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ORIGINAL ARTICLE

Evaluation of phagocytic activity and nitric oxide generation by molluscan haemocytes as biomarkers of inorganic arsenic exposure

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Abstract

The natural habitats of the freshwater bivalve *Lamellidens marginalis* face the risk of contamination by the toxic metalloid arsenic. Haemocyte-mediated non-self phagocytosis and generation of nitric oxide (NO) as reactive nitrogen intermediate were examined to establish the reliability of the parameters as biomarkers of sodium arsenite-induced stress on the animal at sublethal concentrations. The studies suggest imposition of a remarkable immune compromise/immune suppression on the animal by the natural contaminant. The animal expressed partial recovery in its phagocytic potential and NO generation over a period of 30 days. Quantitation of phagocytic efficiency and intrahaemocyte NO generation indicates the possibility of the parameters be accepted as cellular biomarkers to estimate and characterize the vulnerability of the freshwater organisms to sodium arsenite-induced stress.

Keywords: *Lamellidens marginalis; haemocyte; sodium arsenite; phagocytosis; nitric oxide; biomarker*

Introduction

The freshwater wetland systems of India support a rich biodiversity of molluscs. *Lamellidens marginalis*, a filter feeding bivalve, is a common dietary item of the rural mass of the eastern India and a well-recognized poultry feed (Chakraborty et al. 2008). The species is not cultured and is harvested indiscriminately from its natural habitats. Occasionally, natural pearl is found in the species which emphasizes its commercial potentiality. Bivalves have the natural ability to increase sediment homogenization (McCall et al. 1979) and provide clear substratum for the colonization of epiphytic and epizoic biota (Beckett et al. 1996) thereby increasing their importance as a member of freshwater ecosystem.

The natural habitat of *L. marginalis* is threatened by the risk of contamination by arsenic, a major water pollutant of the Gangetic basin of India and Bangladesh (Acharyya et al. 1999). Biochemical leaching of the earth crust sediment cause mobilization of arsenic in water (Islam et al. 2004). Extensive use of arsenic-contaminated groundwater for agriculture is in practice in India; arsenic-laden agricultural run-offs contaminate the field adjacent freshwater bodies (Islam et al. 2000) – the natural habitat of many organisms including *L. marginalis*. Moreover, stratifications of lakes in summer cause a 10–20% increase in arsenic concentration in water (Aggett & O'Brien 1985). In water of the interstitial lake, 90% arsenic exists as arsenite, a highly toxic trivalent form of arsenic (Aggett & Kriegmann 1988). The effects of arsenic toxicity in the mammalian system have so far been well documented but the report of its effect on freshwater bivalves in the Indian subcontinent is limited.

Haemocytes, the circulating cells of the bivalves, act as the major immune effector cells (Cheng 1977, Adema et al. 1991b). Haemocyte-mediated non-self-phagocytosis provides natural immunity in the bivalves

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(Lopez et al. 1997). Nitric oxide (NO) is produced as a reactive nitrogen intermediate during formation of L-citruline from L-arginine by nitric oxide synthase (Ischiropoulos et al. 1992). This NO has the ability to kill foreign microorganisms by combining with superoxide (O_2^-) to form peroxynitrite, a strong bactericidal agent (Arumugan et al. 2000).

In this study, the phagocytic potency and the rate of NO activity of the haemocytes of *L. marginalis* was estimated by exposing the animals to sublethal concentrations of sodium arsenite ($NaAsO_2$) in controlled laboratory conditions so as to validate the immune parameters as biomarkers of aquatic toxicity in contaminated habitats.

Methods

Collection and treatment of animals

Fresh specimens of *L. marginalis* with an average size of 7.022 x 4.135 cm were collected from some selected freshwater ponds of the district of 24-Parganas (South) of West Bengal in India. The collected animals were transported to the laboratory and acclimated for 5–7 days in well-aerated tanks. The animals were reared and maintained according to Raut (1991) with necessary modifications. Aqueous solutions of sodium arsenite (E. Merck, Germany; 99% pure; CAS no. 7784-46-5) were prepared in borosilicate glass containers at concentrations of 1, 2, 3, 4 and 5 ppm. The pH of the solution was maintained at 7.2. Each experimental set consisted of ten animals of the same length. They were exposed to a volume of 5 l of sodium arsenite solution. For the control, a set of animals was kept in arsenic-free analytical grade water. The experiments were carried out in a static water environment and fresh solutions of sodium arsenite were replenished in every 12 h.

