells/ml) in a test tube and allowed to react with 1ml of NBT solution (0.03%) for 30 mins at 37°C. The reaction was terminated by removing the NBT solution and addition of absolute methanol. After proper washings with 70% methanol, the cells were treated with a solution of KOH (1ml, 2M) and DMSO (1ml) to dissolve the cytoplasmic formazan. The optical density of the dissolved formazan was estimated spectrophotometrically at 630 nm and the generation was expressed as absorbance (optical density) at 630 nm/min/10^6 cells.

Nitric oxide assay

The generation of nitric oxide (NO) was measured as the amount of the nitrite released from the tissue suspension (digestive tubule) with Griess reagent after [14]. The concentration of the tissue suspension (1×10^6cells/ml) was incubated with equal volume of Griess reagent (1% sulphanilamide. 0.1% naphthyl ethylenediamine dihydrochloride and 5% orthophosphoric acid) at 37°C 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 / 10^6 cells/ ml.

RESULTS AND DISCUSSION

Phagocytic response

Haemocytes of invertebrates were able to phagocytose yeast particles (Fig 1) under proper elicitation. In the controlled population, percentages of phagocytic haemocytes were maximum after 6 hours of incubation with yeast in short term culture (Fig 1). *B. bengalensis* receiving *in vivo* exposure of 0.5ppm/7days of chlorpyrifos expressed a lowest value of percent phagocytic haemocyte as compared to control value (Fig 5). A sharp decrease of phagocytic haemocyte percent was recorded against 0.5 ppm of chlorpyrifos for 1,2,3,4 and 7 days of *invivo* exposure (Fig 5).

Digestive tubule

The digestive tubules of *Bellamya bengalensis* are located near the anterior siphon on either side of the gut. Stored food particles in the stomach pass through the primary ducts that branch into multiple secondary ducts and end in masses of blind tubules forming the left and right diverticula. Digestive tubules of untreated *B. bengalensis* in the control exhibited no pathological signs (Fig 2). In gastropod, exposed to chlorpyrifos, the thickness of the epithelium of the digestive tubules was drastically reduced. Appearance of large and lightly stained vacuoles was noticed in the digestive cells (Fig 3). The digestive tubules of treated specimen exhibited a sign of cellular dissolution associated with partial loss of cellular integrity (Fig 4).

Superoxide anion production

The control set showed an average activity of superoxide anion production. Chlorpyrifos of 0.5 ppm exposure for 7 days showed a maximum elevation of activity of superoxide anion as compared to control (Fig 6). Superoxide anion production expresses a dose dependent response with higher production in *Bellamya bengalensis* exposed to 0.5 ppm exposure of pesticide compared to control for all span of exposure (Fig 6).

Nitric oxide production

A decrease in nitric oxide (NO) production expressed a dose dependent pattern against 0.05, 0.1 and 0.5 ppm of chlorpyrifos exposure (Fig 7). Exposure to 0.05 and 0.1 ppm of pesticide for varied span of days also show a dose dependent decrement in nitric oxide activity in compared to control (Fig 7).

