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Application of primary haemocyte culture of *Penaeus monodon* in the assessment of cytotoxicity and genotoxicity of heavy metals and pesticides

Seena Jose [a], P. Jayesh [a], A. Mohandas [a], Rosamma Philip [b], I.S. Bright Singh [a,*]

[a] National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Kochi 682016, India
[b] Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Fine Arts Avenue, Kochi 682016, India

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ABSTRACT

Lack of shrimp cell lines has hindered the study of pollutants which adversely affects shrimp health and its export value. In this context a primary haemocyte culture developed from *Penaeus monodon* was employed for assessing the cytotoxicity and genotoxicity of two heavy metal compounds, cadmium chloride and mercuric chloride and two organophosphate insecticides, malathion and monocrotophos. Using MTT assay 12 h IC_{50} values calculated were 31.09 ± 16.27 μM and 5.52 ± 1.16 μM for cadmium chloride and mercuric chloride and 59.94 ± 52.30 mg l^{-1} and 186.76 ± 77.00 mg l^{-1} for malathion and monocrotophos respectively. Employing Comet assay, DNA damage inflicted by these pollutants on haemocytes were evaluated and the pollutants induced DNA damage in >60% of the cells. The study suggested that haemocyte culture could be used as a tool for quantifying cytotoxicity and genotoxicity of aquaculture drugs, management chemicals and pollutants.

1. Introduction

Cell lines are widely utilised for assessing the toxicity of major aquatic pollutants such as heavy metals and pesticides. Effluents from industries and runoff from agricultural fields may contain such pollutants, which on reaching aquatic environments lead to impairment of the ecosystem. Needless to say, the bio-accumulation and persistence of these pollutants in the aquatic environment constitute a serious threat to biological life and also to human beings indirectly by virtue of their involvement in the food chain (Binelli and Provini, 2004).

Among heavy metals, cadmium and mercury are the most highly toxic elements owing to their persistence in the environment. They are redox inactive which challenge antioxidant defence by binding to thiols in cells such as reduced glutathione (GSH) (Stohs and Bagchi, 1995; Ercal et al., 2001). They cause oxidative and nitrosative stress (Stohs and Bagchi, 1995; Ercal et al., 2001; Pompella et al., 2003) and damage macromolecules in cells. Apoptosis or necrotic cell deaths are the usual consequences (Pulido and Parrish, 2003). Toxicity of pesticides on non - target organisms and ecosystems is of world wide concern (Pimentel et al., 1998). Among pesticides, organophosphate pesticides are stable enough to remain in their toxic form for relatively shorter periods of time (Poovala et al., 1998). They act primarily by inhibiting the enzyme acetylcholine esterase at cholinergic junctions of the nervous system (Gallo and Lawryk, 1991) leading to functional changes at the tissue/organism level (Pope et al., 2005).

Heavy metals and pesticides are known genotoxicants, as they have the capacity to interact with and damage the structure of the DNA molecule, often with a concomitant adverse effect on biological integrity (Shugart, 1995, 1998). Many genotoxicants may cause DNA strand breakage by the formation of free radicals or abasic sites which can result in the breakage of phosphodiester linkages within the DNA molecule (Shugart, 2000).

Attempts to develop cell cultures from marine invertebrates date back about a century (Gomot, 1971; Rannou, 1971). Yet for unidentified reasons all efforts to develop continuous cell lines from these organisms including crustaceans have been ineffective so far (Mothersill and Austin, 2000; Rinkevich, 2005). *Penaeus monodon* is one among the important cultured crustacean in the world. Lack of shrimp cell lines has been hampering progress of research in testing the effects of drugs and management chemicals prior to their administration in aquaculture systems. This has also hindered the study on the effects of pollutants such as heavy metals and pesticides in the aquatic environment, which adversely affects shrimp health

* Corresponding author. Tel./fax: +91 484 2381120.
E-mail address: bsingh@md3.vsnl.net.in (I.S. Bright Singh).

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and its export value. Even though development of primary cell cultures (Chen et al., 1986; Fan and Wang, 2002; Maeda et al., 2003) and their application in the study of viral pathogens (Wang et al., 2000; Maeda et al., 2004; Jiang et al., 2005) have been reported, their utility in the assessment of cytotoxicity and genotoxicity of heavy metals and pesticides has not been attempted. In the study undertaken here primary haemocyte culture of *P. monodon* was utilised for examining cytotoxicity and genotoxicity of two heavy metal compounds, cadmium chloride ($CdCl_2.2\frac{1}{2}H_2O$) and mercuric chloride ($HgCl_2$) and two organophosphate insecticides, malathion ($C_{10}H_{19}O_6PS_2$) and monocrotophos ($C_7H_{14}NO_5P$) which reach aquaculture systems through run off. Malathion and monocrotophos are also used as insecticides in paddy fields and aquaculture ponds. Comet assay was employed to evaluate the DNA damage caused by these pollutants. The work was conducted to assess the usefulness of primary haemocyte culture for the study of cytotoxicity and genotoxicity of aquatic pollutants.

