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Alteration of Morphology, Phagocytic Behaviour and Aggregation of Insect Haemocytes Exposed to Contaminated Food with Arsenic and Lead

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Abstract : Grasshopper species may provide good systems to evaluate the toxic effects of some environmental contaminants. Arsenic and lead are widespread heavy metals that are released into the environment from different sources. Their accumulation in the soils can become dangerous to all kinds of organisms, including plants, animals and human life, causing many adverse effects. The aim of the present study was to determine the toxic effect of lead and arsenic on the cytomorphology of haemocytes of *Gesonula punctiforns* and to evaluate its potential as a bio monitor for detecting a heavy-metal polluted environment. Microscopic analysis of normal haemocytes showed different stages of phagocytosis like attachment of charcoal particle on cell surface, internalization of charcoal by cell. The haemocytes in treated group were not able to phagocytose the charcoal particles. As and Pb inhibited the degree of haemocyte aggregation. Significant number of treated haemocytes showed trypan blue positive response. Mean mortality index was significantly increased in treated group. Treated cells showed apoptosis or necrosis or paraptosis (vacuolation) like features. Morphological analyses suggest an irretrievable destruction of normal morphology of haemocyte. Sublethal toxicity of arsenic and lead is reported to affect the insect population by reducing its environmental fitness thus increasing its vulnerability to higher degree of disease, parasitamia and predation.

Keywords: Haemocytes, Insects, Phagocytosis, Aggregation, Cellular Death, Arsenic (As), Lead (Pb).

Introduction

Heavy metals are among the most problematic causes of water, soil and plant pollution. Genetic and biochemical effects of pollutants on organisms are important in establishing species as bioindicators for environmental hazards (1). Terrestrial insects that develop in the soil are also exposed directly to metal ions present in the soil. Grasshopper species may provide good systems to evaluate the toxic effects of some environmental contaminants (2, 3). Arsenic and lead are widespread heavy metals that are released into the environment from different sources. Their accumulation in the soils can become risky to all kinds of organisms, including plants, animals and human, causing many adverse effects (4, 5).

Morphologically, haemocytes are distinct variety of cells comparable to the vertebrate leucocytes and macrophages (6, 7), which constitute the important components of haemolymph of insects as well as in other arthropods and invertebrates (8, 9).

In view of this, in the present research attempt has been made to unify the haemocytes classification. The aim of the present work was to determine the toxic effect of lead and arsenic on the cytomorphology of haemocytes of *Gesonula punctifrons* and to evaluate its potential as a bio monitor for detecting a heavy-metal polluted environment.

Materials and methods

Treatment

The food was supplied to the adult grasshoppers (*Gesonula punctifrons*) for seven days observation. *Gesonula punctifrons* were fed on treated plant [their stems were previously immersed for 24 hrs in distilled water containing 0.025 mg/L and 50 mg/L of sodium arsenite ($NaAsO_2$) and lead nitrate $Pb(NO_3)_2$ respectively to allow the plant to absorb contaminated water] and on untreated plants (10,11).

Haemolymph collection

Haemolymph samples were withdrawn from the insects by means of incision made near the 3rd coxae. Thirty insects were used for each sample. The haemolymph was mixed with anticoagulant solution of citrate buffer/EDTA (pH 4.5). The drop of haemolymph then drawn into a thin film by the edge of another slide and the film air dried before staining. For phagocytosis study activated charcoal particles suspended in normal saline (0·67% NaCl) was injected into insect leg and the aspirate was taken for haemocytes study.

Staining of haemocytes

Haemolymph was placed and smeared directly on sterilized glass slides and were stained by giemsa, methylene blue, leishmans eosin solution and observed under light microscope. Different stages of phagocytosis of charcoal particles by haemocytes on glass slides were determined in both control and treated insects. Cellular morphology was examined.

Counting of haemocytes

We used haemolymph for cell counting by hemocytometer.

Micrometric analysis

In the present study, the cellular diameters (CD) of the cells were measured using calibrated eyepiece (Ocular) micrometer.