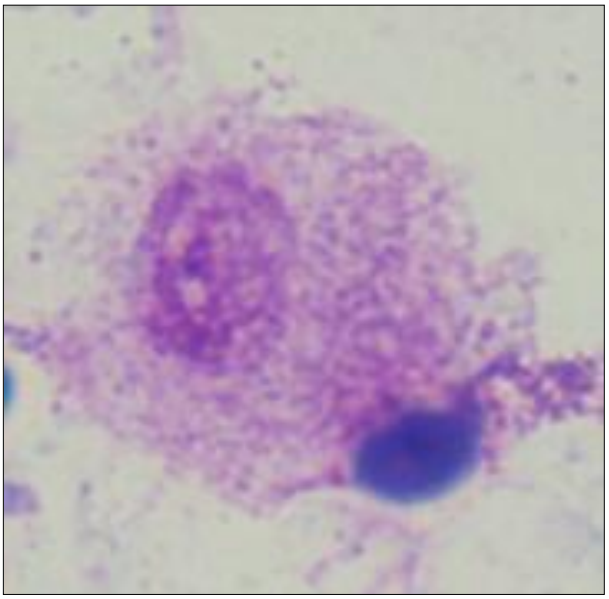

Fig 1 Phagocytosis of yeast (Y) by haemocyte of *B. bengalensis* x400

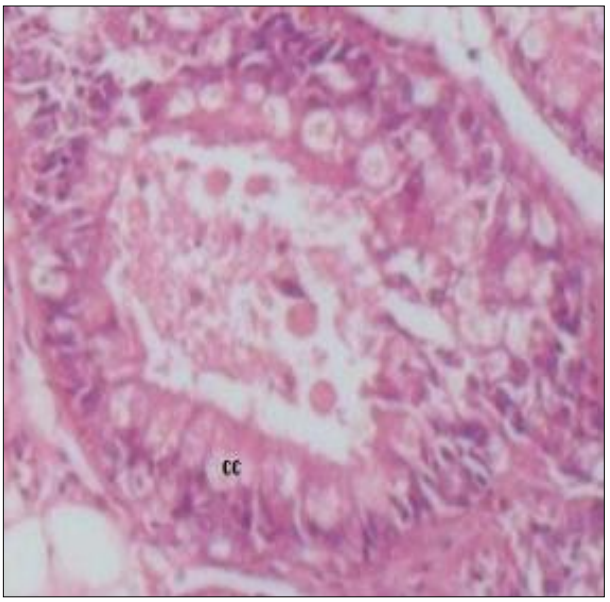

Fig 2 Transverse section of the digestive tubule of *B. bengalensis* lined with non-ciliated columner cells (cc).x1000

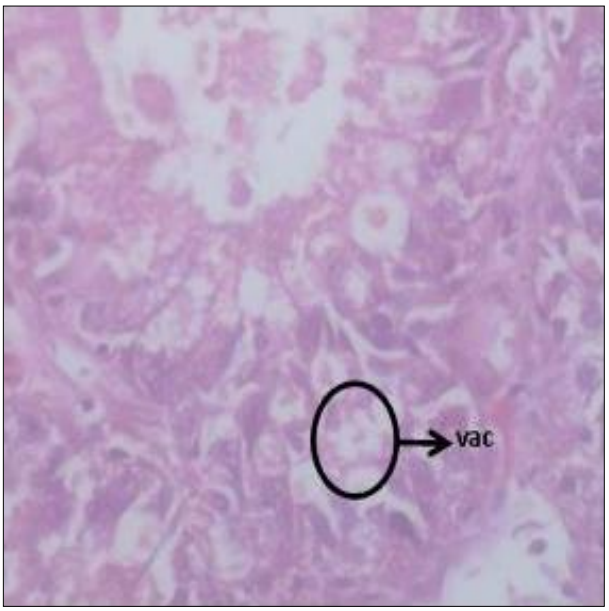

Fig 3 Transverse section of the digestive tubule of *B. bengalensis* exposed to chlorpyrifos (0.1ppm/ 7days) in vivo exhibiting intense vacoulation (vc) and disruption.x1000

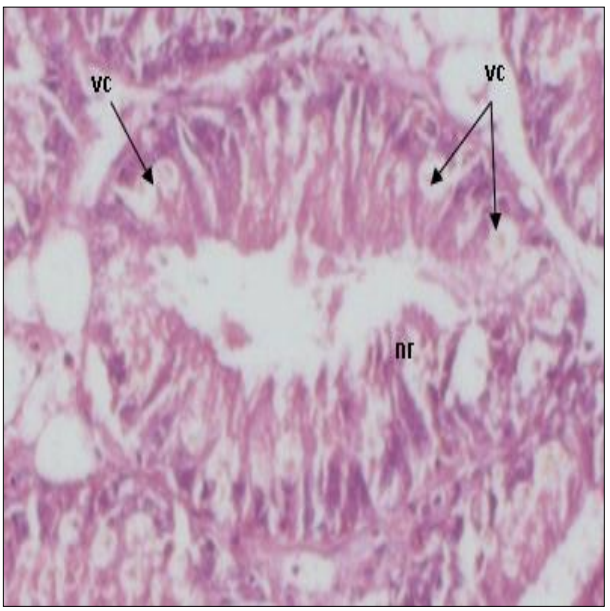

Fig 4 Transverse section of the digestive tubule of *B. bengalensis* exposed to chlorpyrifos (0.5 ppm/ 7days) *in vivo* exhibiting vacoulation (vc) and necrosis (nr) x1000

