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Effect of lead (Pb) toxicity on hemocytes of freshwater molluscs, *Bellamya bengalensis* with emphasis to cell aggregation

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Abstract

The hemolymph of aquatic invertebrates is the target of many environmental contaminants. The alteration of hemocyte aggregation is thought to reflect an impairment of internal defense. In the freshwater molluscs, *Bellamya bengalensis*, after the lead (Pb) treatment, both paraptosis and apoptosis like features were noticed in the hemocytes. Pb inhibited the degree of hemocyte aggregation. Poorly organized loose clump/ aggregates were noticed in lead treated group. Mean number of compact aggregates was found to be less than smaller loose clump/ aggregates after treatment. Mean number of cells in each clump/ aggregate was also decreased after Pb treatment which may affect "encapsulation response" and hemocyte–hemocyte adhesion (microaggregation).

Key words : Hemocytes, loose clump, Cell aggregation.

Innate immunity of invertebrates, including molluscs consists of humoral components (agglutinins, lysosomal enzymes, antimicrobial peptides) and cellular defense performed by hemocytes and is considered to be an evolutionary ancient defense system[3]. Hemocytes represent the first line of internal defense against pathogens and non-self materials in molluscs. Their function is highly sensitive to external factors like temperature, salinity and pollutants of the environment[13,14].

Environmental pollutants can alter hemocyte functions such as aggregation which is supremely important for encapsulation of foreign particles[1]. Live freshwater molluscs are natural biological purifiers, accumulate pesticides and heavy metal residues in the body from their habitat, and are used to assess the health of environment of freshwater system. Various anthropogenic activities like habitat contamination are the causative factors for death of freshwater molluscs like *Bellamya*

bengalensis. *B. bengalensis* has demand to the people of all the economic classes, more particularly to poor communities. Meat of these freshwater molluscs has a good market value in different districts of West Bengal and provides an essential source of income for poor women collectors [7]. Crushed *B. bengalensis* with shell is used for protein requirements of farmed ducks in different districts of West Bengal. Others use boiled *B. bengalensis* with added salt to feed ducks[7].

Bellamya bengalensis were manually collected from the selected sites of North 24 Parganas (West Bengal) and were kept for acclimatization for a week. Some specimens (n=17) were exposed to 50 mg/L of Lead Nitrate in water medium[8]. Control specimens were untreated by any toxic metals. Hemolymph was collected from *B. bengalensis*[12]. The syringe with a needle was inserted in foot, through the operculum and drops of hemolymph were aspirated and smeared on glass slides and stained by Giemsa, Leishmans Eosin Methylene blue solution and observed under light microscope. The phagocytic efficiencies of hemocytes were determined by challenging the hemocytes with activated charcoal and were incubated for 1 h at 37°C and fixed with methanol, stained with Giemsa and the phagocytic index was calculated[8]. Cells were treated with 50 µl of 0.25 % trypan blue dye solution for 5 minutes. The percentage of blue-stained cells represented a mortality index[8,11].

After the Pb treatment, the hemocytes cytoplasm displayed vacuoles (paraptosis like features) and membrane blebbing (apoptosis like features). Lead (Pb) inhibited the degree of hemocyte aggregation which may affect "encapsulation response" and hemocyte–hemocyte adhesion (microaggregation). Mean mortality index was significantly increased in treated group. Mean phagocytic index was significantly reduced in treated group.

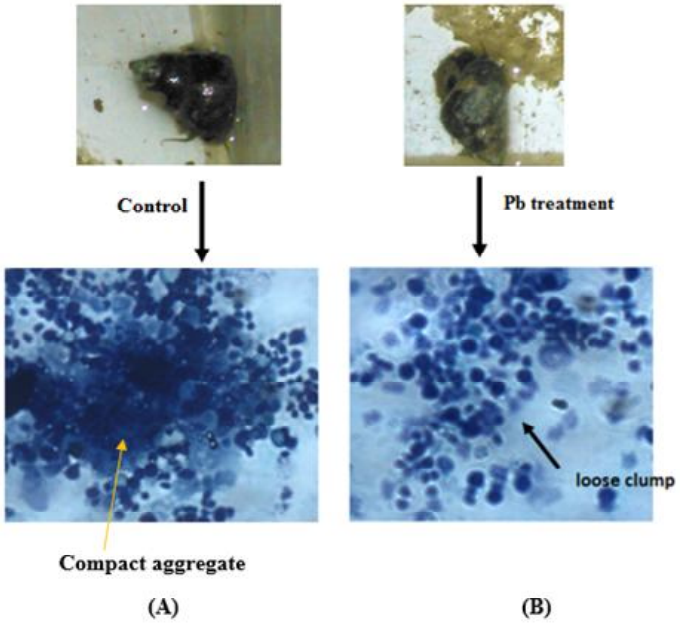


Fig. 1. Leishmans Eosin Methylene blue solution stained typical aggregation of hemocytes (compact aggregates contained cells that tightly cohere together, and the individual cells were difficult to distinguish) of control molluscs (A),
Pb inhibited the degree of hemocyte aggregation. Poorly organized loose clump/ aggregates were noticed (B).