2. Materials and methods

2.1. Experimental animals

WSSV and Monodon Baculo virus (MBV) negative *P. monodon* larvae obtained from a local hatchery were stocked and reared in a recirculating shrimp rearing system integrated with nitrifying bioreactors (Kumar et al., 2009) in sea water having a salinity of 15 g l^{-1}. Water quality was maintained by the addition of a probiotic Detrodigest TM (NCAAH, India) to manage detritus and Enterotrophotic TM (NCAAH, India) to control *Vibrio*. Water quality parameters measured were pH (7.5–8.5), temperature (25 ± 1 °C), NH_4^+-N (<0.1 mg l^{-1}) and alkalinity (60–75 mg l^{-1}). The larvae were fed three times daily with commercially available pelleted feed having 40% protein (Higashimaru, India) made offish meal, wheat gluten, squid meal, clam meal, wheat flour, yeast, soy lecithin, krill meal, marigold flower petal extract, refined fish oil, cholesterol, minerals and vitamins. They were confirmed WSSV negative by nested PCR (WSSV detection kit, Genei, India) when they grew to 8–12 g after a culture period of three months, and used for all experiments.

2.2. Development of primary haemocyte culture

The animals were sacrificed by immersing in crushed ice and disinfected by maintaining in 800 mg l^{-1} sodium hypochlorite solution prepared in ice cold sea water (salinity 15 g l^{-1}) for 10 min. Subsequently, they were washed 5 times in sterile ice cold sea water, dipped in 70% alcohol and rinsed in ice cold sea water. Haemolymph was withdrawn asceptically using capillary tubes containing 100 µl anticoagulant (Tris HCl 0.01 M (pH 7), Sucrose 0.25 M, Tri Sodium Citrate 0.1 M) from rostral sinus and diluted to obtain 5 × 10^5 cells ml^{-1} using modified 2X L-15 (Leibovitz) medium supplemented with 2% glucose, MEM vitamins (1X), tryptose phosphate broth (2.95 g l^{-1}), 20% FBS, N-phenylthiourea (0.2 mM), 0.06 µg ml^{-1} chloramphenicol, 100 µg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin, and aliquots of 200 µl were dispensed into 96 well micro plates (Greiner Bio-One) and incubated at 25 °C (Jose et al., 2010) for 12 h. Haemolymph from 5 animals were pooled and diluted to seed a microwell plate.

2.3. Cytotoxicity of cadmium chloride ($CdCl_2.2\frac{1}{2}H_2O$) and mercuric chloride ($HgCl_2$)

Cadmium chloride and mercuric chloride were used as representatives of aquatic pollutants. Stock solutions of cadmium chloride (Qualigens) and mercuric chloride (Merck) were prepared in cell culture medium. A range finding test was carried out before the definitive test and different concentrations of the compounds were added to the wells to get the final concentrations ranging from 0.49 to 500 µM for cadmium chloride and 0.49 to 62.5 for mercuric chloride respectively. Replicates were maintained for each concentration. Cells with the growth medium were kept as control. After 12 h incubation, wells were observed under phase contrast microscope (Leica, Germany) and MTT assay was performed. Percentage inhibition of cells at each concentration of the compounds was calculated based on the formula,

Percentage inhibition of haemocytes = [100 − (Average absorbance (MTT assay) of haemocytes at a particular concentration of the compound/Average absorbance of control haemocytes without the compound) × 100)].

2.4. Cytotoxicity of malathion [S-(1, 2-dicarboethoxyethyl) O, O-dimethyl phosphorodithioate];($C_{10}H_{19}O_6PS_2$)) and monocrotophos (E)-(dimethyl1-methyl-3-(methylamino)-3-oxy-propenyl phosphate); ($C_7H_{14}NO_5P$))

Commercially available preparations of malathion (50% w/w) and monocrotophos (53% w/w) were used for toxicity studies. Insecticides were dissolved in DMSO and diluted with medium to get stock concentrations of 500 mg l^{-1} and 1000 mg l^{-1} of malathion and monocrotophos respectively. Concentrations were fixed based on the range finding test and different concentrations of the compounds were added to the wells to get final concentrations ranging from 0.76 to 185.2 mg l^{-1} for malathion and 0.98–1000 mg l^{-1} for monocrotophos maintaining replicates for each concentration. Cells with the growth medium were kept as control. After 12 h incubation wells were observed under phase contrast microscope (Leica, Germany) and MTT assay was performed. Percentage inhibition of cells at each concentration of the compounds was calculated.

2.5. MTT assay

The assay is a colorimetric method based on the determination of cell viability utilising the reaction of a tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, MTT) with the mitochondria of metabolically active cells. The reduction of the tetrazolium salt by nicotinamide adenine dinucleotide dehydrogenase (NADH) and nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) within the cells produces insoluble purple formazan crystals, which are later solubilised yielding a purple-coloured solution (Mosmann, 1983).

After replacing the medium, 50 µl of MTT (Sigma) solution (5 mg ml^{-1} in PBS; 720 mOsm) were added to each well and incubated for 5 h in dark. Blank consisted of medium alone with MTT added. After incubation, the medium was removed and MTT-formazan crystals were dissolved in 200 µl dimethylsulfoxide. Absorbance was recorded immediately at 570 nm in a micro plate reader (TECAN Infinite Tm, Austria).

