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Arsenic and Mercury Induce Death of *Anabas testudineus* (Bloch) Involving Fragmentation of Chromosomal DNA

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ABSTRACT

Heavy metals are considered as devastating environmental pollutants that cause serious pollution of water bodies affecting aquatic inhabitants, including fishes. The objective of this work was to examine the toxicological effects of two major heavy metal pollutants, sodium arsenite ($NaAsO_2$) and mercuric chloride ($HgCl_2$), on fresh water climbing perch, *Anabas testudineus* (Bloch). $HgCl_2$ was found to be more toxic than $NaAsO_2$ and when fishes were exposed to different concentrations of these two metals, they required less time to induce fish death as their concentration increased. The highest concentration (1 mM) tested in this study induced fish death as early as 2 hours in $HgCl_2$ and 8 hours in $NaAsO_2$ treatments. Both heavy metals decreased total protein content of the exposed fishes in a concentration-dependant manner; however, no significant change was observed in fat, moisture and ash content. Liver cell viability was reduced to about 32 and 48% by $HgCl_2$ and $NaAsO_2$, respectively. The death of the liver cells was accompanied by chromosomal DNA fragmentation. We later investigated whether the heavy metals could induce any change in protein expression and found that both heavy metals induced higher expression of a relatively high molecular weight protein detected on the upper portion of the gel. We conclude that mercury and arsenic showed their toxic effect by causing death of the fishes or fish cells involving fragmentation of chromosomal DNA and expression of certain high molecular weight proteins.

Keywords: apoptosis, climbing perch, heavy metals, liver cell, protein expression

INTRODUCTION

Bangladesh, as a developing country, is at a high risk of environmental pollution. Uncontrolled industrial effluents and indiscriminate use of inorganic fertilizers and chemicals in agriculture are mostly responsible for the accumulation of various pollutants, including heavy metals, in inland water bodies causing the highest aquatic pollution problem in Bangladesh (D'Monte 1996). Among different toxicants of water, heavy metals are gaining importance for their non-degradable nature. Contamination of water bodies with heavy metals may have devastating effects on the ecological balance of the aquatic environment, and it may limit the diversity of aquatic organisms (Suzuki *et al.* 1988). Mercury, lead and arsenic are the top three toxic pollutants of environmental concern (Gonzalez *et al.* 2006). Arsenic is a metalloid element that is widespread in the aquatic environment as a result of both geogenic processes and anthropogenic disturbances (Bears *et al.* 2006; Gonzalez *et al.* 2006) whereas mercury is primarily introduced into aquatic systems through atmospheric deposition and anthropogenic pathways, including effluent containing dyes, fungicides, mining, industrial wastes, combustion of fossil fuels, municipal and medical wastes (Clarkson 1990; Porcella 1994; Watras 1994).

In aquatic environments, several species of microorganisms make arsenic and mercury biologically available to organisms, including fish (Duker *et al.* 1995; Gonzalez *et al.* 2006). *In vitro* experiments have shown multiple effects at the molecular level following arsenic and mercury exposure including differential expression of genes involved in cell cycle regulation, signal transduction, stress response, apoptosis, cytokine production, growth-factor and hormone-receptor production (Chen *et al.* 1992; Shen *et al.* 2002; Yang *et al.* 2002; Hossain *et al.* 2003; Scholz *et al.* 2005; Tabellini *et al.* 2005). Several studies indicate that exposure of both metals has deleterious effects on aquatic organisms. For example, dietary arsenic exposure resulted in pathological injury to both the liver and gallbladder in lake white fish, *Coregonus clupeaformis* (Pedlar *et al.* 2002) and in rainbow trout (*Onchorhynchus mykiss*) (Cockell *et al.* 1991), while waterborne arsenic decreased hemoglobin and packed cell volume in walking catfish, *Clarius batrachus* (Tripathi *et al.* 2003). Embryos of medaka (*Oryzias latipes*) exposed to arsenic had a reduction in hatching success as well as reduction in time to hatching (Ishaque *et al.* 2004). Arsenic exposure can interfere with the normal expression of glucocorticoid receptor-mediated gene in the common killi fish, *Fundulus heteroclitus* (Bears *et al.* 2006) and increase morphological abnormalities in their offspring (Gonzalez *et al.* 2006). Depending upon different parameters like dose and exposure route the toxicological effects of mercury includes organ lesions (kidney, liver, and lung), neurological effects and haematological alterations (Kotsanis *et al.* 2000; Spalding *et al.* 2000; Iliopoulou-Georgudaki *et al.* 2001; Sweet *et al.* 2001). Immunological effects of mercury toxicity have also been described, both in mammals (Thuvander *et al.* 1996; Spalding *et al.* 2000; Institoris *et al.* 2001) and in fish (Voccia *et al.* 1994; MacDougal *et al.* 1996). Among immunological effects in fish, alterations of MLR and blastic transformation (Low *et al.* 1996), phagocytosis and respiratory burst (Voccia *et al.* 1994; MacDougal *et al.* 1996).