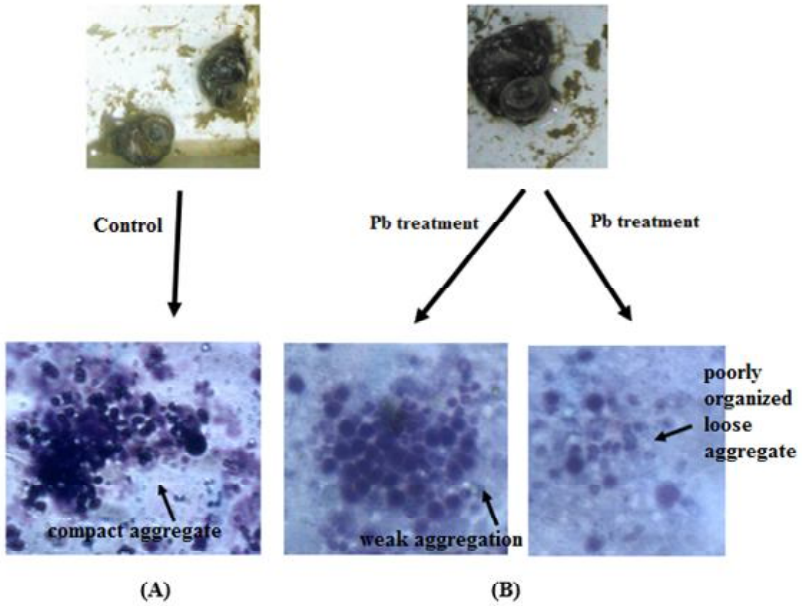


Fig. 2. Giemsa stained aggregation response of hemocytes (compact aggregates also called cohesive aggregation) of control molluscs (A),
Pb inhibited the degree of hemocyte aggregation. Weak aggregates, smaller irregular in shape, poorly organized loose clump/ aggregates were noticed (B).

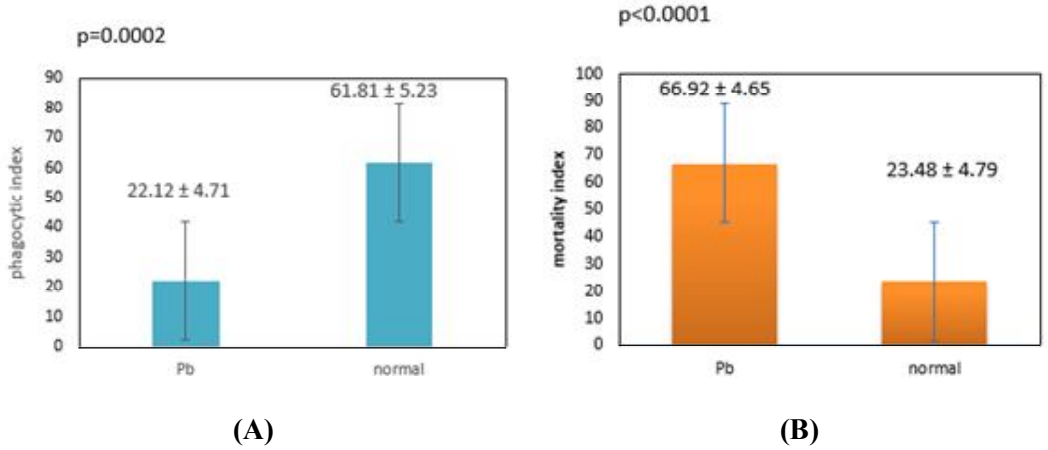
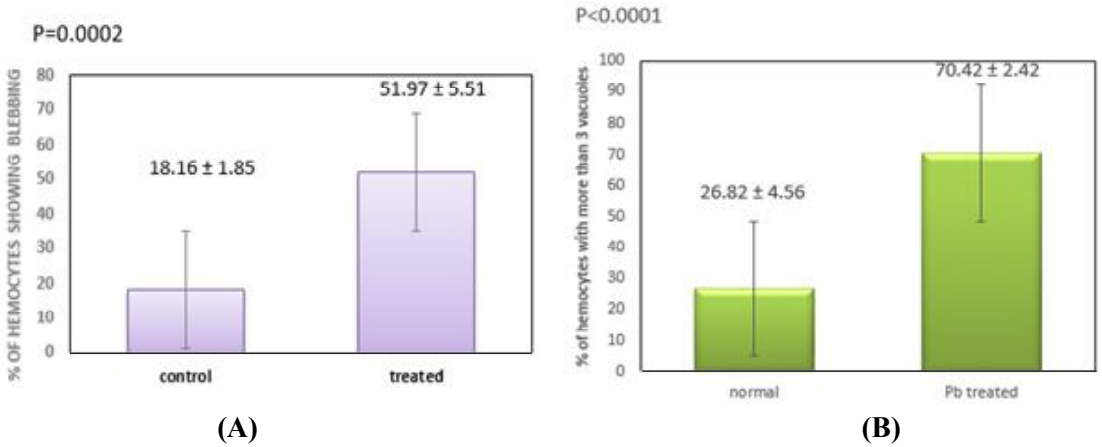


