Chapter 7

RESTORATION OF HAEMOCYTE DENSITY & FUNCTION

7.1 Introduction

Analysis of haemocyte population dynamics in relation to total haemocyte count (THC) is important to judge the health and vigour of an invertebrate organism (Jones, 1962; Wheeler, 1963; Shapiro, 1979; Arnold and Hinks, 1976). An understanding of the types and functions of haemocyte in molluscs under natural ambient conditions is essential in studying basic cell responses in relation to: (a) environmental change (Fisher et al., 1989; Pamparinin et al., 2002) (b) handling of the animals (Ballarin et al., 2003; Malham et al., 2003) and (c) infections (Beckmann et al., 1992; Ford et al., 1993; Cochennec-Laureau et al., 2003). Marked variation in the density of haemocytes is often related to haemocytes release from haemocytopoietic organs into the circulation (Hoffmann, 1973; Crossley, 1975). Haemocytes act as the major immune effector cells (Cheng, 1977; Adema et al., 1991b) and mediate non-self phagocytosis that provides natural immunity in the bivalves (Lopez et al., 1997a, b).

Nitric oxide (NO) is considered as an important immune molecule for invertebrates including bivalve molluscs (Conte and Ottaviani, 1995; Bogdan, 2001) as it has the ability to kill pathogens itself or by combining with superoxide (O$_2^-$) to form peroxynitrite, a strong bactericidal agent (Arumugam et al., 2000).

Since bivalves have an open circulatory system, haemocytes are susceptible targets of many contaminants, with serious consequences for their homeostatic capacity (Auffret and Oubella, 1997). The response and functioning of the immune system is a complex process and is highly susceptible to disruption by environmental stressors. In bivalves, exposure to pollution has often caused an increase in the number of circulating haemocytes (Pipe et al., 1995b; Fisher et al., 2000; Oliver et al., 2001). In addition, long-term exposure to contaminant or short acute exposure often inhibits the phagocytic capacity of haemocytes (Sami et al., 1992; Pipe et al., 1995b; Cajaraville et al., 1996; Dyrynda et al., 2000; St-Jean et al., 2003). While many studies have been conducted on the response of the immune system to pollution, very few have investigated the recovery capacity of the immune system when environmental health is restored. Dyrynda et al. (2000) observed a rise in total haemocyte count in parallel with a decrease in phagocytic capacity one month after an oil spill.
One of the aims of toxicological study is the determination of the biosafe level of a particular toxicant or pollutant in the environmental exposures. Below these reference values, a model should not experience adverse health effects, whatever their exposure conditions. Determination of NOEL or "No Observed Effect Level" of a particular toxin with respect to a particular biological parameter is one such process of estimation of a biosafe level of exposure of a particular toxic substance (Crane and Newman, 2000). The NOEL is the highest concentration in a test with a mean response not differing significantly from the mean response of the control if compared statistically. The perceived advantage of the NOEL relative to regression- derived estimates, such as the LD$_{50}$, is that it is easy to calculate, easy to understand, and is an important component of current chemical and effluent risk assessment procedures (Campbell and Hoy, 1996; de Bruijn and Hof, 1997).

In the present study, attempts have been made to examine the possibility of recovery of density and selected functions of the haemocytes of _L. marginalis_ pre-exposed to different concentrations of sodium arsenite. Estimation of NOEL for determination of biosafe level of sodium arsenite exposure to _L. marginalis_ with respect to haemocyte structure and function would provide baseline information about the functional response of the chief immune effector cells of the molluscs. The information would pave path towards formulation of a sustainable strategy for conservation of the mollusc and similar freshwater organisms in their natural habitat and might prove essential for conservation of the habitat itself.

### 7.2 Materials and methods

#### 7.2.1 Total haemocyte count

Haemolymph was collected aseptically from the heart of the large sized animals and was stored in prechilled glass vials. The collected haemocytes were resuspended in sterile snail saline (SSS)(5mM HEPES, 3.7mM NaOH, 36 mM NaCl, 2 mM KCl, 2mM MgCl$_2$, 2H$_2$O, 4mM CaCl$_2$, 2H$_2$O; pH 7.8) (Adema _et al._, 1991a) and stored at 4°C. A portion of the fresh haemolymph was placed on clean, sterilized, glass slides in moist chamber and the hemocytes were allowed to settle for 15-20 min at room temperature. The adherent haemocytes were fixed with 1% glutaraldehyde (Sigma, USA, synthesis grade) for 5 min and rinsed with sterile snail saline. After fixation the monolayer of the haemocytes was stained with Giemsa’s stain (Himedia, India). The slides were then observed under a light microscope (Axiostar Plus, Zeiss, Germany). The total count of the haemocytes of each specimen was carried out according to Brousseau _et al._ (1999) using a Neubauer haemocytometer. Each experiment was repeated for at least 5 times.
7.2.2 Phagocytosis of nonself particulate