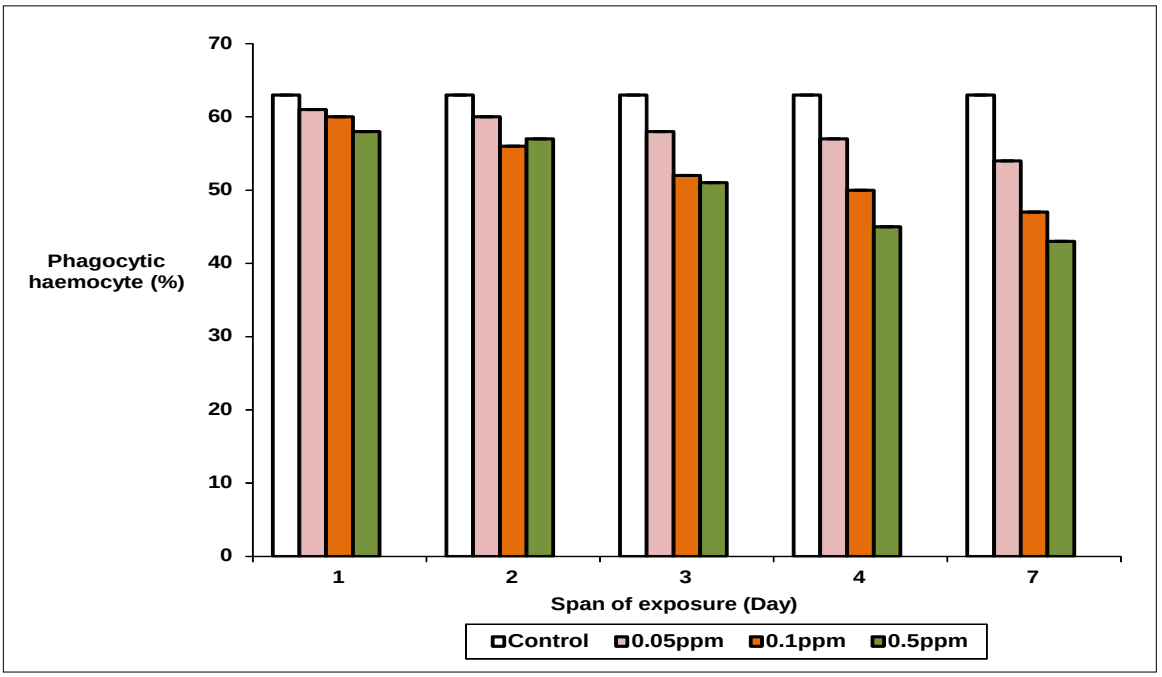

Fig 5 Phagocytic response of haemocytes (challenged with yeast) of *B. bengalensis* exposed to chlorpyrifos *in vivo*

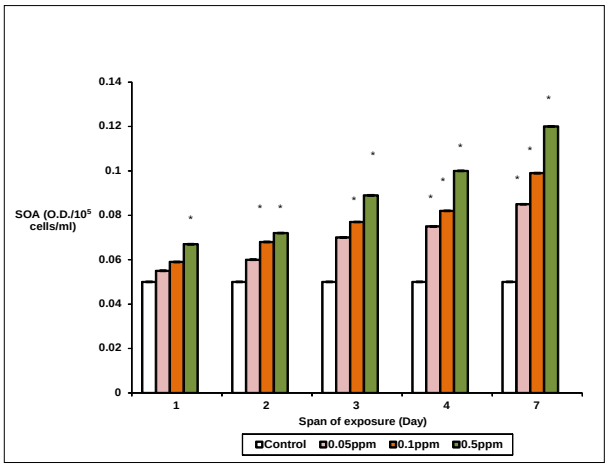

Fig 6 Activity of superoxide anion of digestive tubule of *B. engalensis* exposed to chlorpyrifos *in vivo*. Data is represented as Mean ± S.D. Statistical significance is shown at P<0.05*