Trypan blue dye exclusion test

Cells were treated with 50 µl of 0.25 % trypan blue dye solution for 5 minutes. Cells that have taken up the dye are dead, since the dye is normally excluded by the membranes which maintain their semi permeability intact and therefore, the percentage of blue-stained cells represents a mortality index.

$$\text{Mortality index} = \frac{\text{Number of cells with blue stained cytoplasm}}{\text{Total number of cells}} \times 100$$

Study of Phagocytosis

Different stages of phagocytosis of charcoal particles by haemocytes were determined in both control and treated insects. The phagocytic index was calculated as per the following formula.

$$\text{Phagocytic index} = \frac{\text{Number of cells with phagocytosed charcoal particle}}{\text{Total number of cells counted}} \times 100$$

Result

Normal cytomorphological profile of haemocyte of grasshopper

Normal cell morphotypes were noticed in control insects. Prohaemocytes (PRs), Plasmatocytes (PLs), Granulocytes (GRs), Vermicytes (VEs) and Podocytes (POs) were categorically recognised (Figure 1).

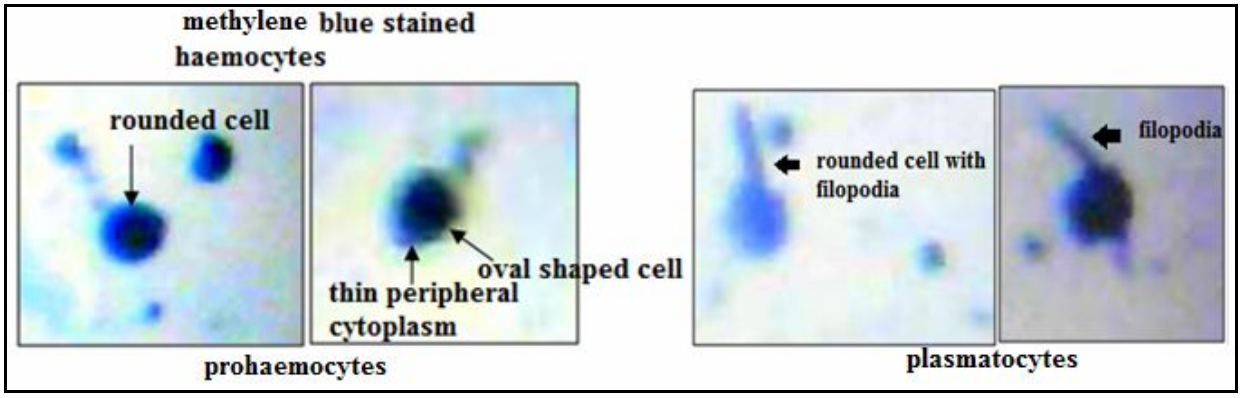


Figure1. Normal cytomorphological profile of some selected haemocytes

Phagocytic behaviour of haemocytes

Normal haemocytes showed different stages of phagocytosis like attachment of charcoal particle on cell surface, phagocytosis of charcoal by cell or internalization of charcoal by cell. Formation of small cytoplasmic process, food cup and filopodias were noticed (Figure 2A and B).