Baker's yeast (Saccharomyces cerevisiae, West Mill Foods, Maidenhead, Berks, UK) was cultured in YM broth (Difco, E. Molesly, Surrey, U.K.) overnight at 25°C in a shaking water bath. The cultured cells were killed by boiling for 1 h and the resulting cell suspension was washed thrice in TBS/Ca2+ (20mM Trizma base, 77 mM NaCl, 10 mM CaCl2) at pH 7.4 by centrifugation at 650 x g for 10 min. The washed cells were then resuspended at a concentration of 10⁷ cells/ml in Grace's Insect Medium (GIM; Himedia). The phagocytic efficiency of the hemocytes was examined by challenging them with yeast suspension over glass slides.

For the animals exposed in vivo to 1, 2, 3, 4 and 5 ppm sublethal doses of sodium arsenite, aseptical collection of haemolymph was done from the heart of the animals after Brousseau et al. (1999). Haemolymph was stored in prechilled glass vials. The concentration of haemocytes was adjusted to 10⁶ cells/ml with SSS. A portion of the haemolymph was smeared on clean, sterilized, glass slides in moist chamber to obtain a haemocyte monolayer on the glass surface. The haemocytes were allowed to settle for 15–20 min at room temperature. To the adherent monolayer of haemocytes, 10 µl of yeast (1 x 10⁷ cells/ml) suspension was added and incubated at 37°C in a humid chamber for 3 h. After incubation, the monolayer was washed with sterile snail saline, stained with Giemsa's stain and observed under microscope (Axiostar Plus, Zeiss, Germany). A negative control for the assay was set using 2% sodium azide, a known phagocytic inhibitor. From each slide, 200 fields were counted on an average to estimate the number of phagocytic haemocyte, total number haemocyte and number of yeast particles engulfed by each haemocyte. The data were calculated and represented in terms of phagocytic index (PI), where, PI= (Percentage Phagocytosis x Average number of engulfed particles) (Elssner et al., 2004). The entire experiment was repeated for at least 5 times.

7.2.3 Estimation of nitric oxide generation

The generation of nitric oxide was measured as the amount of the nitrite released from the haemocytes with Griess reagent after Green et al. (1982) with minor modifications. The concentration of hemocytes was adjusted to 10⁶ cells/ml and 1 ml of the haemocyte suspension was incubated with equal volume of Griess reagent (1% Sulphanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 5% orthophosphoric acid) at 37°C for 30 min in a humid chamber. The absorbance was recorded in a spectrophotometer (CECIL-CE 4002, Germany) at 550nm against a standard blank. The generation of nitric oxide was determined using a standard curve of sodium nitrite. The entire experiment was repeated for at least 5 times.
7.2.4 Determination of NOEL

For estimation of NOEL, large sized animals (10 per batch) were exposed to sublethal concentrations of 0.0005, 0.0006, 0.0007, 0.0008, 0.0009, 0.001, 0.002 and 0.003 ppm of sodium arsenite \textit{in vivo}. The pH of the solution was maintained at 7.2. Each experimental batch of animals was exposed to a volume of 5 l of sodium arsenite solution in borosilicate glass containers. The experiments were carried out in static water environment and fresh solution of sodium arsenite were replenished every 12 h for 30 days. Aseptical collection of haemolymph was done from the heart of the animals after Brousseau \textit{et al}. (1999) and was stored in prechilled glass vials.

7.2.4.1 NOEL for haemocyte count and dimension

The THC of each specimen was carried out according to using a Neubaue haemocytometer. The entire experiment was repeated at least for 10 times. Haemocytometer suspension of 200μl was placed on clean sterile glass slide and the cells were left to adhere on the glass surface for 3 h at 37°C in a humid chamber. For analysis of the adherent cell morphology, the slides were washed with sterile snail saline (SSS), stained with Giemsa's stain and observed under microscope (Axiostar Plus, Zeiss, Germany). Agranular haemocyte diameters were measured with the help of an oculometer and the readings were compared with a stage micrometer. Nearly 200 fields/slide was observed for accounting the cell dimensions.