Limited data are available on the impact of agriculture, domestic and industrial pollution, especially heavy metals, on the aquatic environment. In Bangladesh the concentration of heavy metals in aquatic animals, water and sediment were studied, which covered mostly coastal area, parts of the GBM river system and some rivers of central parts (Ahmed *et al.* 2002, 2003; Haque *et al.* 2006; Ahmed *et al.* 2009). In Bangladesh, the fisheries sector is now considered

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as an emerging potential sector and, in particular, has an invaluable contribution of 4.92% to the GDP (BBS 2004). However, to date no systematic study yet has been carried out at the molecular level on the impact of heavy metals on fish.

Fish are considered ideal organisms for toxicological studies due to economic considerations. Fish models can be used to establish biomarkers of aquatic pollutants because their genomes have many sequence similarities with humans (Ballatori and Villalobos 2002). We performed experiments to examine the toxic effect of arsenic ($NaASO_2$) and mercury ($HgCl_2$) in fresh water climbing perch, *Anabas testudineus*, whose liver we chose to focus on because it is a significant site of heavy metal accumulation and bio-transformation (Pedlar and Klaverkamp 2002). We also examined the toxic effects of $NaASO_2$ and $HgCl_2$ on muscle proximate composition and on the chromosomal DNA as well as the cellular protein profile of liver cells.

MATERIALS AND METHODS

$HgCl_2$ and $NaAsO_2$ exposure

Fishes (climbing perch, *Anabas testudineus*) were either purchased from the local market or collected from a fish farm, and almost same sized fish (length 12.05 ± 0.21 cm, weight 19.05 ± 0.35 g) were used for the experiment. All fishes were acclimatized for a week in well aerated tap water with no food and the water was changed once daily. After acclimatization, only the healthy fishes were selected for the experiment. Small aquaria of $1m^3$ with 1L water (same water which used in acclimatization) were used as experimental unit. Three different doses of $HgCl_2$ and $NaAsO_2$ (BDH, UK) (0.1, 0.5 and 1.0 mM) each with three replicates were prepared using the experimental water and then inoculated in the experimental units. After mixing the dose one climbing perch, *Anabas testudineus* per unit was exposed to it and the physiological responses were observed. The survival period (period between exposures to death) of fishes were recorded for each concentration of heavy metal used.

Determination of proximate composition

The muscles were collected from the dorsal, ventral and caudal portions of the fishes being died due to $HgCl_2$ and $NaAsO_2$ exposures as soon as possible and macerated in a blender to have a homogenous mass. Moisture and ash content of fish muscle were determined by the AOAC (1965) method on a wet weight basis. Crude protein content and fat were determined as total nitrogen by the Kjeldhal method and ether extractions of the dry material method, respectively (AOAC 2006).

Preparation of single cell suspension

Fish liver was isolated after being killed by exposure to $HgCl_2$ and $NaASO_2$. The fishes were dissected by cutting the ventral aorta; the liver was aseptically removed and pushed through 70 µm nylon mesh (BD Falcon™, USA) to make single cell suspension with normal saline (sterile isotonic saline).

Liver cell viability assessment

An equal volume of 0.2% Trypan blue was added to the cell suspension and mixed well. The resultant cell suspension (~10 µl) was added into the counting chamber of a haemocytometer (Neubauer®, Germany) covered with a cover slip.While observing under the light microscope, the cells which take up the blue stain of Trypan blue, were considered as dead cells, whereas those having yellow nuclei were considered as viable cells. The subsequent cell concentration per ml was determined using the following formula:

$$\text{Cell per ml} = \frac{\text{The average count per square} \times \text{dilution factor}}{\text{Depth of fluid undercover slip} \times \text{area counted}}$$

The percentage viability is calculated as follows:

$$\text{Percentage viability} = \frac{\text{No. of unstained cells} \times 100}{\text{Total no. of cells counted}}$$