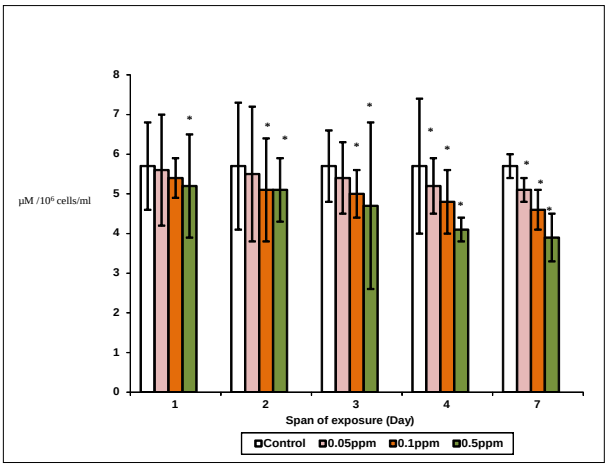

Fig 7 Activity of nitric oxide of digestive tubule of *B.bengalensis* exposed to chlorpyrifos *in vivo*. Data is represented as Mean ± S.D. Statistical significance is shown at P<0.05*

Haemocytes of invertebrates phagocytose invading microorganisms and pathogens under proper elicitation [15]. Chlorpyrifos induced impairment of phagocytic response (Fig 5) indicated a physiological state of immune suppression. Phagocytosis is considered the primary line of cellular defense in molluscs and therefore any factor that affects this activity would greatly influence the general immune status of the animal [16].

Digestive tubule is the principal site of detoxification and multiple metabolic activities of molluscs [17]. Digestive tubule of the treated animal expressed thinning of the epithelial wall, appearance of numerous vacuoles and formation of necrotic foci at the tubule channel associated with tubular lysis (Fig 3). Chlorpyrifos has a deleterious effect on the tissue composition and tissue architecture of the digestive tubules of *B. bengalensis* (Fig 4).

Exposure to chlorpyrifos had increased the production of superoxide anion for all three concentrations, which expressed a dose dependent response (Fig 6). Chlorpyrifos contamination resulted in an induction of generation of reactive oxygen species in the digestive tubule which is indicative of increased capability of tissues for intracellular killing of pathogen. This response of selected tissue may be a reflection of cellular stress under the indefinite period of pesticide exposure. Dose dependent response of digestive tubule in generation of reactive oxygen species is suggestive of the parameter which may be considered as biomarker of pesticide toxicity. Superoxide anions are extremely toxic, powerful and hyperactive killing agents which are capable of producing cellular and tissue damage of self [18].

Sublethal concentrations of chlorpyrifos suppressed the generation of nitric oxide production in digestive tubule (Fig 7). The trend of immunological suppression in relation to nitric oxide production persists with increase in toxin exposure under prolonged period of exposure. Pesticide contamination resulted in suppression of reactive nitrogen species (RNS) in the selected tissue, which is suggestive of decreased capability of haemocytes for intracellular killing of pathogen through phagocytosis [3] (Chakroborty *et al.*

2009). Chlorpyrifos induced impairment of phagocytic response indicated a state of immunological suppression and possible reduction of ecological fitness of *B. bengaensis* in its natural habitat.

CONCLUSION

In the biological unsafe environment, the molluscs are capable of immunological response against invading pathogens. This response of mollucs help the animals to combat with toxic pathogens abundantly distributed in their natural habitat. Pesticide induced alteration in cellular and tissue function may modulate metabolic activity that lead to opportunistic growth of parasites and pathogen in the blood and tissue of *B. bengalensis.* This alarming situation may lead to rapid loss of this important edible species from freshwater ecosystem of India.

Acknowledgements

The author takes this opportunity to acknowledge the cooperation of Officer-In Charge and Principal, Bidhannagar College for extending the facility of the department for research.

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