7.2.4.2 NOEL for intrahaemocyte nitric oxide generation

The generation of intrahaemocyte nitric oxide was measured with Griess reagent after Green \textit{et al}. (1982). The concentration of haemocytes was adjusted to 10^6 cells/ml and 1 ml of the haemocyte suspension was incubated with equal volume of Griess reagent at 37°C for 31 min in a humid chamber. The absorbance was recorded in a spectrophotometer (CECIL- CI 4002, Germany) at 550nm against a standard blank. The generation of nitric oxide was determined using a standard curve of sodium nitrite. The nitric oxide activity was expressed as μM/10^6 cells/min. The entire experiment was repeated for at least 12 times.

7.2.5 Statistical analysis

For the recovery assays, the statistical data analysis was carried out using Student's \textit{t}-test (Sokal and Rohlf, 1973). Differences were considered significant at \( P < 0.05 \), \( P < 0.01 \), \( P < 0.001 \). Data was presented as the mean ± standard error (S.E). For estimation of NOEL, data was analyzed by single tailed ANOVA (Dunnett’s test). Differences were considered significant at 0.05.
7.3 Results

7.3.1 Partial restoration of total haemocyte count

The total restoration of the haemocyte count was not attained in all sets of post-treated animals even after maintenance in toxin free water for a maximum period of 30 days. The maximum recovery in total count was recorded in the animals exposed to 2 ppm/96 h as 37.10% in 30 days (Figure 1b). The lowest level of restoration was noted as 2.69% in 5 ppm / 24 h in 15 days (Table 1).

![Graph](image)

Figure 1. Restoration of THC of post-treated large sized *L. marginalis* after maintenance in arsenic free water for (a) 15 and (b) 30 days. Data are mean + SE. (P<0.05).

Table 1. Percentage of restoration of THC of post-treated large sized *L. marginalis* after maintenance in arsenic free water for 15 and 30 days.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Sodium arsenite (ppm)</th>
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<tbody>
<tr>
<td>15 days</td>
<td>30 days</td>
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<tr>
<td>24</td>
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<tr>
<td>12.36 %</td>
<td>16.66 %</td>
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<td>48</td>
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<td>3.22 %</td>
<td>9.67 %</td>
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<td>72</td>
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<td>10.21 %</td>
<td>18.28 %</td>
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<tr>
<td>96</td>
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<tr>
<td>19.89 %</td>
<td>34.95 %</td>
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7.3.2 Inhibition in restoration of phagocytosis

Partial restoration in PI of the haemocytes was observed in toxin free water with presence of residual toxic effect of inhibition (Figure 2). In the long term exposure experiments, trend of recovery was identical with other sets and full recovery of phagocytic efficacy was not achieved.

![Figure 2. Recovery in phagocytic index (PI) of the haemocytes of post-treated large sized L. marginalis after maintenance in arsenic free water for 30 days. Data presented as mean ± S.E. *P < 0.05, **P < 0.01.](image)

7.3.3 Restoration of nitric oxide generation

The animals exposed to sublethal concentrations of sodium arsenite for 24 h and maintained in arsenic free water for 24 h recovered fully in terms of generation of intrahaemocyte nitric oxide. Partial restoration in intrahaemocyte nitric oxide generation was observed in hemocytes of rest of the post-treated animals even after maintenance in toxin free water for a maximum period of 30 days (Figure 3).

![Figure 3. Recovery in intrahaemocyte nitric oxide (NO) activity of post-treated large sized L. marginalis after maintenance in arsenic free water for 30 days. Data presented as mean ± S.E. *P < 0.05, **P < 0.01, ***P< 0.001.](image)
7.3.4 No observed effect level (NOEL)

The NOEL value of sodium arsenite with respect to the THC of large sized *L. marginalis* was enumerated as 0.0009 ppm for 30 day exposure. The NOEL value of sodium arsenite with respect to size of the adherent agranular haemocytes was enumerated as 0.0007 ppm for 30 day exposure. The NOEL value of sodium arsenite with respect intrahaemocyte nitric oxide generation of large sized *L. marginalis* was enumerated as 0.0007 ppm for 30 day exposure.