As for the recovery assay, sets of ten animals were first exposed to solutions of sodium arsenite. After exposure for 24, 48, 72 and 96 h and 30 days, the animals were transferred to arsenic-free analytical grade water for examining possible restoration activity. The temperature of the water was maintained between 24 and 26°C.

Collection of haemolymph

Aseptic collection of haemolymph was done from the heart of the animals after Brousseau et al. (1999) 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 min at room temperature.

Culture of yeast

Baker's yeast (*Saccharomyces cerevisiae*, West Mill Foods, Maidenhead, Berks, UK) was cultured in YM broth (Difco, E. Molesly, Surrey, UK) 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 three times in pH 7.4 TBS/Ca^{2+} (20 mM Trizma base, 77 mM NaCl, 10 mM $CaCl_2$) by centrifugation at 650*g* for 10 min. The washed cells were then resuspended at a concentration of 10^7 cells ml^{-1} in Grace's Insect Medium (GIM; Himedia).

Cell viability

The viability of the haemocytes of *L. marginalis* of all experimental variants was tested with 2% Trypan blue for 10 min following the dye-exclusion principle.

Phagocytosis

The phagocytic efficiency of the haemocytes was examined by challenging them with yeast suspension in a prestandardised ratio of 1:10 *in vitro* over slide. To the adherent monolayer of haemocytes, 0.1 µl of yeast (1×10^7 cells ml^{-1}) was added and incubated at 37°C in a humid chamber for 3 h. After incubation, the monolayer was washed with sterile snail saline (5 mM HEPES, 3.7 mM NaOH, 36 mM NaCl, 2 mM KCl, 2 mM $MgCl_2.2H_2O$, 4 mM $CaCl_2.2H_2O$; pH 7.8) (Adema et al. 1991a), stained with Giemsa's stain and observed under microscope (Axiostar Plus, Zeiss). A negative control for the assay was set using 2% sodium azide, a known phagocytic inhibitor. Not less than 200 fields were examined for each slide and the following data were recorded: number of phagocytic haemocytes, total number of haemocytes and number of yeast particles engulfed by each haemocyte. The data were calculated and represented in terms of phagocytic index (PI), where PI = (particles/cell x phagocytic cells/100) (Elssner et al. 2004). The entire experiment was carried out for five times.

Estimation of NO generation

The generation of NO 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 haemocytes was adjusted to 10^6 cells ml^{-1} 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)

at 550 nm against a standard blank. The generation of NO was determined using a standard curve of sodium nitrite and was expressed as µM of NO generated per 10^6 cells. For assay of NO during phagocytosis, a suspension of 10^6 haemocytes ml^{-1} was challenged with yeast spores at an optimum ratio of 1:10 and was incubated in humid chamber at 37°C for 3 h. The NO activity of the incubated cell samples were estimated with Griess reagent and the absorbance was spectrophometrically recorded at 550 nm. The entire experiment was repeated five times.

Statistical analysis

The statistical data analysis was carried out using Student's *t*-test. Differences were considered significant at $p<0.05$, $p<0.01$, $p<0.001$. Data are presented as the mean ± standard deviation.

Results

Cell viability

The average viability of haemocytes ranged from the highest value of 96.30 ± 2.65% under an exposure of 3 ppm of sodium arsenite for 24 h to the lowest value of 86.50 ± 1.89% under an exposure of 5 ppm of sodium arsenite for 96 h (Table 1). The lethal response to arsenic of haemocytes of the animals maintained in an arsenic-free environment for a maximum time of 30 days (Table 2) was identical to the arsenic-exposed set.

Inhibition of phagocytic response

The phagocytic response of the haemocytes (Figure 1) challenged with yeast was suppressed upon exposure of the animals to 2, 3, 4 and 5 ppm sodium arsenite as revealed from the decline in PI (Figure 2).