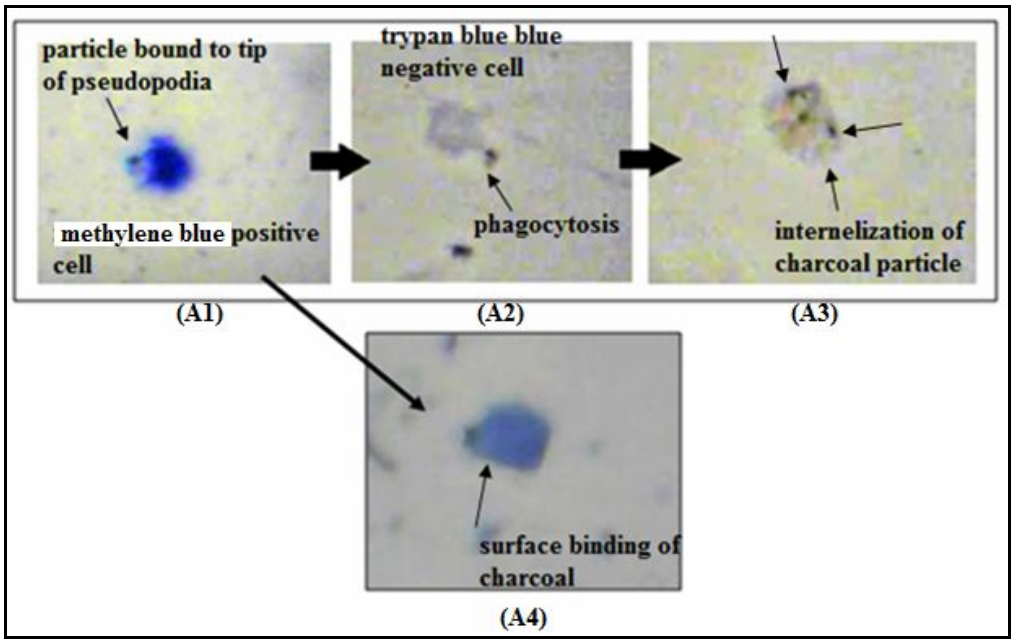


Figure2. (A) Different stages of phagocytosis in normal grasshopper haemocytes stained by methylene blue and trypan blue. A1-A2: attachment of charcoal particle on cell surface, A3: internalization of particle, A4: phagocytosis of charcoal by cell.

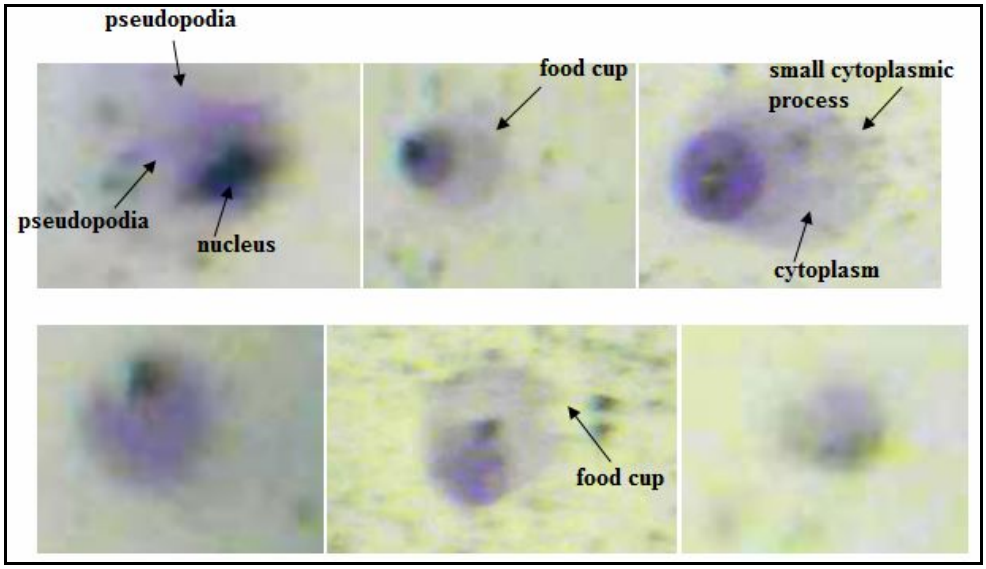
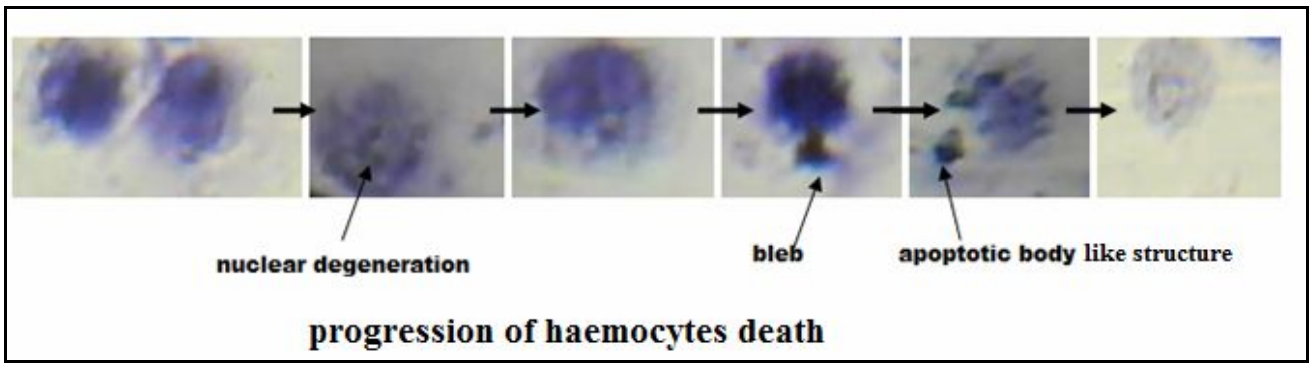