2.6. Comet assay

Haemocytes were exposed to cadmium chloride (140 µM), mercuric chloride (17 µM), malathion (60 mg l^{-1}) and monocrotophos (186 mg l^{-1}) for 4 h. These concentrations were chosen because more than 80% viability of cells was recorded after 4 h exposure by trypan blue viability assay as dead cells might give rise to false positive results in Comet assay. After the exposure to the compounds, cells were detached using a cell scraper (Greiner Bio-One), pelletised, washed and suspended in PBS. Negative control consisted of cells without any treatment. Added 150 µl normal agarose solutions (Sigma, USA) (0.75% in PBS) to a frosted slide and

covered with a cover slip (22 × 50 mm) to get a uniform layer, and allowed to solidify. To prepare the second layer, 10 μl cell suspension was mixed with 70 μl low melting agarose (Sigma, USA) (0.75% in PBS) and, the suspension was layered over the one prepared initially after removing the cover slip. The preparation was kept undisturbed after replacing the cover slip until agarose got solidified. A third layer of low melting agarose of the above strength was applied over the second layer after removing the cover slip and replaced the same subsequently. The slides were treated with pre chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1.5 M NaOH, pH 10 with 1% Triton X 100 and 10% DMSO) for 1 h at 4 °C after removing the cover slips. On completion of the process, the slides were incubated in electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min at 4 °C for unwinding of DNA, and were electrophoresed for 40 min at 25 V and 300 mA in the same buffer. Subsequently, the slides were neutralised in 0.4 M Tris (pH 7.5), stained with ethidium bromide (2 μg ml^{-1}) and observed under fluorescent microscope (Olympus, Germany) at 400× magnification, and the cells were photographed. For each sample, 25 cells from each slide were analysed for DNA damage using Comet Score™ (TriTek Corp., Summerduck, USA), of the Comet scoring software. Experiments were repeated four times. Percentage of nuclei with tails, tail length, percentage DNA in the tail and tail moment (arbitrary units) were used to estimate DNA damage. Tail moment is the tail length multiplied by fraction of DNA in the tail.

2.7. Statistical analysis

The results in the figures are average values of 4 replicates ± standard deviation. The results of the cytotoxicity assay were analysed by probit analysis using the SPSS software (SPSS Inc., USA). The effects of treatments were statistically analysed by analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Cytotoxicity of cadmium chloride and mercuric chloride

Control haemocytes appeared spherical or elliptical initially, and within a few hours of culturing two distinct morphological types such as round to elliptical and small and large spindle shaped cells, with granules, could be seen in the modified 2X L-15 medium (Fig. 1). At higher concentrations of cadmium chloride (>125 μM) and mercuric chloride (>15.6 μM) round cells were few and spindle shaped cells were totally absent. More round cells and few spindle shaped cells were present at lower concentrations (<15.6 μM for cadmium chloride and <1.95 μM for mercuric chloride) (Fig. 1). At still lower concentrations number of spindle cells was higher. Toxicity of cadmium chloride and mercuric chloride in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay is presented in Figs. 2 and 3. IC_{50} values were 31.09 ± 16.27 μM and 5.52 ± 1.16 μM (7.10 ± 3.72 mg l^{-1} and 1.50 ± 0.31 mg l^{-1}) for cadmium chloride and mercuric chloride respectively.

3.2. Cytotoxicity of malathion and monocrotophos

At higher concentrations of malathion and monocrotophos (>61.7 mg l^{-1} and >166 mg l^{-1} respectively) only round cells were present and the number of spindle shaped cells increased as the concentrations decreased (Fig. 1). Toxicity of malathion and monocrotophos in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay is presented in Figs. 4 and 5. IC_{50} value of malathion was found to be 59.94 ± 52.30 mg l^{-1} and of that of monocrotophos 186.76 ± 77.00 mg l^{-1}. Malathion was found to be non toxic at ≤6.86 mg l^{-1} and monocrotophos at ≤15.6 mg l^{-1}.

3.3. Genotoxicity of cadmium chloride, mercuric chloride, malathion and monocrotophos

All pollutants tested induced DNA strand breakage and thereby Comets in the haemocytes (Fig. 6). In the negative control most of the nuclei were circular without any visible tail while short tails were observed in 11.46 ± 7.89% of cells (Table 1). Percentage of cells with tails was significantly higher ($P < 0.05$) than the control for cadmium chloride, mercuric chloride, malathion and monocrotophos (>60%). Tail length of the Comets in mercuric chloride treatment was the lowest (23.09 ± 6.83 pixels) while for cadmium chloride, malathion and monocrotophos length of the tail ranged from 28–33 pixels. Percentage tail DNA for all the pollutants were significantly higher ($P < 0.05$) but no significant differences ($P > 0.05$) were obtained between that of cadmium chloride and mercuric chloride, and malathion and monocrotophos. Tail moment was found to be lesser for mercuric chloride (11.82 ± 5.50) ($P < 0.05$) when compared to the other three and the values were highest for cadmium chloride (17.17 ± 6.22). In the control tail moment was significantly lower (0.04 ± 02) ($P < 0.05$).