The toxicity of arsenic on haemocyte was maximal under an exposure of 30 days. The PI of the haemocytes exhibited a dose-dependent suppression.

Inhibition in NO generation

The lowest concentration of sodium arsenite resulted in an elevation of NO generation and the exposure to higher concentrations resulted in a steady decrease in generation of intrahaemocyte NO expressing mild hormesis at certain concentrations (Figure 3). Long-term exposure to sodium arsenite for 30 days resulted in an acute decrease in intrahaemocyte NO generation (Figure 3).

Generation of NO in haemocytes exposed to yeast was initially high followed by a decrease in NO activity under 30 days exposure to higher concentrations of sodium arsenite (Figure 4).

Inhibition in restoration of phagocytosis and NO generation

Partial restoration in PI of the haemocytes was observed in arsenite-free water with the presence of a residual toxic effect of inhibition (Figure 5).In the long-term exposure experiments, the trend of recovery was identical to that

Table 1. Viability (%) of haemocytes of *L. marginalis* on exposure to 1, 2, 3, 4 and 5 ppm of sodium arsenite for 24, 48, 72, 96 h and 30 days. Data are presented as mean ± standard deviation ($n=5$).

Concentration of $NaAsO_2$ exposure	Duration of exposure of *L. marginalis* in inorganic arsenic				
	24 h	48 h	72 h	96 h	30 days
Control	96.23 ± 2.58	96.05 ± 2.11	95.83 ± 1.98	96.45 ± 3.25	95.57 ± 1.25
1 ppm	95.45 ± 3.44	95.2 ± 2.65	94.7 ± 1.56	91.00 ± 3.68	90.87 ± 1.50
2 ppm	94.50 ± 2.11	93.25 ± 2.99	91.5 ± 3.45	90.4 ± 2.77	88.23 ± 2.54
3 ppm	96.30 ± 2.65	95.5 ± 4.12	91.4 ± 3.87	92.5 ± 3.21	89.45 ± 1.46
4 ppm	89.60 ± 2.21	87.4 ± 3.89	93.25 ± 0.96	95.4 ± 2.33	89.12 ± 2.87
5 ppm	88.00 ± 2.45	87.1 ± 2.60	88.10 ± 1.55	86.50 ± 1.89	87.54 ± 1.98

Table 2. Viability (%) of haemocytes of post-treated *L. marginalis* maintained in arsenic-free water for 30 days. Data are presented as mean ± standard deviation ($n=5$).

Concentration of $NaAsO_2$ exposure	Duration of maintenance of post-treated *L. marginalis* in arsenic-free water				
	24 h	48 h	72 h	96 h	30 days
Control	94.33 ± 1.75	93.57 ± 2.45	95.49 ± 2.87	93.22 ± 1.85	93.34 ± 1.75
1 ppm	94.05 ± 2.53	94.54 ± 1.89	93.67 ± 2.55	94.45 ± 1.73	92.77 ± 2.11
2 ppm	94.10 ± 2.07	92.44 ± 3.2	92.83 ± 2.64	93.56 ± 1.79	91.22 ± 3.07
3 ppm	92.20 ± 1.34	90.95 ± 2.74	91.66 ± 2.03	89.24 ± 1.83	90.29 ± 1.99
4 ppm	91.27 ± 2.54	89.40 ± 1.79	90.54 ± 2.54	92.35 ± 2.10	88.88 ± 1.47
5 ppm	92.36 ± 1.95	89.96 ± 1.75	91.58 ± 3.12	88.26 ± 2.11	89. 53 ± 2.47