Figure2. (B) Different stages of formation of pseudopodia, small cytoplasmic process, food cup and filopodia in grasshopper haemocytes

Effect of toxic metal on cell-structure of haemocytes

Toxic metal treatment led to vacuolization in the cytoplasm of haemocytes. Significant changes were observed in the cytomorphology of haemocytes when compared with the control group under light microscopy. Treated insects exhibited cellular damage. Higher magnification of lead (Pb) treated cells showed different phases of cellular death like formation of membrane blebs, rupture of plasma membrane and degeneration of nuclei (Figure 3).

Note the large cytoplasmic vacuoles found in arsenic (As) treated haemocytes. Normal ultra structural morphology was predominately found in the control cells, showing a well-defined plasma membrane and intact nucleus. After arsenic treatment, the cell cytoplasm displayed vacuoles (Figure 4). The haemocytes in treated group were not able to phagocytose the charcoal particles (Figure 4). A significant percentage of haemocytes become pyknotic in treated condition.



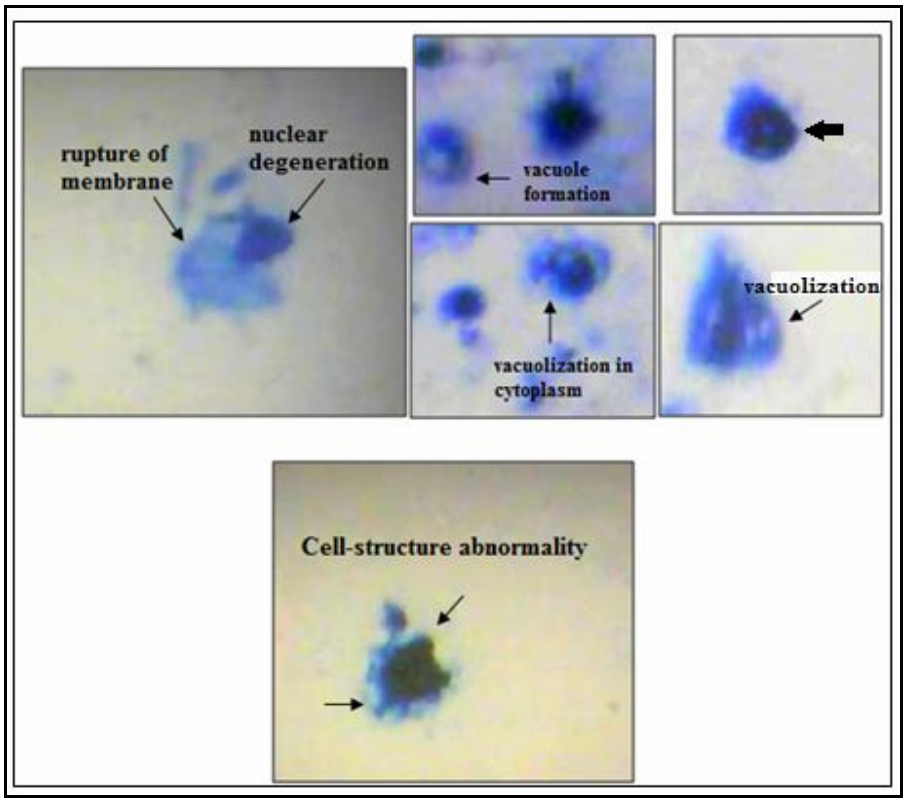


Figure3. Methylene blue stained lead treated cell showed altered cell surface indicating cellular apoptosis or necrosis or vacuolation (x 400)