Analysis of DNA fragmentation by electrophoresis

100 µl of liver cell suspension (from 1×10^8 cells/ml) was added to an equal volume of hypotonic lysing buffer [50 mM Tris-HCl (SIGMA, St. Louis, USA), 10 mM EDTA (Merk, Germany), 0.5% SDS (LOBA, India)] followed by the addition of 2 µl of proteinase K (20 mg/ml) (SIGMA) and 6 µl of RNAse (10 mg/ml) (SIGMA) according to Akhand *et al.* (1998). 10 µl of the DNA sample mixed with 1 µl dye was loaded on a 1% agarose gel (SIGMA) with 0.1 µg/ml ethidium bromide (SIGMA). The sample was run for about 1 h at 50 mV. Gels were observed under UV light for viewing DNA bands and photographs were taken (Kodak, EDAS290).

SDS-PAGE and cellular protein analysis

Liver cell suspension (1×10^8 cells/ml) was placed in an Eppendorf tube and washed twice with equal volume (100 µl) of PBS through centrifugation (Mikro 22R; Hettich Zentrifugen) at 4°C for 5 min at 1500 rpm. An equal volume of a two-fold concentrated sample buffer [125 mM Tris-HCl, pH 6.8; 20% w/v glycerol (Robinson Wagner Co., New York, USA); 4% w/v SDS; 0.02% bromophenol blue (SIGMA); 10% 2-mercapto ethanol (Nacalai Tesque, Kyoto, Japan)] was added with cell suspension to lyse the cells and dissolve the cellular proteins. Thereafter the dissolved proteins were denatured by heating for 3 min in boiling water. Liver protein samples were run at 30 mA on a 10% polyacrylamide gel (SIGMA) using PAGE buffer (196 mM glycine (BDH), 0.1% SDS and 50 mM Tris-HCl pH 8.3). The gel was stained with Coomassie Brilliant Blue (Merck, Germany) followed by destaining with destaining buffer [methanol 25% (Merck), water 65% and glacial acetic acid 10% (Merck)] and finally a photograph of the dried gel was taken by a digital camera (Nikon D60, Japan).

Experimental design and data presentation

Three replicates for each treatment of both heavy metals were performed in this study. Treatments were distributed following a completely randomized design (CRD) among the 18 (3 × 3 × 2) experimental tanks. Data were expressed as the mean ± standard deviation of three measurements for each treatment. Differences between the treatments (dose levels) were compared by one way ANOVA with Tukey's *post-hoc* for multiple comparisons. Statistical software for windows SPSS version 12 was used to analyze the data with the levels of significance at $p<0.05$.

RESULTS AND DISCUSSION

$HgCl_2$ was more toxic than $NaAsO_2$

While treating fish with different concentrations of $HgCl_2$ and $NaAsO_2$ it was found that both heavy metals have significant toxic effect on the survivability of the exposed fishes, $F(5,12) = 913.581$, $p < 0.05$. However, $HgCl_2$ was more toxic than $NaAsO_2$ to induce death of the fishes (**Fig. 1**). When fishes were exposed to lower concentration of $HgCl_2$ (0.1 mM), time required for induction of death was about 9 h. The same concentration of $NaAsO_2$ (0.1 mM) induced fish death in more than 18 h. As the concentrations of the heavy metals were increased, the time required for death induction was decreased. The highest concentration (1 mM), tested in this study, induced death of the fishes as early as 2 h by $HgCl_2$ and 8 h by $NaAsO_2$.

Muscle total protein, fat, moisture and ash content of the exposed fishes

Little variation was observed in the level of fat, moisture, ash content of the exposed fishes, however it was found that both the heavy metals had significant toxic effect on the muscle total protein level of *Anabas testudineus* ($F(7,16) =$

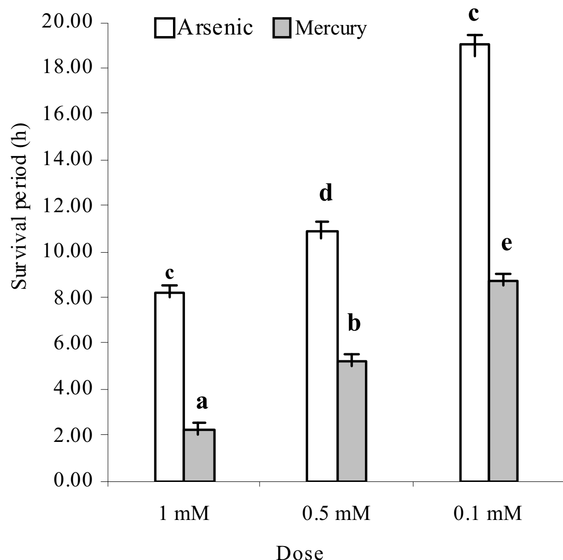

Fig. 1 Survival periods (h) of *A. testudineus* on $HgCl_2$ and $NaAsO_2$ treatments. Fishes were exposed to either $HgCl_2$ or $NaAsO_2$ with indicated concentrations and their survival periods were recorded. Bars representing the mean ± SD of triplicate assays with different letters are significantly different ($p < 0.05$).