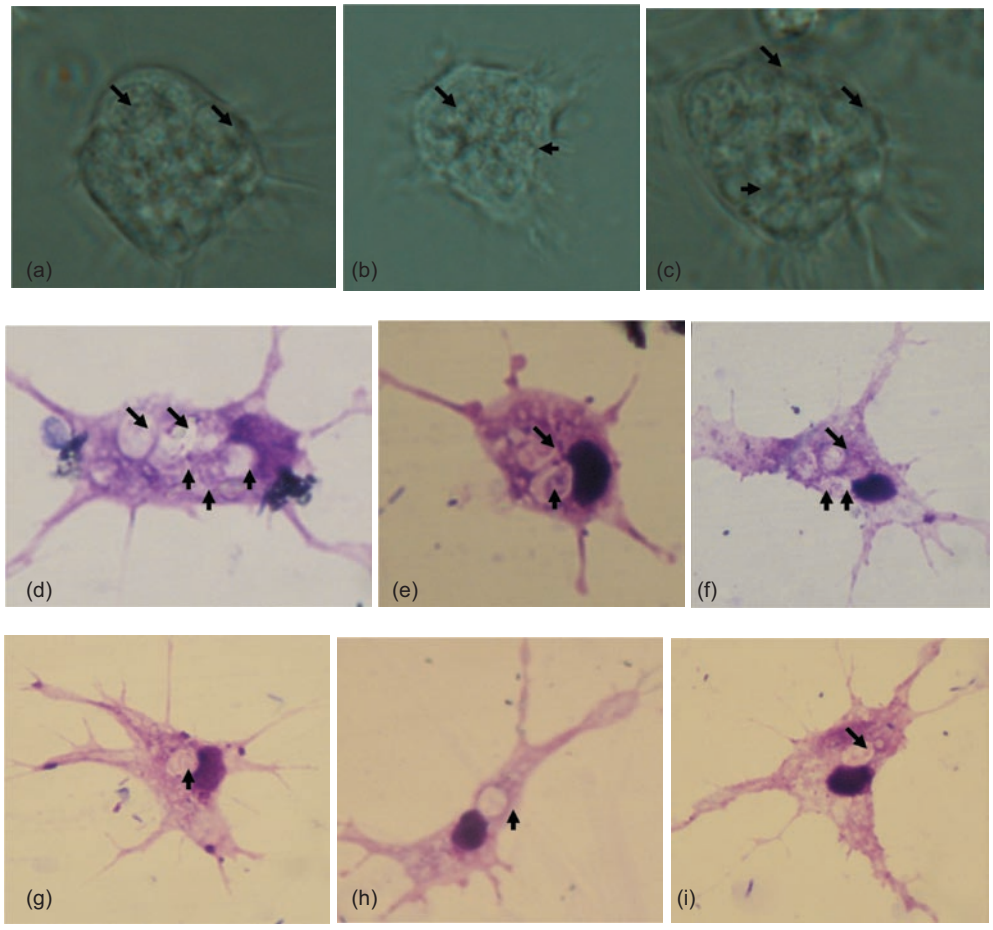

Figure 1. Haemocytes of *L. marginalis* phagocytosing yeast particles: (a–c) phase-contrast images and (d–f) Giemsa-stained light micrographs of haemocytes of the control animals showing high phagocytic efficiency; (g–i) haemocytes from animals exposed to 2 ppm of sodium arsenite for 48 h showing low phagocytic efficiency. Bar = 10 μm, magnification x 1000.