4. Discussion

Deleterious effects of environmental pollutants may result from direct toxic action on the tissues or from subtle alterations in the homeostatic mechanisms such as the immune system (Auffret and Oubella, 1997). The immune defence of shrimp is comprised of cellular and humoral-mediated mechanisms, in which haemocytes play a key role and this makes haemocyte culture an ideal system for evaluating the effects of environmental pollutants. Primary cell cultures of penaeid shrimp have been developed from different tissues such as lymphoid (Chen and Wang, 1999; Lang et al., 2002), heart (Tong and Miao, 1996; Chen and Wang, 1999), ovary (Shimizu et al., 2001; Maeda et al., 2003) and embryo (Toullec et al., 1996; Fan and Wang, 2002) while haemocyte culture was attempted only by a few researchers in *Penaeus vannamei*, *Penaeus aztecus* (Ellender et al., 1992), *Penaeus japonicus* (Itami et al., 1999) and *Penaeus chinensis* (Jiang et al., 2005). In the primary haemocyte culture developed, round to elliptical cells could be regarded as hyaline cells and small and large spindle shaped cells with granules could be regarded as granular or semi-granular cells based on the morphology and presence of granules. The metabolic activity of the culture was almost the same during the second, third and fourth days and the activity started declining only subsequently. The culture remained viable for upto 8 d. Even though dividing cells were not observed, DNA synthesis was detected in 22 ± 7% of cells by means of 5-Bromo-2′-deoxyuridine (BrdU) assay at 24 h (Jose et al., 2010). Studies on the application of haemocyte culture or any other primary cultures of shrimp in cytototoxicity and especially the genotoxicity of drugs, management chemicals and pollutants in aquaculture systems have been rarely cited in literature. The *P. monodon* heamocyte culture has been applied for assessing cytotoxicity of N- methyl-1-hydroxyphenazine, a proposed aquaculture drug (Preetha et al., 2010) and benzalkonium chloride, an aquaculture management chemical (Jose et al., 2010) employing MTT assay. Sung et al. (2003) examined the effects and toxicity of phthalate esters on the isolated haemocytes of *Macrobrachium rosenbergii* utilising haemocytic adhesion, pseudopodia formation, superoxide anion production and phenoloxidase activity.