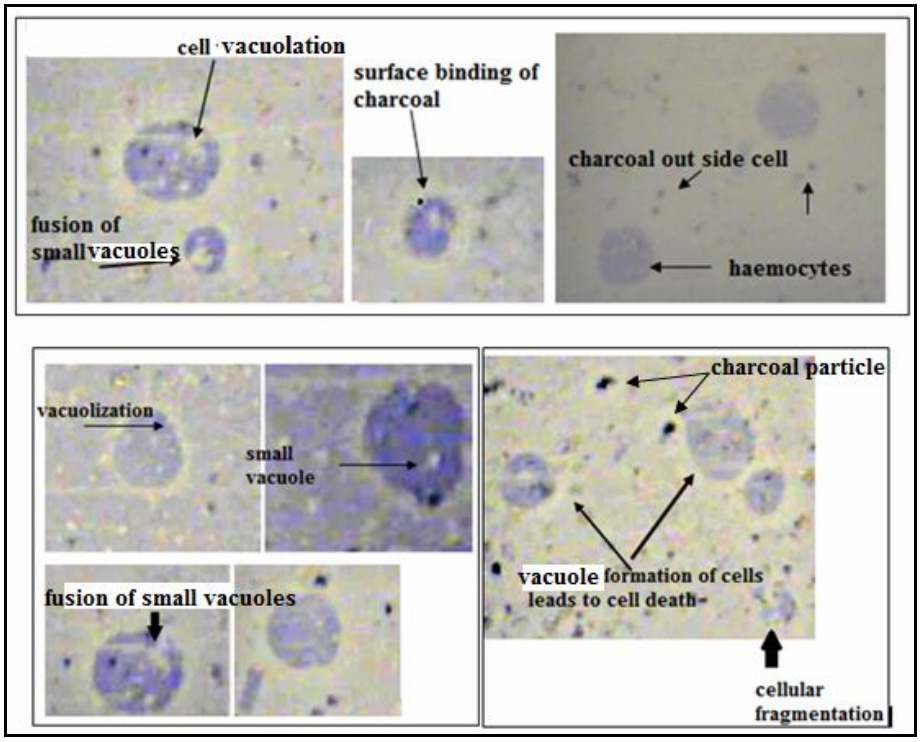
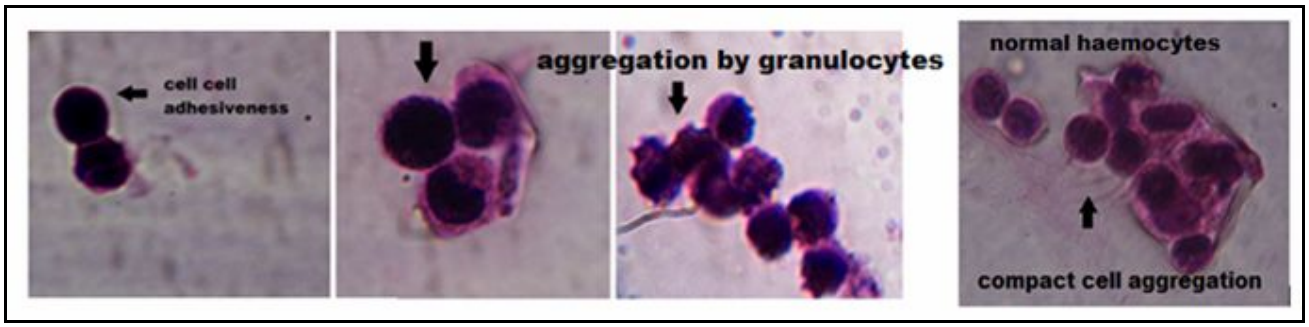


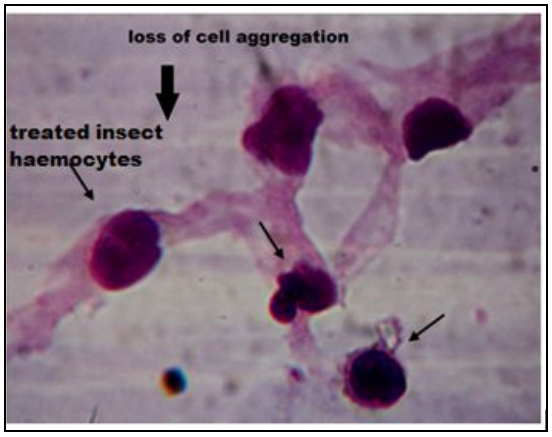
Figure4. Arsenic treated haemocytes with different phases of cytoplasmic vacuole formation (x 400).V= Vacuole. Note the charcoal particles remain outside the cells.