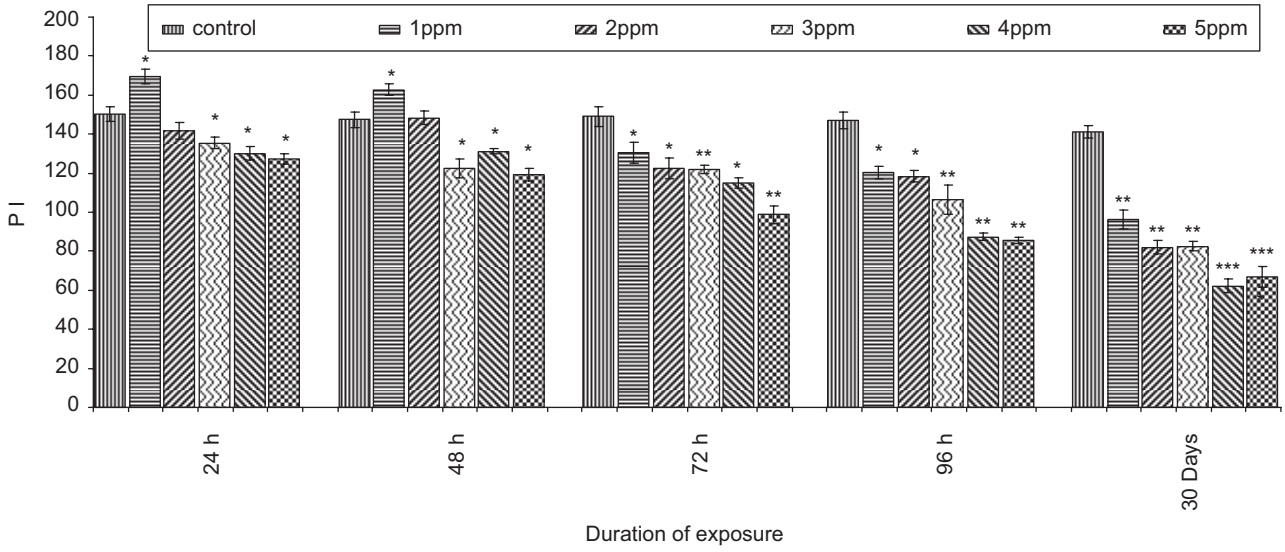

Figure 2. Phagocytic index (PI) of haemocytes of *L. marginalis* after exposure to 1, 2, 3, 4, 5 ppm of sodium arsenite for 24, 48, 72, 96 h and 30 days under the challenge of yeast. Data are presented as mean ± standard deviation ($n = 5$). *$p < 0.05$, **$p < 0.01$, ***$P < 0.001$.

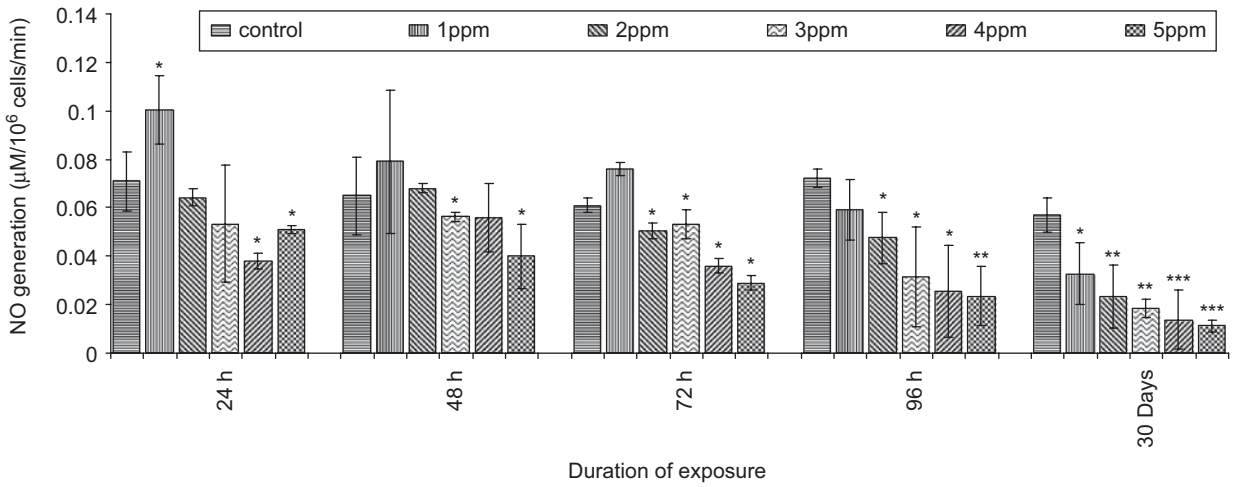

Figure 3. Intrahaemocyte NO generation of *L. marginalis* exposed to 1, 2, 3, 4, 5 ppm of sodium arsenite for 24, 48, 72, 96 h and 30 days. Data are presented as mean ± standard deviation ($n = 5$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