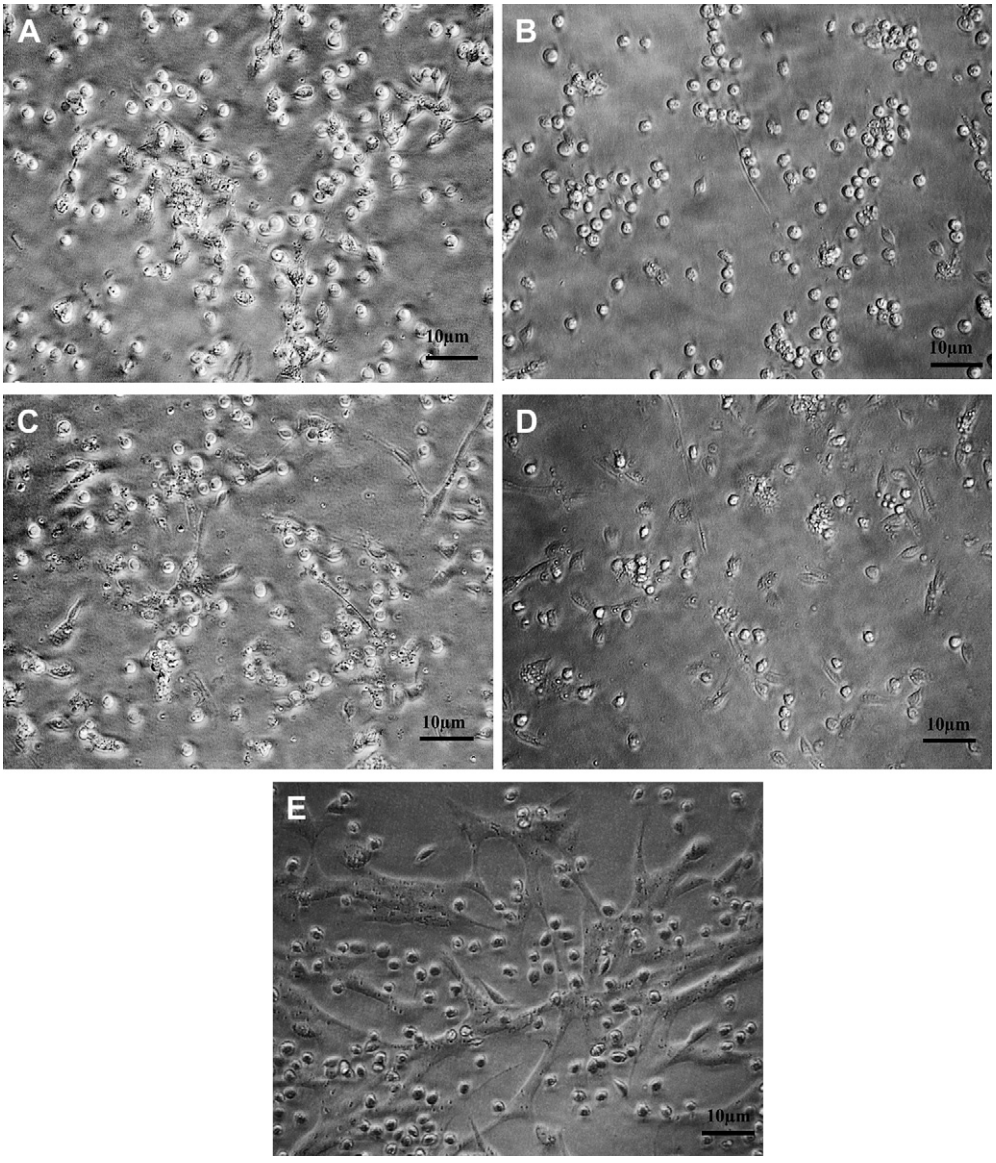

Fig. 1. Primary haemocytes after 12 h exposure to A) Cadmium chloride (15.63 µM), B) Mercuric chloride (1.95 µM), C) Malathion (61.7 mg l^{-1}), D) Monocrotophos (100 mg l^{-1}), and E) Control.

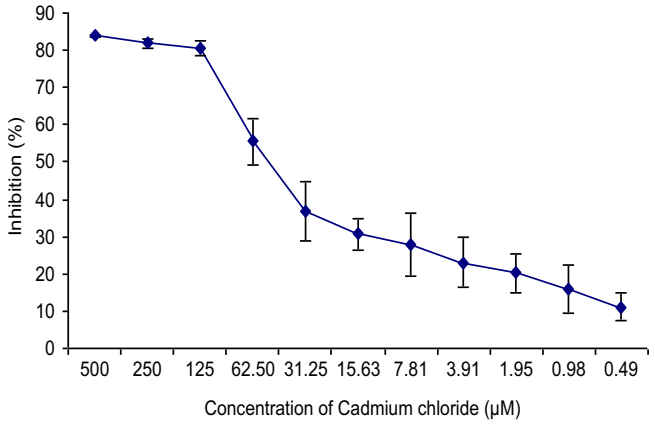

Fig. 2. Toxicity of cadmium chloride in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay ($n = 4$).

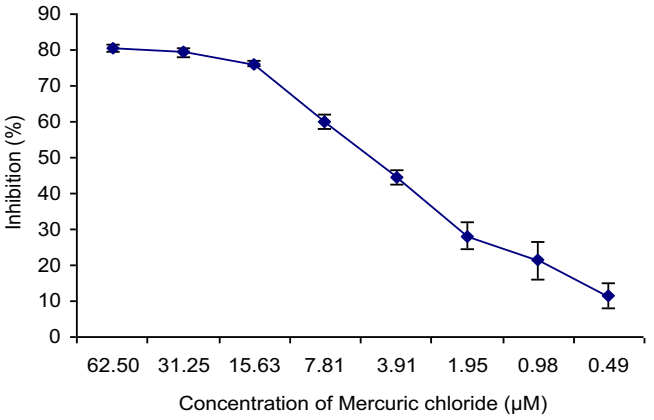

Fig. 3. Toxicity of mercuric chloride in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay ($n = 4$).

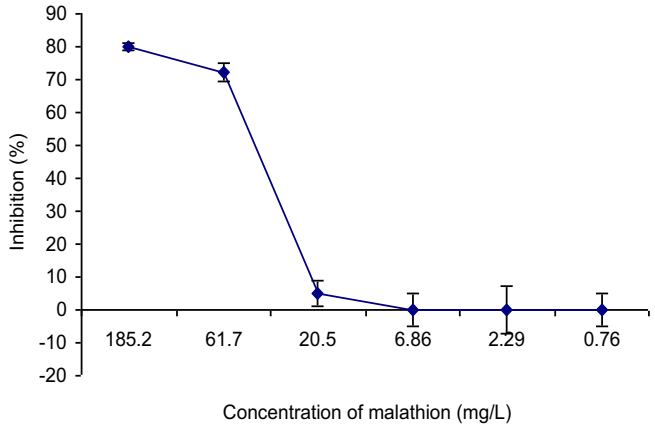

Fig. 4. Toxicity of malathion in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay ($n = 4$).

Toxicity of cadmium chloride and/or mercuric chloride has been evaluated in vivo on different aquatic organisms such as fishes (Pereira et al., 2009), sea urchin (Filosto et al., 2008), shrimp (Lee et al., 2000; Espericueta et al., 2001) and crab (Botton, 2000). In our study toxicity was tested in vitro on primary haemocyte culture of *P. monodon* and 12 h IC_{50} values for cadmium chloride and mercuric chloride were 31.09 ± 16.27 μM and 5.52 ± 1.16 μM, respectively. Mercuric chloride was found to be more toxic than cadmium chloride and concentration dependent increase was observed in the toxicity of both heavy metals in haemocytes. Espericueta et al. (2001) reported the LC_{50} values as 2.49 and 1.23 mg l^{-1} for cadmium and mercury, respectively in post larvae of *Litopenaeus vannamei* after 96 h exposure while in *Limulus polyphemus* embryos IC_{50} was reported to be >1000 mg l^{-1} and 12.80 mg l^{-1} for Cd and Hg after 24 h exposure (Botton, 2000). In the present study, MTT assay which measured the metabolic activity as well as viability of cells was utilised to estimate the cytotoxicity rather than trypan blue assay which measured only the viability. IC_{50} value of malathion was found to be 59.94 ± 52.30 mg l^{-1} and that of monocrotophos was 186.76 ± 77.00 mg l^{-1}. Cytotoxicity increased with concentration for both the pesticides, and malathion was more toxic when compared to monocrotophos. Similarly, concentration dependant increase in the toxicity of malathion was detected in grass carp cell line (Chen et al., 2006) and human liver carcinoma cell line (Moore et al., 2010). IC_{50} of malathion ranged from 37.94 ± 1.93 mg l^{-1} for 12 h to 3.04 ± 0.27 mg l^{-1} for 72 h in