7.4 Discussion

Haemocytes are characterised by diverse subpopulation of cells of morphological and functional discreteness namely granulocyte, agranulocyte, hyalinocyte, blast-like cell, astrocyte. Granulocytes and hyalinocytes are capable of performing nonself phagocytosis, encapsulation, cytotoxicity (Smith and Söderhäll, 1983; Söderhäll *et al.*, 1985) and play important role in mounting immunological response against invading parasites and pathogens. Cytoplasmic granule is less in agranulocytes and astrocytes which perform phagocytosis as chief immunological response. Hemocytes with blast-like morphology are considered as haemocyte progenitors (Hine, 1999; Cima *et al.*, 2000) to other cell types. It is reported that haemocytes appeared to recover, at least partially, from a long-term exposure to industrialization once mussels were transferred to a cleaner environment (Mayrand *et al.*, 2005). Partial restoration of total haemocyte count in post-treated animals in toxin free water suggests that the toxic effect is persistent in nature. The alteration in the total count of hemocytes of *L. marginalis* implies a possible immune compromise in the animal that reflects a potential risk to the species in arsenic contaminated habitat. It has also been demonstrated that the phagocytic capacity, which at the time of collection was significantly low in mussels native to polluted area, rapidly rose when transferred and maintained in healthy environment (Mayrand *et al.*, 2005). Phagocytosis is considered as a classical immune response of the invertebrates including molluscs. Haemocytes are reported as chief phagocytes capable of generating nitric oxides - a potential cytotoxic agent. Exposure to inorganic arsenic affected the phagocytic efficiency and generation of nitric oxide in haemocytes. Sublethal concentration of sodium arsenite had suppressed these primary defence responses in the bivalve leading to a state of immune compromise. The effect of the natural toxin in all the studied concentrations was threatening over prolonged exposures and efforts to restore the normal parameters proved futile even after 30 days. Such shift in immunological status of *L. marginalis* may lead to decline of the population of the species due
to possible opportunistic growth of microbes and parasites. Sublethal concentrations of sodium arsenite not only suppressed the nitric oxide activity in *L. marginalis*, its residual toxic effect enabled the post-treated animals to restore the normal activity. Although the result is indicative of a hormetic effect of sodium arsenite on nitric oxide generation, such stimulation in immune function is quite usual under induction of low concentrations of metallic toxins (Cheng and Sullivan, 1984; Barnier, 1995). But the trend remains as immune suppression with further increase in toxin concentration on prolonged time of exposure. Since nitric oxide plays the dual role of scavenging superoxide anions and production of bactericidal peroxynitrite molecules, low production of the immune molecule would increase oxidative stress on the animal and make the animal vulnerable to opportunistic microbial attack. Situation may lead to gradual loss of biodiversity in the freshwater ecosystem of selected regions of India. In this study, the NOEL values of sodium arsenite for the THC, haemocyte dimension and generation of intra-haemocyte cytotoxic molecules of *L. marginalis* was found to be very low signifying the sensitivity of the parameters at feeble concentrations of arsenic exposure. Immune effector subpopulation of haemocytes of the *L. marginalis* appears to be an important biomarker of aquatic pollution in relation to phagocytosis and generation of nitric oxide. Aquatic ecosystem of this subcontinent supports a wide range of biodiversity which is under threat of environmental contamination. The alterations in the total count of haemocytes of *L. marginalis* imply a possible immune compromise in the animal. Phagocytosis and generation of intra-haemocyte nitric oxide are major immune parameters of the species that regain partial normalcy even in 30 days. The observation clearly reflects that the toxin slowed the resilience of the animal against the immunological stress which might prove detrimental for the species population and other inhabitants in arsenic contaminated natural habitat. Sublethal toxicity is reported to affect a biological population by reducing its fitness thus increasing its vulnerability to higher rate of disease, parasitamia and predation (Oliver and Fisher 1999; Fournier et al. 2000). Even if a toxin retards the developmental event rather killing the members of some population, the impact of such event on the ecology probably remains even more far reaching. With the increased development and industrialisation of the world, the likelihood of water pollution increases. While it is essential that we have clean fresh drinking water for drinking and cooking, it becomes the duty of every civilized mind to take care of their surrounding environment in a eco-friendly and sustainable manner. The assays clearly demonstrate the feasibility of the parameters to be adopted for monitoring the health of the freshwater aquasystems of India. Although the toxicity of sodium arsenite was persistent, the resilience exhibited by the animals provides hope that in restored environments they are likely to be surviving. The
increase in density of these efficient filter feeders would stabilize the biodiversity of the freshwater habitats of India and conserve the habitability of the water bodies in a sustainable manner.

7.5 Bibliography


Studies on the toxicity of arsenic in Lamellulens marginalis (Lamarck)

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