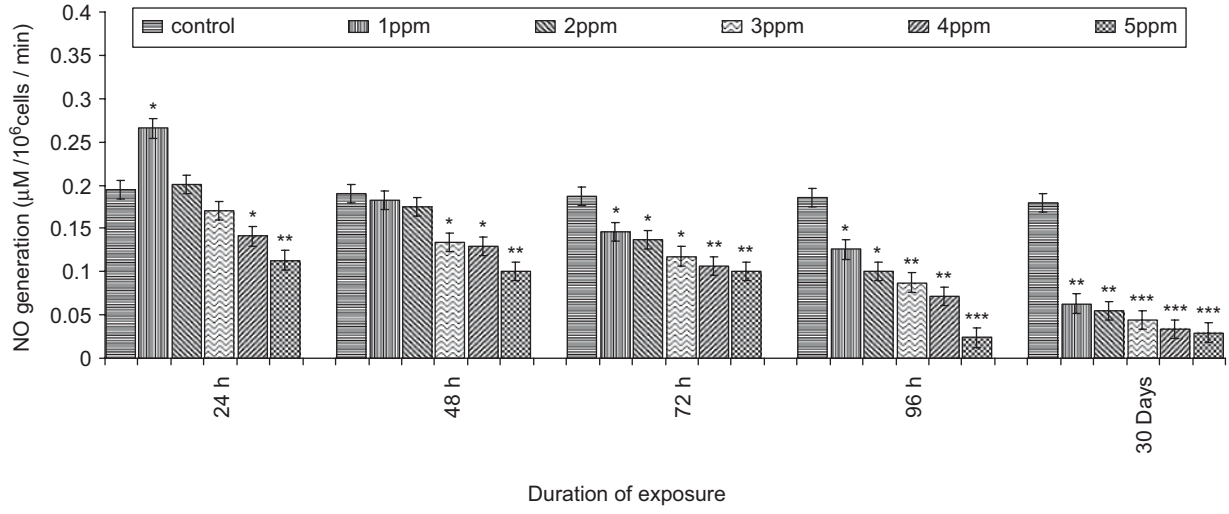

Figure 4. Intrahaemocyte NO generation of *L. marginalis* after exposure to 1, 2, 3, 4, 5 ppm of sodium arsenite for 24, 48, 72, 96 h and 30 days under the challenge of yeast. Data are presented as mean ± standard deviation ($n = 5$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

with other sets and full recovery of phagocytic efficacy was not achieved.

The NO generation of the animals was restored to normal level conditions in the animals exposed to 1 ppm sodium arsenite for up to 96 h of exposure for both yeast-challenged and unchallenged conditions. However, the animals maintained for 30 days in arsenic-free water exhibited a lower level of intrahaemocyte NO generation both in the presence and absence of yeast (Figure 6a, b).

Discussion

Phagocytosis is a widely acknowledged immunological parameter and considered as an effective biomarker of aquatic pollution (Oliver & Fisher 1999). The present study provides a convincing proof of the inhibitory effect of sodium arsenite on the phagocytic potency of the haemocytes of *L. marginalis* at various sublethal concentrations. The effect of the natural contaminant in all the studied concentrations was threatening over prolonged exposures and efforts to restore the normal parameters proved futile even after 30 days. 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 NO in haemocytes. Sublethal concentrations of sodium arsenite had suppressed these primary defence responses in the bivalve leading to a state of immune compromise.