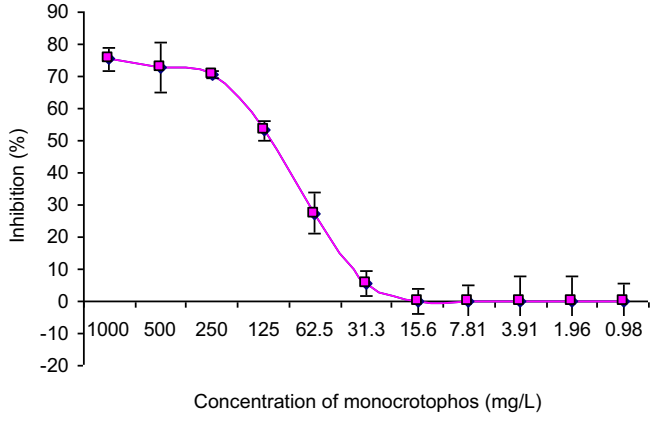

Fig. 5. Toxicity of monocrotophos in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay ($n = 4$).

grass carp cells (Chen et al., 2006). In the nauplii (48 h old) of *Artemia salina* 24 h LC_{50} of monocrotophos was estimated to be 262.68 ± 17.30 mg l^{-1} (Rao et al., 2007). These values are comparable to those in the present study. Semi-granular or granular cells were more sensitive to heavy metals and pesticides than the hyaline cells and the number of these cells increased as the pollutant concentration decreased. According to Holmblad and Soderhall (1999) semi-granular cells appear to be the most sensitive and react first during an immune response, by degranulating. This degranulation stimulates the granular cells also to degranulate as well. The advantage of using haemocyte culture over explant cultures for cytotoxicity studies is the easiness in quantification of cells to be seeded to maintain uniform cell number in micro plates, a prerequisite for quantal assays, and the rapidity in obtaining monolayer of cells.

Different methods have been developed for detecting DNA strand damage such as micronucleus and sister chromatid exchange assays which are based on the enumeration of downstream aberrations after DNA damage (Lee and Steinert, 2003). A more recent method is the Comet assay which detects the DNA strand breaks and alkali labile sites by measuring the migration of DNA from immobilised nuclear DNA (Singh et al., 1988). A large number of studies report that Comet assay is more sensitive when compared to sister chromatid exchanges or micronucleus test (Mitchelmore and Chipman, 1998; Lee and Steinert, 2003; Frenzilli et al., 2009). The main advantages of the Comet assay include the collection of data at the level of the individual cell, the need for a small number of cells per sample and the sensitivity for detecting DNA damage (Lee and Steinert, 2003; Jha, 2004). Tail length is the basic parameter used for quantifying DNA damage in the Comet assay. However, the tail length levels off after migrating to a certain distance. After the maximum length is attained, the Comet tail will not be extending in length, however grow in intensity as compared to that with the head portion (Bowden et al., 2003). Therefore, in addition to tail length, percentage tail DNA and tail moment were also calculated using Comet scoring software.

Comet assay has become one of the most popular tools for detecting DNA stand breaks in aquatic animals (Frenzilli et al., 2009) and has been employed to assess the DNA damage in aquatic vertebrates such as *Cyprinus carpio* (Arabi, 2004), *Carassius auratus* (Masuda et al., 2004), *Danio rerio* (Kosmehl et al., 2008) and *Liza aurata* (Pereira et al., 2009), and invertebrates such as *Limnoperna fortunei* (Villela et al., 2007), *Dreissena polymorpha* (Juhel et al., 2007), *Polycelis feline* (Horvat et al., 2005), *Palaemonetes pugio* (Lee et al., 2000, 2008), *Mytilus edulis* (Rank and Jensen, 2003; Cheung et al., 2006) and *Crassostrea virginica* (Bissett et al., 2009). For in vivo studies in aquatic invertebrates such as oysters, mussels, clams and shrimp, cells from haemolymph, embryos, gills and digestive glands were used for Comet assay (Lee and Steinert, 2003). In vitro studies of genotoxicants were reported in haemocytes, gill cells and digestive gland cells from various mollusks namely *Mytillus edulis*, *Nassarius tegula* and *Musculista senhousia* (Sastre et al., 1997; Mitchelmore et al., 1998; Wilson et al., 1998). Studies of genotoxicants in shrimp cell cultures were not reported while Lee et al. (2000) studied the effects of genotoxicants such as chromium (III) chloride, sodium chromate, mercuric chloride, and 2-methyl-1,2-naphthoquinone (MNQ) on grass shrimp, *P. pugio*, embryos employing Comet assay. DNA damage in a variety of aquatic animals has been associated with reduced growth, abnormal development and reduced survival of embryos, larvae and adults (Steinert, 1999; Lee et al., 2000). In grass shrimp embryos Comet assay was used to provide a sensitive measure of DNA strand breakage exposed to genotoxicants, highway run off sediments, sediments with coal ash and phototoxicants (Lee et al., 2000, 2008; Lee and Kim, 2002; Kim and Lee, 2004).