Effect on cellular aggregation

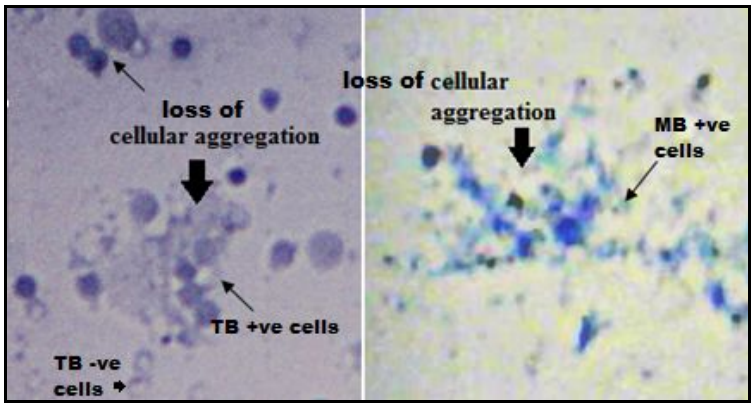
As and Pb inhibited the degree of haemocyte aggregation (Figure5).



(A)



(B)



(C)

Figure5. (A) Typical aggregation response of haemocytes (giemsa stained) of a normal grasshopper. (B) Arsenic inhibited the degree of haemocyte aggregation. (C) Lead partially inhibited the degree of haemocyte aggregation. MB= Methylene Blue, TB= Trypan Blue

Trypan blue staining of haemocytes

Significant number of treated haemocytes showed trypan blue positive response. Dead cells were blue in colour whereas the viable cells of controls were white (Figure 6A & B).

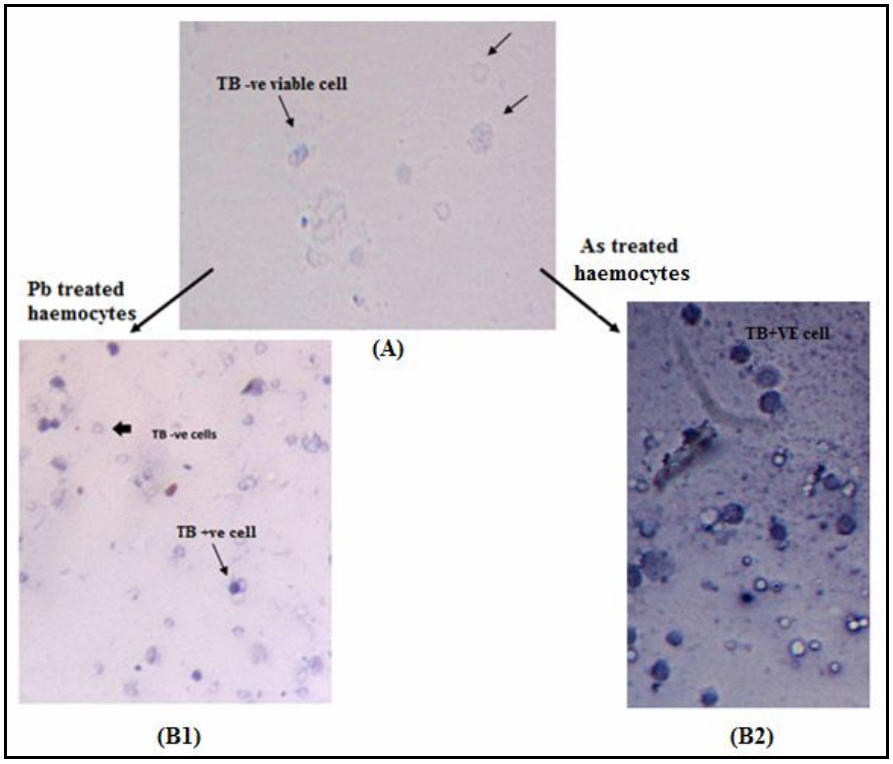


Figure6. (A) Normal insect viable haemocytes and (B1 & B2) lead and arsenic treated dead haemocytes.

Calculation of mortality index

Mean mortality index was significantly increased in treated group (Figure7).