Fig.3. Mean phagocytic index in normal (control) and treated group in *Bellamya bengalensis* (A),
Mean mortality index in normal (control) and treated group in *Bellamya bengalensis* (B).
Values are expressed as Mean ± SEM. P-Value < 0.05 is considered to be statistically significant.



(A) **(B)**

Fig. 4. Mean percentage of hemocytes with blebbing in normal and Pb treated group of *Bellamya bengalensis* (A), Mean percentage of hemocytes with more than 3 vacuoles in normal and treated group of *Bellamya bengalensis* (B). Values are expressed as Mean ± SEM. P-Value < 0.05 is considered to be statistically significant.

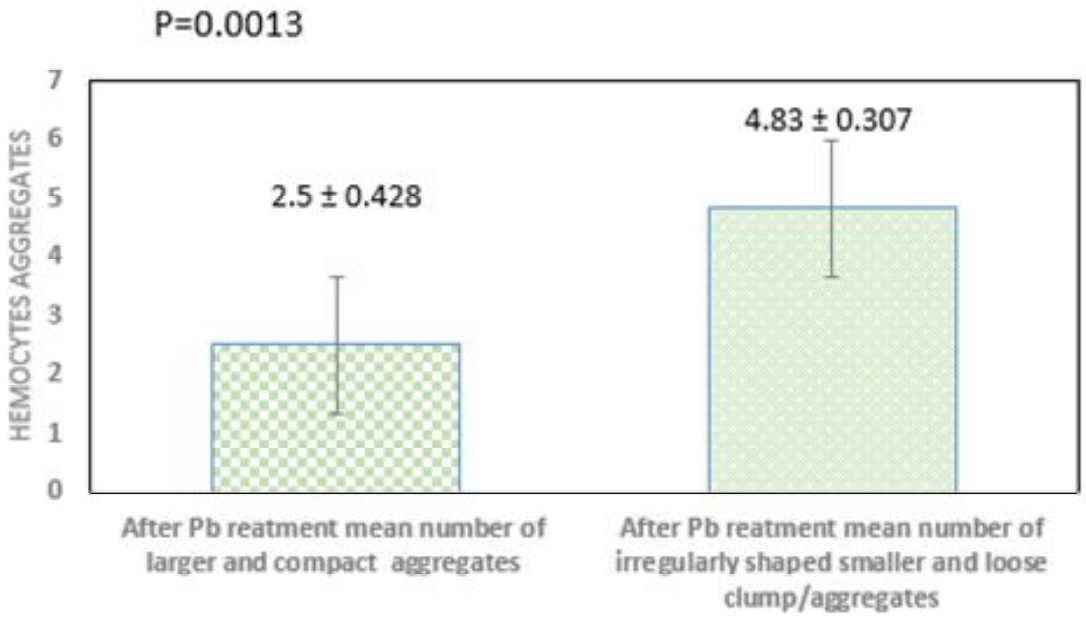


Fig. 5. Mean number of larger, compact aggregates and smaller irregular in shape, poorly organized loose clump/ aggregates noticed on glass slides after lead treatment. Values are expressed as Mean ± SEM. P-Value < 0.05 is considered to be statistically significant.

