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Density shift, morphological damage, lysosomal fragility and apoptosis of hemocytes of Indian molluscs exposed to pyrethroid pesticides

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ABSTRACT

Bellamya bengalensis (Gastropoda: Prosobranchia) and *Lamellidens marginalis* (Bivalvia: Eulamellibranchiata) are the molluscs of Indian freshwater ecosystem and important biological resources. These edible species bear economical, ecological, nutritional and medicinal importance. Natural habitat of these organisms is under the ecological threat of contamination by cypermethrin and fenvalerate, the common pyrethroid pesticides of India. Hemocytes are chief immunoeffector cells of molluscs which exhibit responsiveness against environmental toxins and perform diverse immunological functions including phagocytosis, encapsulation and cytotoxicity. Experimental exposure of cypermethrin and fenvalerate resulted in significant shift in density and morphological damage in hemocytes of *B. bengalensis* and *L. marginalis* respectively. Pyrethroid induced fragility and destabilization of hemocyte lysosomal membrane was recorded and proposed as an indication of toxin induced stress in molluscs. Apoptosis is an immunologically important cellular response which is modulated by environmental toxins. Pyrethroid exposure suppressed the physiological level of apoptosis and necrosis in hemocytes of *B. bengalensis* and *L. marginalis* indicating possible impairment of apoptosis mediated immunoprotection. Differential responses of *B. bengalensis* and *L. marginalis* hemocytes may be due to species specificity, toxin specificity, nonidentical immune strategies of Gastropoda and Bivalvia, specific habitat preference and related ecological niches.

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1. Introduction

Mollusca is the second largest invertebrate Phylum which represents around 200,000 extant species [1] inhabiting terrestrial, marine, freshwater and estuarine ecosystem. The success of molluscan evolution and widespread distribution depended on their unique body plan [2], behavior, adaptation and efficient immunological system. Freshwater molluscs like *Bellamya bengalensis* and *Lamellidens marginalis* are the important aquatic resources of India. These two taxonomically distant species are sources of indigenous food and ethnomedicines for rural and tribal population of India [3,4]. They occupy separate benthic habitats of freshwater ecosystem without overlapping of respective ecological niches and evolved two dissimilar reproductive strategies. *B. bengalensis* exhibits viviparity and internal mode of fertilization whereas in *L. marginalis*, fertilization of gamets occur externally.

Toxic contamination of the waterbodies and habitat destruction due to urbanization and industrialization have been identified as potential ecological threats of molluscs of India. Natural habitat of *B. bengalensis* and *L. marginalis* are often contaminated by different immunotoxins including cypermethrin and fenvalerate, the two common pyrethroid pesticides [5]. These pesticides are widely used by the Indian farmers to control insect pests of paddy, wheat, jute and vegetables. Unrestricted application of cypermethrin and fenvalerate appears to be physiologically detrimental for the several nontarget species like *B. bengalensis* and *L. marginalis*, the inhabitants of accumulated water of agricultural fields and adjacent waterbodies. Moreover, during monsoon and flood, agricultural run-off laden with cypermethrin and fenvalerate often contaminates the natural habitat of molluscs and expected to create toxicity in them. Toxicity of pyrethroids is well reported in mammal [6] but their toxicity in molluscs is not studied in detail.

Molluscs, in general, lack adaptive immunity and antibody and depend on innate immune system to combat invading parasites, pathogens and toxin exposure [7]. *B. bengalensis* and *L. marginalis* exhibit open mode of circulation of hemolymph carrying varied populations of hemocytes to different organs. Hemocytes or blood cells play a pivotal role in molluscan cell mediated immunity. They are capable of performing diverse immunological functions like nonself recognition, phagocytosis, encapsulation

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and cytotoxicity under the exposure of toxins and pathogens [8,9]. Functional attributes of hemocytes of Indian molluscs is a less studied area of research with very limited information. Ray et al. (2013) reported morphological variations and immunological reactivity of hemocytes of *B. bengalensis* and *L. marginalis* with reference to generation of cytotoxic agents like superoxide anion, nitric oxide, phenoloxidase and their phagocytic potential to engulf nonself yeast particulates [10]. We investigated immunotoxicity of cypermethrin in *B. bengalensis* and fenvalerate in *L. marginalis* in relation to dynamics of hemocyte count, morphological damage of hemocyte morphotypes, stability of hemocyte lysosomal membrane and apoptosis. Field survey carried out by us in the state of West Bengal of India revealed cypermethrin and fenvalerate as the major contaminants of the most of the waterbodies distributed near the agricultural fields. On this basis, cypermethrin and fenvalerate were selected for investigating their toxicity in *B. bengalensis* and *L. marginalis* respectively.