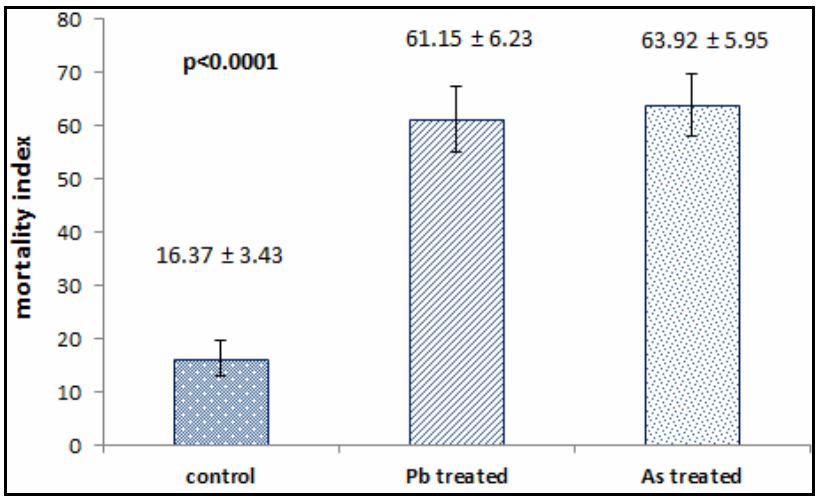


Figure7. Mean mortality index in normal and treated group. Values are expressed as Mean ± SEM. P-Value < 0.05 is considered to be statistically significant.

Phagocytic Index

Mean phagocytic index was significantly reduced in treated group (Figure8).

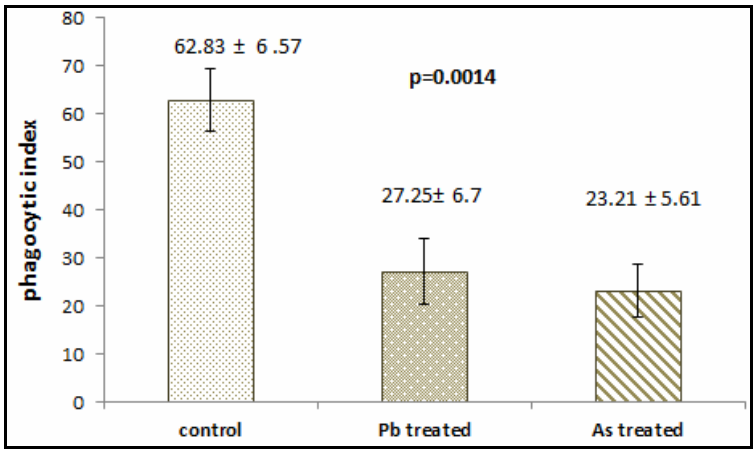


Figure8. Mean phagocytic index in normal and treated group. Values are expressed as Mean ± SEM.

Haemocytes diameter

Mean cell diameter was significantly increased in treated group (Figure9).

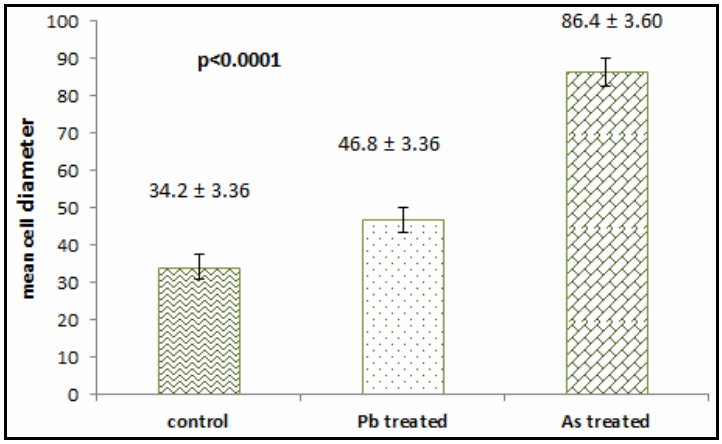


Figure9. Mean cell diameter in normal and treated group. Values are expressed as Mean ± SEM.