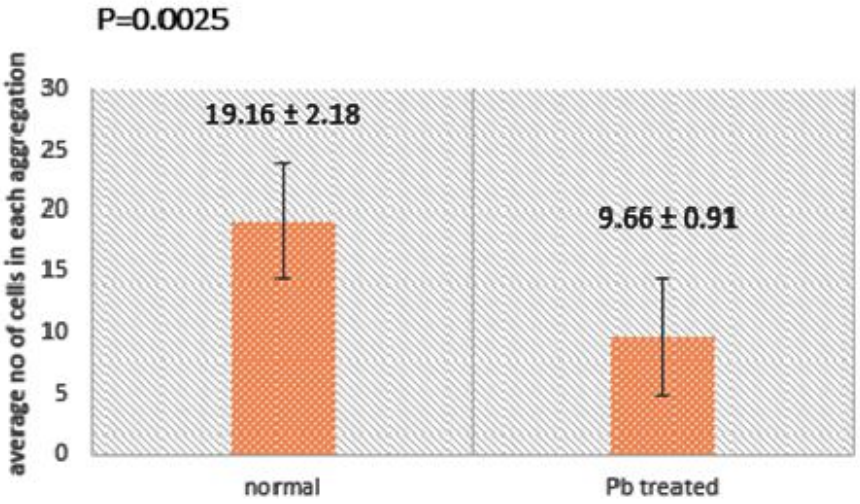


Fig. 6. Mean number of cells in each clump/ aggregate noticed on glass slides. Values are expressed as Mean ± SEM. P-Value < 0.05 is considered to be statistically significant.

Chen[4] stated when non-self particles invade molluscs or are introduced experimentally, phagocytosis, cell aggregation or encapsulation are induced[4]. If particles are too large to be phagocytosed, they may be gradually surrounded by hemocytes and encapsulation occurs[4].

The first report of molluscan cell clumping was published by Geddes 1880, cited by Narain[15]. Geddes described cell clumps as "plasmodia" because he observed hemocytes fused together to form clots[15]. In fact, clotted hemocytes only attached to each other by their filopodia[5]. Small clumps of cells then aggregate to form larger masses[6]. In molluscs, cell clump/aggregate formation occurs in vivo in response to microbial injection[2]. Such aggregation is also induced *in vitro*[15]. Chen[4] stated The aggregation of California mussel hemocytes in vitro can be categorized into three levels[4], like no aggregation (cells accumulate at the central area, but no aggregation formed), weak aggregation (individual cells are distinguishable, especially those located at the edge of the aggregate) and cohesive aggregation (due to compact nature of such aggregates, cells cannot be seen individually)[4].

In the present study Pb inhibited the degree of hemocyte aggregation. Smaller irregular in shape, poorly organized loose clump/ aggregates were noticed in lead treated group. (Fig. 1 and 2). In the present study mean number of larger, compact aggregates were less than smaller poorly organized loose clump/ aggregates on glass slides after lead treatment (Fig. 5) (two tailed p value is 0.0013). Mean number of cells in each clump/ aggregate was also decreased after Pb treatment (Fig. 6) (two tailed p value is 0.0025).

Mean phagocytic index in treated group in *Bellamya bengalensis* was significantly reduced (Fig. 3A) (two tailed p value is 0.0002). Mean mortality index in treated group in *Bellamya bengalensis* was significantly increased (Fig. 3B) (two tailed p value is <0.0001).

Cell death images in the present study showed membrane blebbing which was the indication of apoptosis whereas some cells revealed cytoplasmic vacuolation suggesting paraptosis type of cell death (pictures not shown). Mean percentage of hemocytes with membrane blebbing was significantly increased in treated group (Fig. 4A) (two tailed p value is 0.0002) and mean percentage of hemocytes with more than 3 cytoplasmic vacuoles (suggesting paraptosis) was significantly increased in treated group of *B. bengalensis* (Fig. 4B), (two tailed p value is <0.0001).

Sokolova *et al*.,[16] stated, cadmium exposure in oysters resulted in significant cytotoxicity and elevated levels of apoptosis and necrosis in hemocytes[16]. High rates of apoptosis due to heavy metal exposure may have important implications for the immune defense of the molluscs, resulting in the weakened immune system due to hemocyte loss[16]. Industrial pollutants might induce apoptosis in molluscan hemocytes by reactive oxygen intermediates (ROIs), through stress signal[17].

Guria *et al*.,[11] reported the effect of arsenic and lead on hemocytes of grasshopper [11] where both As and Pb inhibited haemocyte aggregation. Guria S, 2018 reported that lead (Pb) treatment led to cell death of hemocytes of *Lamellidens marginalis* [10] and Guria S, 2020 showed hemocytes of *Pila* sp. yielded multiple cellular aberrations upon treatment with arsenic[9]. These reports suggested nature of cell death may be used as an effective biomarker of pollution[10]. The present study corroborated the earlier studies.

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