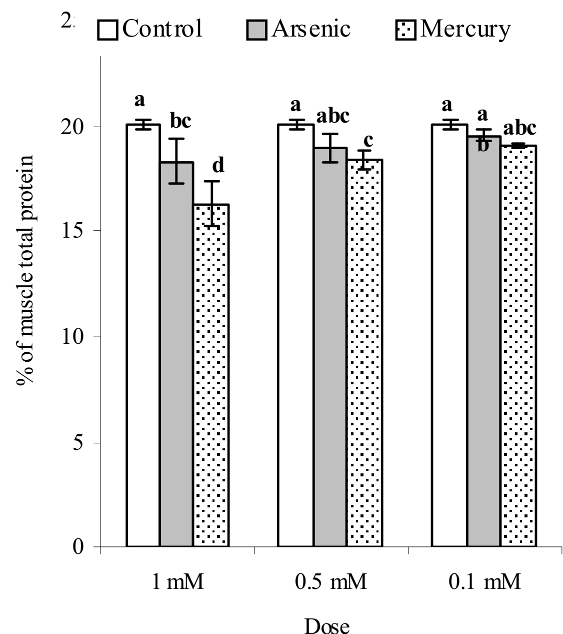

Fig. 2 Effects of $HgCl_2$ and $NaAsO_2$ on muscle total protein content of *Anabas testudineus* (Bloch). Fishes were treated with or without $HgCl_2$/ $NaAsO_2$ followed by isolation of muscle total protein by the Kjeldahl method (2006). Bars representing the mean ± SD of triplicate assays with different letters are significantly different ($p < 0.05$).

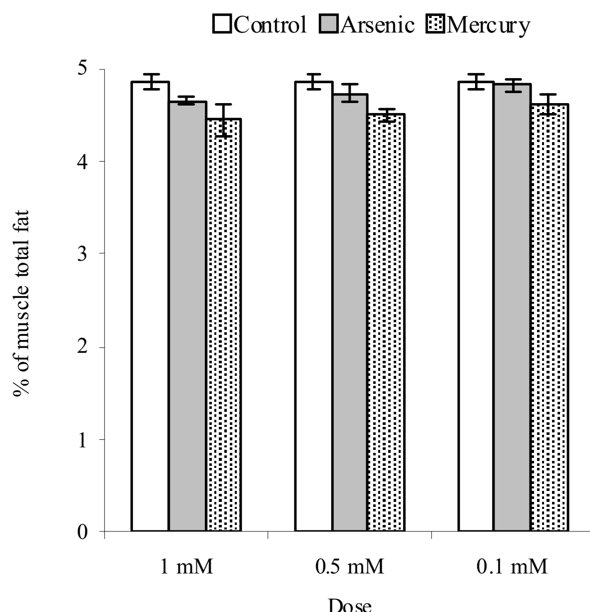

Fig. 3 Effects of $HgCl_2$ and $NaAsO_2$ on muscle total fat content of *A. testudineus*. Fishes were treated with or without $HgCl_2$/$NaAsO_2$ followed by isolation of total fat by the ether extraction method AOAC (2006). Bars representing the mean ± SD of triplicate assays with no letters are not significantly different ($p < 0.05$).