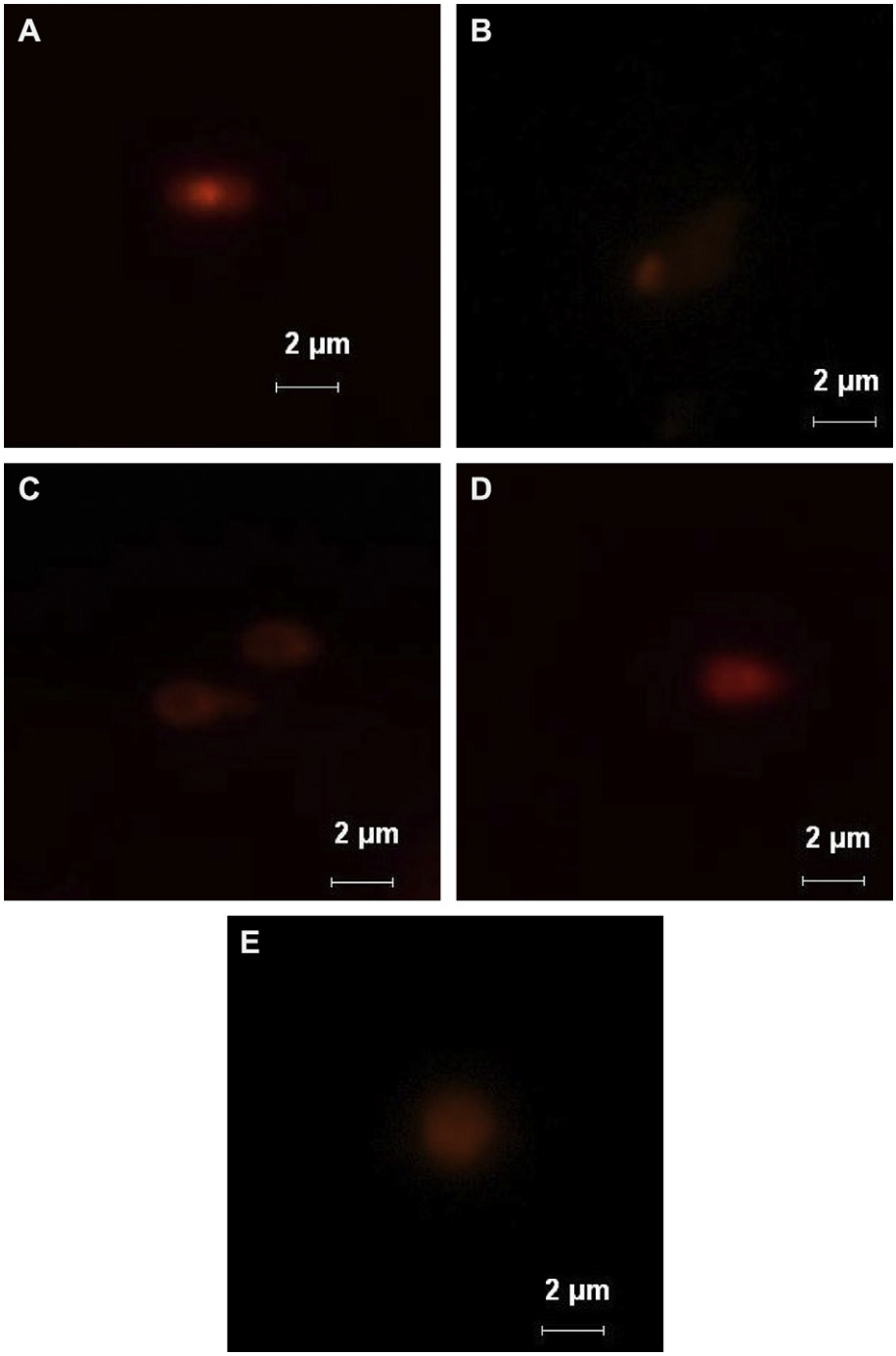

Fig. 6. Comets formed by haemocytes after exposure to A) Cadmium chloride, B) Mercuric chloride, C) Malathion, D) Monocrotophos, and E) Control.

In the concentrations tested cadmium chloride and mercuric chloride induced Comets in 72% and 61% of the cells respectively after an exposure over a period of 4 h. Genotoxicity of cadmium was reported in rat hemispheres and cerebellum (Klimova and Misurova, 2002) in rat Leydig cells (Yang et al., 2003), in *P. pugio* embryos (Hook and Lee, 2004), in *M. edulis* (Pruski and Dixon, 2002; Emmanouil et al., 2007) and in *L. vannamei* (Chang et al., 2009). The mechanism of metal induced carcinogenesis is still

Table 1
DNA damage induced by heavy metals and pesticides in the primary haemocyte culture of *P. monodon*. Mean values and standard deviation obtained from average of 25 cells per experiment (Total 4 experiments for each compound).