Discussion

Previous studies showed that plasmatocytes were responsible for cellular immune responses in many insect (12). The number of haemocytes in circulation can change rapidly in response to environmental stress, wounding or infection (13). It is possible that the number of haemocytes was directly altered by the change in food. Our result showed that significant number of treated haemocytes were dead as they showed trypan blue positive response (Figure 6 B1 & B2). Toxicity of arsenic was investigated in the cytoarchitecture of haemocyte of insect by exposing the animals to the sublethal concentrations of sodium arsenite. Treated cells showed apoptosis or necrosis or paraptosis (vacuolation) like features (Figure 3 & 4). Increased cell diameter in treated group may be the indication of early stage of cell rupture (Figure 9). Arsenic induced haemocytic disruption in relation to shape, size of cell and nuclear morphology is indicative to possible dysfunction of cell. Blood cells or haemocytes are reported as chief immune effector cells of invertebrates and are capable of performing multiple immunological functions including non self adhesion, aggregation, phagocytosis and generation of cytotoxic agents (14). The toxic exposure may impair or alter the innate immune response of haemocyte of the animal that may lead to decline of biodiversity. This study would help to be selected grasshopper as bio indicator species. Invertebrates including arthropods rely on innate immune defences (14). They have complex and efficient host defence systems that can identify and eliminate potential pathogens efficiently. Neutrophils, macrophages and dendritic cells of vertebrate are professional phagocytes and have a regulatory role in adaptive immunity by producing costimulatory molecules and immunomodulatory cytokines (15). Phagocytosis is a primordial aspect of innate immunity and is conserved in all arthropods. In Drosophila, a single cell type that

resembles the mammalian monocyte/macrophage lineage exerts this function is plasmatocyte, a type of haemocyte (16).

Phagocytosis, in general, is considered a classical innate immune response reported in the majority of the invertebrate phyla. It is an established immunological response and is considered as a biomarker of any pollution (17). We reported the inhibitory effect of sodium arsenite and lead on the phagocytic response of insect haemocytes under the challenge of charcoal particle. Mean phagocytic index was significantly reduced in treated group (Figure 8). The haemocytes in treated group were not able to phagocytose the charcoal particles (Figure 4). Phagocytic index showed the impairment in the phagocytic potential of the haemocytes of arsenic-treated insects may lead to compromisation of the immune status of the animals distributed in the contaminated habitat. Arsenic and lead induced altered reactivity of haemocytes may affect the propagation and survival of insect population by increasing its vulnerability to higher rate of disease and parasite attack.

Cellular aggregation is a functional feature offered by the haemocytes of invertebrates to prevent the accidental blood loss by formation of biological plug at the site of wound and resist the entry of pathogenic microorganism (18). Hence, cell–cell aggregation is considered as an immunological response for host defence. Aggregation of haemocytes around invaded microorganisms is termed as "encapsulation response" and is considered as an important immunological reaction (19). When successful encapsulation occurs, a host animal can restrict the proliferative and invasive property of a pathogen. Encapsulation reaction is mediated by specific population of immunoactive blood cells and is effective in cell-mediated immunity of invertebrates. As inhibited the cellular aggregation response in arthropod haemocytes (Figure 5B). Workers apprehend such scenario in the natural environment may lead to a decline in the population of insects and loss of grassland biodiversity. Furthermore, a drastic increase in the occurrence of free cells was recorded against As and Pb treatment, which was suggestive to possible role of these chemical agents as inhibitor of cellular aggregation (Figure 5). Therefore, arsenic and lead induced alteration of immune status may impart a state of vulnerability in insect inhabiting the arsenic and lead-polluted environment.

Morphological analyses suggest an irreparable destruction of normal morphology of haemocyte. Sub lethal toxicity of arsenic and lead is reported to affect the insect population by reducing its environmental fitness thus increasing its vulnerability to higher degree of disease, parasitamia and predation. Additionally, present study is also aimed to establish an effective bio indicator by which the health of the grassland ecosystem can rapidly and accurately be screened to protect its important bio resource. From present investigation and generated data, we suggest to adopt necessary measure to minimize the degree of aquatic as well as soil contamination for protection of various economically and ecologically important species.

Conclusion

Recent studies of both the humoral and cellular components of the invertebrate immune system have revealed that invertebrates share many of the fundamental immunological mechanisms with vertebrates, including humans (20). However, both the vertebrate macrophage and the invertebrate haemocyte phagocytic systems are homologous in being concerned with recognition and engulfment of foreign material (20). The mechanism whereby invertebrate phagocytes inherently recognise 'foreignness' in the absence of immunoglobulins is unknown. Due to continuous use of arsenic and lead contaminated water, it may accumulate in soils, can be taken up by plants and thereby enter the food chain as well as food web. Being a component of ecological food chain, this arsenic and lead affected grasshopper may play a significant role in accumulating and further transferring toxic metals to higher trophic levels in the food chain (21).

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgement

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