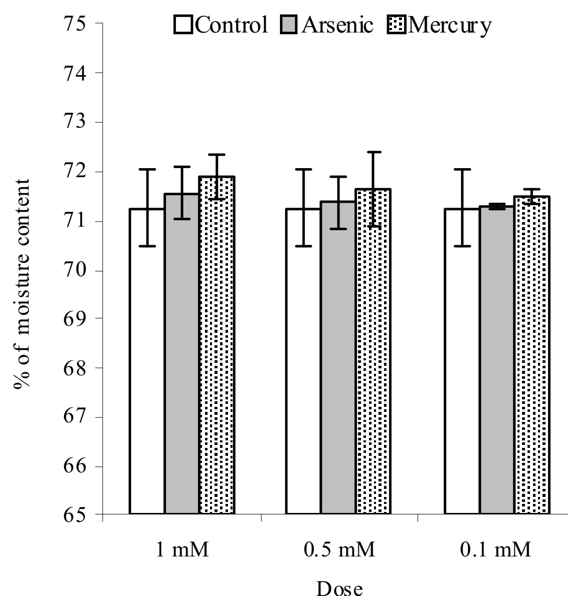

Fig. 4 Effects of $HgCl_2$ and $NaAsO_2$ on muscle total moisture content of *A. testudineus*. Fishes were treated with or without $HgCl_2$/$NaAsO_2$ followed by moisture content measurement by AOAC (1965) method on weight basis. Bars representing the mean ± SD of triplicate assays with no letters are not significantly different ($p < 0.05$).

20.258, $p < 0.05$). The protein content was declined with increasing concentrations of $NaAsO_2$ and $HgCl_2$ (**Fig. 2**). Heavy metal-untreated fish (control fish) contained about 20% muscle total protein, whereas $HgCl_2$ decreased the protein level in a concentration dependent manner. It was observed that a relatively high concentration of $HgCl_2$ (1 mM) decreased the protein level to 16.3%, therefore, around one fifth of the total muscle protein content was reduced. However, the effect of arsenic in reducing protein was less than that of $HgCl_2$. High concentration of arsenic (1 mM) reduced the total muscle protein content to 18.31% that is one tenth of the muscle total protein content was reduced. The muscle total fat content of the control sample was 4.85%. There was a slight reduction in fat content with increasing the concentration of heavy metals but it was not statistically significant ($p < 0.05$) marked change compared with the control (**Fig. 3**). The moisture content of the control sample was near about 71% and in case of both heavy metal treatments, an increasing trend in muscle total moisture content was observed with increasing the treatment dose but it was not statistically significant ($p < 0.05$) (**Fig. 4**). However, a significant ($p < 0.05$) variation in muscle total ash content was found between the treated and untreated samples as shown in **Fig. 5**.

The depletion in protein content was probably caused by the increased metabolism of proteins to overcome the stress in $HgCl_2$/$NaAsO_2$-treated fishes (Janna and Bandhopadhyay 1987; Sivarama and Radha 1998). The increased metabolism of protein might be due to increased activation of metabolic enzymes by mercury and arsenic treatment. Our result is also supported by other reports in which the investigators showed reduction of muscle total protein in

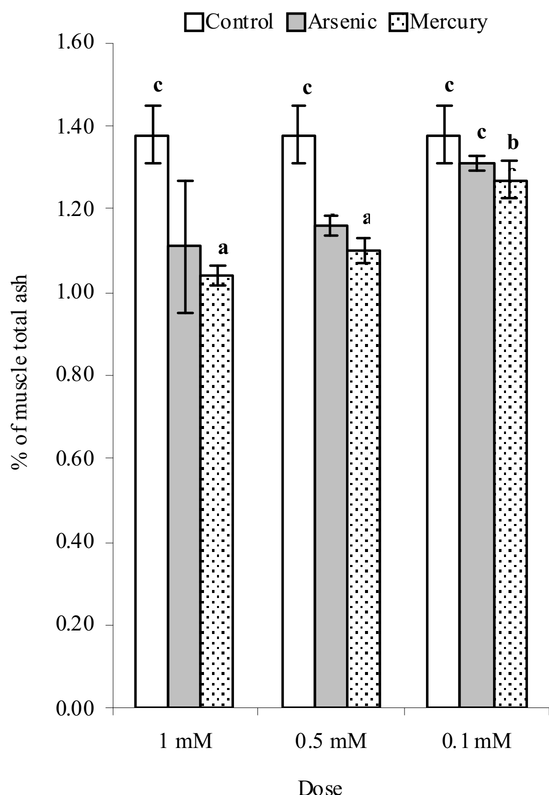

Fig. 5 Effects of $HgCl_2$ and $NaAsO_2$ on muscle total ash content of *A. testudineus*. Fishes were treated with or without $HgCl_2$/$NaAsO_2$ followed by ash content measurement by AOAC (1965) method on weight basis. Bars representing the mean ± SD of triplicate assays with different letters are significantly different ($p < 0.05$).