Parameters	Control	Cadmium chloride (140 µM)	Mercuric chloride (17 µM)	Malathion (60 mg l^{-1})	Monocrotophos (186 mg l^{-1})
% Cells with tail	11.46 ± 7.89	72.20 ± 7.46	60.83 ± 17.72	67.75 ± 13.55	61.55 ± 12.98
Tail length (pixels)	1.3 ± 0.50	33.47 ± 9.24	23.09 ± 6.83	29.13 ± 7.75	28.17 ± 7.87
% DNA in tail	2.04 ± 1.25	44.21 ± 4.76	44.72 ± 7.25	40.81 ± 9.10	38.85 ± 13.68
Tail moment	0.04 ± 0.03	17.17 ± 6.23	11.82 ± 5.50	14.36 ± 5.56	13.86 ± 5.74

unknown, but one possible pathway may involve the interaction of metals with DNA, either directly or indirectly (Hartmann and Speit, 1994). Cadmium induces an oxidative DNA damage leading to DNA strand breaks, DNA-protein cross-linking and inhibitions of DNA repair (Dally and Hartwig, 1997; Valverde et al., 2001). Genotoxicity of mercury was reported in *C. carpio* gill cells (Arabi, 2004), blood cells of *L. aurata* (Pereira et al., 2009), human salivary gland tissue cells and lymphocytes (Schmid et al., 2007) and *P. pugio* embryo (Lee et al., 2000). $HgCl_2$ have a deleterious effect on the membrane integrity and glutathione (GSH) content of gill cell suspensions of carp, *Cyprinus carpio* L. and mercury caused DNA strand breaks at 3000 μM (Arabi, 2004). $HgCl_2$ has been reported to cause DNA damage in *P. pugio* embryos at concentrations as low as 0.003 μM after a 12 h exposure period (Lee et al., 2000). DNA damage was reported by Pereira et al. (2009) in golden grey mullet, *L. aurata* due to environmental exposure of mercury. Clastogenic effects mostly associated with the spindle mechanism disturbance and the generation of reactive oxygen species, accompanied by glutathione depletion contributes to the genotoxicity of mercury (De Flora et al., 1994). The present study demonstrates the potential of cadmium chloride and mercuric chloride to induce DNA damage in primary haemocyte culture.

Giri et al. (2002) evaluated the genotoxicity of malathion using chromosome aberration, sister chromatid exchange and sperm abnormality assays in mice. Genotoxicity was detected in human liver carcinoma cells at 24 mM exposure of malathion. In *Channa punctatus*, sublethal concentrations of malathion (Kumar et al., 2010) and monocrotophos (Ali and Kumar, 2008) induced dose dependant DNA damage in gill, kidney and lymphocytes were reported during a study period of 1–29 d and 1–21 d respectively. Jamil et al. (2005) reported genotoxic effect of monocrotophos in human lymphocytes in vitro employing Comet assay. A concentration dependant increase in the DNA damage was detected in the erythrocytes of *Tilapia mossambica* (Banu et al., 2001) and the gill cells of the bivalve, *Meretrix ovum* (Revankar and Shyama, 2009) after the exposure to monocrotophos. Both malathion and monocrotophos are organophosphorous compounds capable of phosphorylation and alkylation. Although phosphorylation is responsible for the inhibition of acetylcholine esterase and produces corresponding neurotoxicity in target and non-target species, other biological activities such as mutation appear to be due to the alkylation properties (Eto and Ohkawa, 1970). The heterocyclic bases of the nucleic acids may serve as nucleophilic agents for such a reaction, and alkylation may take place on nitrogen atoms possessing high electron density. Alkylation is more likely with the methyl ester group than ethyl and higher alkyl ester groups, and phosphate esters such as monocrotophos are more reactive than the phosphorothionate esters such as malathion. As organophosphorous insecticides are chemical alkylating agents (Wild, 1975), alkylation of DNA bases either directly or indirectly via protein alkylation is probably involved in the DNA damage (Mohan, 1973). According to Wild (1975) the phosphorous moiety in the organophosphates appears to be a good substrate for nucleophilic attack and it causes phosphorylation of DNA, which is an instance of DNA damage. Malathion used in the present study is a commercial grade product and may contain impurities formed during manufacturing and storage. Among the impurities are malaoxon formed from malathion by oxidation and isomalathion formed by isomerisation (Berkman et al., 1993). Malaoxon and isomalathion have been reported to be mutagenic (Flessel et al., 1993; Pluth et al., 1996; Blasiak et al., 1999). These two compounds also could contribute to the genotoxicity of malathion. In the present study malathion and monocrotophos, which are the two commonly used insecticides in India, have proved to be genotoxic to *P. monodon* primary haemocyte culture.

In conclusion, the study has demonstrated the use of primary shrimp haemocyte culture for testing the cytotoxicity of aquatic pollutants such as heavy metals and pesticides employing MTT assay. The primary cell culture system could also be employed to assess the DNA damage induced by genotoxic pollutants in the aquatic environment. The present study confirms the genotoxic potential of two heavy metal compounds, cadmium chloride and mercuric chloride and two organophosphate insecticides, malathion and monocrotophos, and this forms the first report of the demonstration of genotoxicity of pollutants using shrimp cell culture as a model system.

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