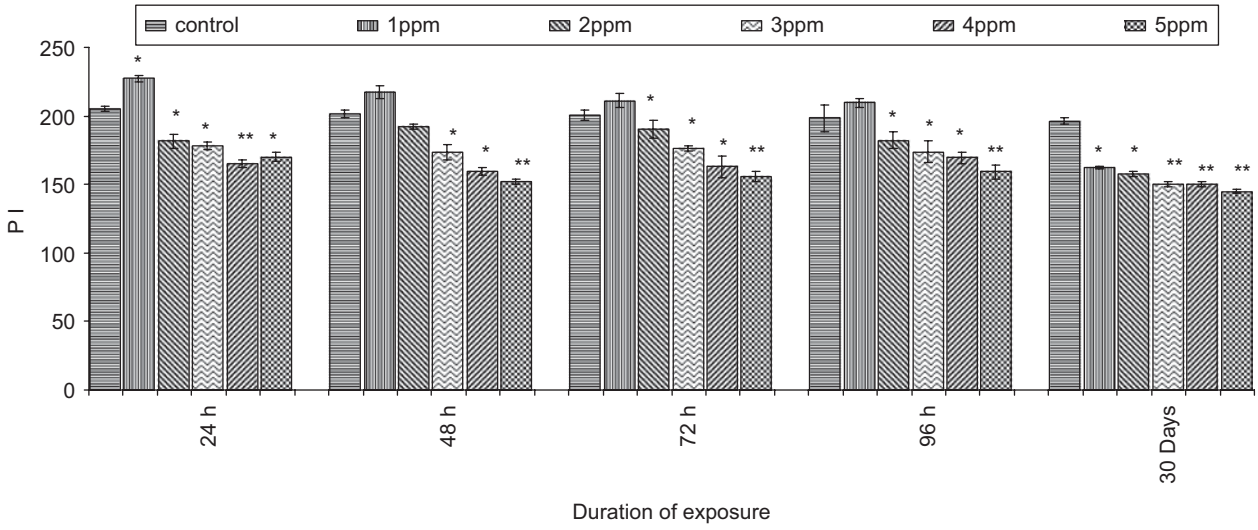

Figure 5. Restoration of phagocytic index (PI) in haemocytes of post-treated *L. marginalis* maintained in arsenic-free water for 24, 48, 72, 96 h and 30 days under the challenge of yeast. Data are presented as mean ± standard deviation ($n = 5$).*$p < 0.05$, **$p < 0.01$.

In the past two decades, NO-mediated pathogen killing and superoxide remediation has earned recognition as an effective immune strategy in the animal world (Bogdan 2001). Sublethal concentrations of sodium arsenite not only suppressed the NO activity in *L. marginalis*, its residual toxic effect prevented the post-treated animals to restore the normal activity. The NO activity was also found to be downregulated by sodium arsenite exposure when the treated haemocytes were challenged with yeast spores. Although the result is indicative of a hormetic effect of sodium arsenite on NO generation, such stimulation in immune function is quite usual under induction of low concentrations of metallic contaminants (Cheng & Sullivan 1984, Barnier et al. 1995). However the trend remains as immune suppression with a further increase in contaminant concentration on prolonged time of exposure. As NO plays the dual role of scavenging O_2^- radicals and production of bactericidal peroxynitrite molecules, low production of the immune molecule affects the animal dually: unscavenged naturally produced O_2^- will pose damage to the conformity of the internal cell and tissue structures of the animal and will make the animal vulnerable to opportunistic microbial attack.

Sublethal toxicity is reported to affect a biological population by reducing its fitness thus increasing its vulnerability to higher rate of disease, parasitaemia and predation (Oliver & Fisher 1999, Fournier et al. 2000).

The use of an innate immune biomarker like phagocytosis is a promising *in situ* tool for investigation because this assay appears to be effective in field conditions and it does not require sensitization of the animals, a critical issue in dealing with wildlife species (Fournier et al. 2000). The present results have confirmed that phagocytosis and generation of intrahaemocyte NO may be considered as an effective immunological biomarker for arsenic-induced stress due to dose-responsive immunomodulation in the experimental circumstances.

In India, the contamination of wetlands by various contaminants affects the aquatic ecosystem adversely. Development of a suitable biomarker to assess the quality of the environment appears to be an important scientific challenge of the country. *L. marginalis* is a widely distributed aquatic bivalve sensitive to diverse forms of xenobiotics (Chakraborty et al. 2008). The immune effector subpopulation of haemocytes of the animal appears to be an important biomarker of aquatic pollution in relation to phagocytosis and generation of NO. Aquatic ecosystem of this subcontinent supports a wide range of biodiversity which is under threat of environmental contamination. Our study is aimed to establish an inexpensive biomarker by which the health of the aquatic ecosystem of India can rapidly and accurately be screened to protect its important bioresource. As such, the assays bear high feasibility to be selected as biomarkers of aquatic contamination and can be considered as significant tools for estimation of contaminant-induced stress in the aquatic molluscs. However, the present study provides an information base of immunological effect of inorganic arsenic on phagocytosis and cytotoxic response in bivalve. Further in depth investigations are needed for the establishment of the species parameters as biomarkers of aquatic contamination.