Maintenance of hemocyte density within physiological limit is a determinant factor of hemocyte mediated immunity of molluscs. Chakraborty et al. (2008) reported dynamics of hemocyte density of *L. marginalis* exposed to arsenic, an environmental contaminant [11]. Arsenic induced shift in hemocyte count is a manifestation of sublethal toxicity and according to them, be considered as biomarker of toxin exposure. Increment and high concentration of hemocytes is reported to be indicative to augmentation of immune defense in the parasitized insect *Drosophila suzukii* [12]. We investigated dynamics of hemocyte density in *B. bengalensis* and *L. marginalis* exposed to environmentally realistic sublethal concentrations of cypermethrin and fenvalerate respectively. Toxin induced morphological damage of molluscan hemocytes is in report. Chakraborty and Ray (2009) reported morphological damage in the hemocytes of *L. marginalis* exposed to sublethal concentrations of arsenic for 30 days of exposure [13]. Authors identified micronucleation, binucleation, pycnosis, nuclear disintegration and cell membrane disruption as major damages in the arsenic exposed hemocytes. Arsenic induced morphological damage of hemocytes was indicative to a possible impairment in the functional attributes of molluscan hemocytes. Morphological damage of the hemocytes of *B. bengalensis* and *L. marginalis* exposed to cypermethrin and fenvalerate respectively was microscopically investigated for assessment of pyrethroid toxicity.

Phagocytosis is a classical immunological response of molluscan hemocytes [9] and well reported in the phylogeny. After phagocytosis, phagocytic vacuoles with engulfed particulates fuse with lysosomes of cells and form phagolysosomes. Lysosome is an important subcellular organelle of molluscan hemocyte involved in intracellular degradation of engulfed particulate. Lysosomal enzymes degrade the foreign particulates leading to their intracellular destruction and inactivation. Internalization of foreign pathogens and their subsequent degradation in phagolysosomes has been of immunological interest. As lysosomes act as 'store house' of various digesting enzymes, effective phagolysosomal degradation of internalized pathogen depends on lysosomal membrane integrity. Chakraborty and Ray (2009) reported impairment of membrane integrity of lysosomes of hemocytes of *L. marginalis* exposed to arsenic as revealed by neutral red retention assay *in vitro* [13]. Arsenic induced fragility of lysosomal membrane yielded leakage of lysosomal enzyme leading to onset of toxicity by possible destruction of the adjoining self cell or tissue. We investigated lysosomal membrane integrity of hemocytes of *B. bengalensis* and *L. marginalis* under the experimental exposure of cypermethrin and fenvalerate respectively. Pyrethroid induced lysosomal membrane fragility was examined by estimating the retention time of neutral red probe within lysosomal compartment [14,15] of control and treated hemocytes.

Apoptosis or programmed cell death presents a wide ranging biological implication during and after development and bears immunotoxicological significance. Apoptosis involves cell injury caused by various stimuli including toxic exposure of xenobiotics [16]. Apoptosis exhibits characteristic cellular changes including membrane blebbing, nuclear condensation, cytoplasmic shrinkage and membrane asymmetry. According to Kiss (2010), the translocation of membrane phosphatidylserine from inner leaflet to outer cell surface is a hallmark of apoptotic process, accounted in majority of mammalian species and few invertebrate species [17]. Sweet et al. (1999) and Kiss (2010) reported apoptosis as phylogenetically conserved cellular response which is modulated by environmental xenobiotics and indicative of cellular stress [16,17]. Hemocytes of a wide range of species exhibit immunological reactivity against environmental toxins [18]. Hemocyte apoptosis is an established immune response of aquatic mollusc *Lymnaea stagnalis* exposed to environmental toxin fomesafen, a herbicide [19]. Leakage of proteases from disintegrated lysosomes results in apoptosis of adjoining self cells [20–22]. Apoptotic and necrotic cell deaths of hemocytes of *B. bengalensis* and *L. marginalis* under the exposure of cypermethrin and fenvalerate respectively were identified and analyzed by employing fluorescence activated cell sorting (FACS) and fluorescence microscopy. Our estimation of apoptosis by FACS is based on staining the hemocytes with FITC conjugated annexin-V, a Ca^{++} dependent 35–36 kDa protein with high affinity for exposed phosphatidylserine in conjunction to a vital dye propidium iodide (PI). However, information of toxicity of cypermethrin and fenvalerate on molluscan hemocytes with reference to lysosomal membrane fragility and apoptosis is absent in current scientific literature. In this paper, we report the toxic effect of cypermethrin and fenvalerate on density, morphology, lysosomal membrane stability and apoptosis of hemocytes of *B. bengalensis* and *L. marginalis* respectively.

2. Materials and methods

2.1. Reagents

Giemsa's stain, trypan blue were obtained from Hi Media, India. Neutral red dye and salts for sterile snail saline were obtained from Sisco Research Laboratories, India. Cypermethrin and fenvalerate were procured from United Phosphorus Limited and Rallis India Limited, India respectively. Methanol was purchased from Merck and annexin-V-FITC apoptosis detection kit was purchased from Abcam plc, UK.

2.2. Laboratory maintenance and acclimation of B. bengalensis and L. marginalis

B. bengalensis and *L. marginalis* of average size of 3.93 × 1.96 cm and 7.02 × 4.14 cm respectively were purchased from animal supplier of University of Calcutta (Pabitra Dev, Kolkata, India). None of these experimental animals had the previous history of exposure to toxin, pathogen and other stressors. Prior to experimentation, *B. bengalensis* and *L. marginalis* were acclimated in the laboratory for 5–7 days in the controlled static water environment at 25 °C, fed with chopped lettuce leaves and received uniform illumination [23]. The water of the storage tanks was changed at every 24 h to remove unutilized food and excretory product that might cause water fouling. This work was designed in accordance with the guidelines of the institutional (University of Calcutta) norms of animal handling and maintenance in controlled laboratory condition.

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