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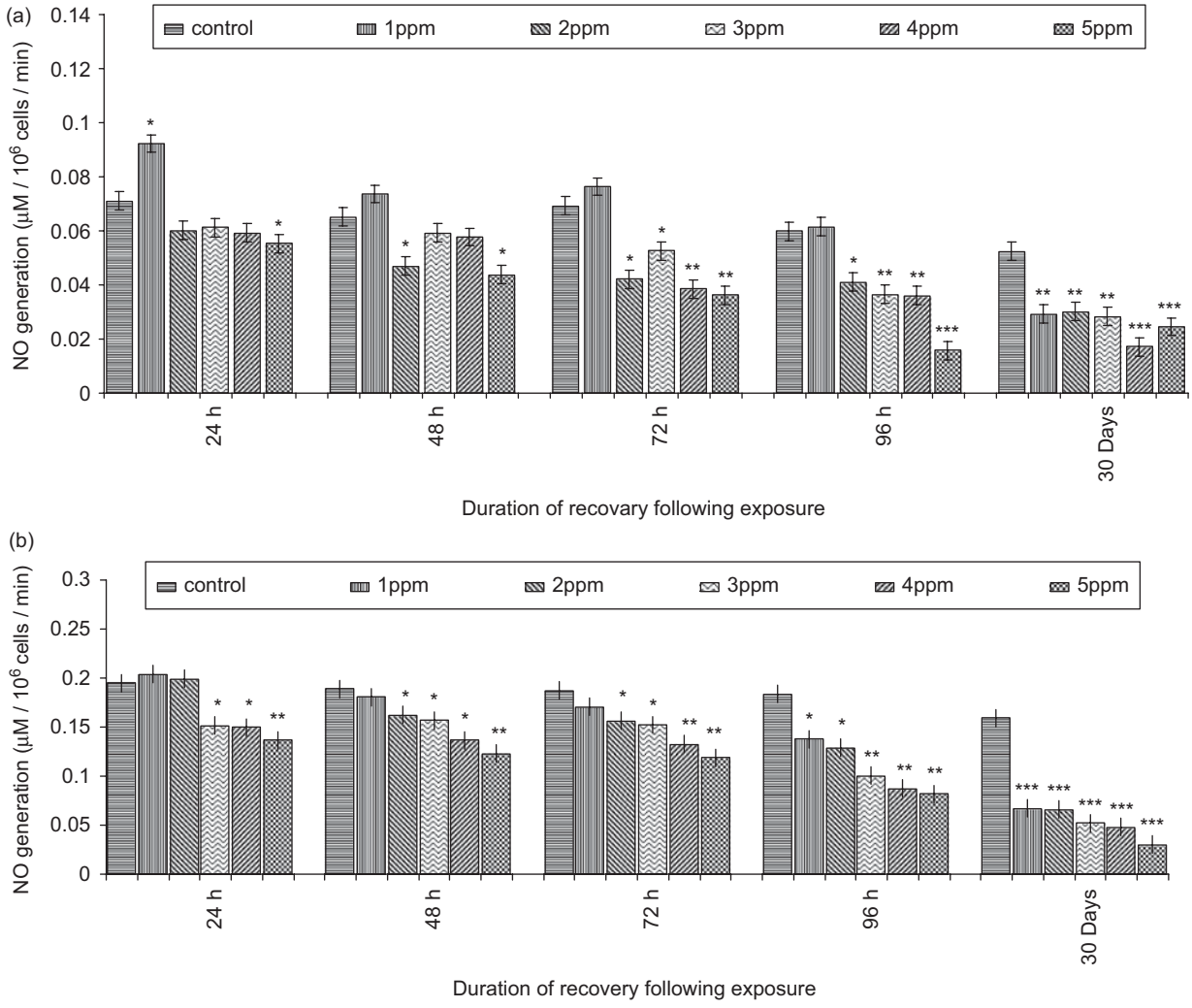

Figure 6. (a) Restoration of NO activity in haemocytes of post-treated *L. marginalis* maintained in arsenic-free water for 24, 48, 72, 96 h and 30 days. Data are presented as mean ± standard deviation ($n = 5$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.(b) Restoration of NO activity in haemocytes of post-treated *L. marginalis* maintained in arsenic-free water for 24, 48, 72, 96 h and 30 days under the challenge of yeast. Data are presented as mean ± standard deviation ($n = 5$).*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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