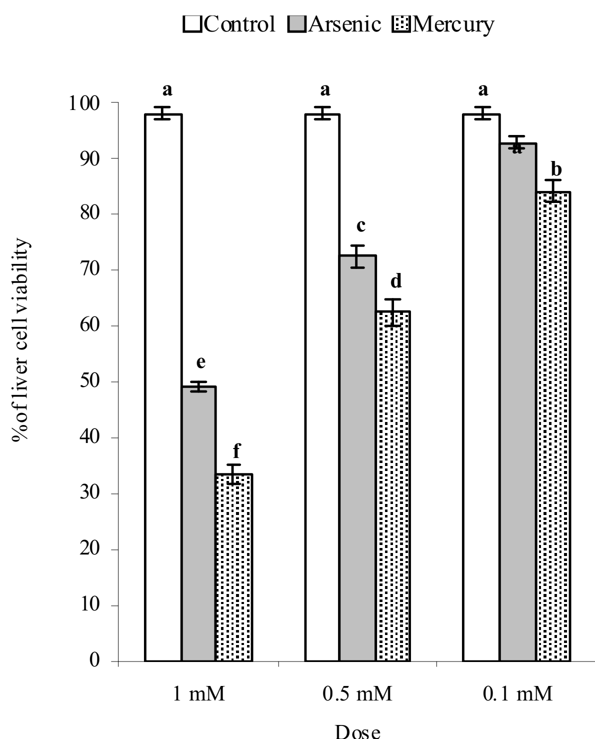

Fig. 6 $HgCl_2$ and $NaAsO_2$ induced liver cell death. Fishes were exposed to either $HgCl_2$ or $NaAsO_2$ with indicated concentrations. Bars representing the mean ± SD of triplicate assays with different letters are significantly different ($p < 0.05$).

Cyprinus carpio treated with Cu, Cd and Zn and in *Notopterus notopterus* with mercury (Verma and Tonky 1983; Shalaby 2000). The reduction in muscle total protein might also be attributed to the great energy demands and cellular damage occurred in the tissue of toxicated fish exposed to heavy metals. With declination in muscle total protein content, an increasing trend was observed in moisture content of the treated fishes compared to untreated fishes. This might be due to increased metabolism of muscle protein as exposed fishes required energy to fight with the toxic environment. However, we observed a little variation in muscle total fat content after exposure to the heavy metals. This result could be explained by the fact that activation of enzymes related to fat metabolism might not be affected by mercury and arsenic or the fat metabolism process might need more time to get a visible change.

Liver cell death due to $HgCl_2$ and $NaAsO_2$ toxicity

$HgCl_2$ and $NaAsO_2$ significantly induced liver cell death of the exposed fishes, $F(7,16)= 695.485$, $p < 0.05$. The control liver cells (collected from fishes that were not exposed to $HgCl_2$ and $NaAsO_2$) were almost all (98%) alive (**Fig. 6**). Viable cell number gradually increased with decreasing concentrations of $HgCl_2$ (for 0.5 mM = 62% viable cells and for 0.1 mM = 84% viable cells), however the count decreased drastically to 32% after exposure of the fishes to 1 mM of $HgCl_2$. Viable cell number gradually increases with decreasing concentrations of $NaAsO_2$ (for 0.5 mM = 72% viable and for 0.1 mM = 92% viable cells); however, it decreased to 48% after the exposure of the fishes to 1 mM of $NaAsO_2$.

Characterization of liver cell death by $HgCl_2$ and $NaAsO_2$

Liver cell DNA was isolated to investigate whether or not $HgCl_2$/$NaAsO_2$-induced liver cell death was accompanied by DNA fragmentation. The isolated DNA was resolved on agarose gel and chromosomal DNA of control fish sample was detected in relatively on the upper portion of the gel, whereas, the chromosomal DNA of either mercury or arsenic exposed fishes was fragmented as revealed by the appearance of the DNA bands in lower portion of the gel (**Fig. 7**). The effect of 0.5 mM $HgCl_2$ in inducing DNA fragmentation was most extensive as demonstrated by the detection of the DNA band in the lowest position. On the other hand, 1mM concentration of $NaAsO_2$ induced DNA fragmentation, although the fragmented band appeared relatively on a higher position to that of $HgCl_2$ (0.5 mM). Lower concentrations of both $HgCl_2$ (0.1 mM) and $NaAsO_2$ (0.5 mM) also induced fragmentation of DNA, however, the fragmented band appeared relatively closer to that of control. When the fishes were exposed to 1 mM of $HgCl_2$, no DNA fragmentation was observed (data not shown). This is probably due to the necrotic death of the cells by the toxic effect of the higher concentration. Usually cell death due to apoptosis is accompanied by fragmentation of DNA show-

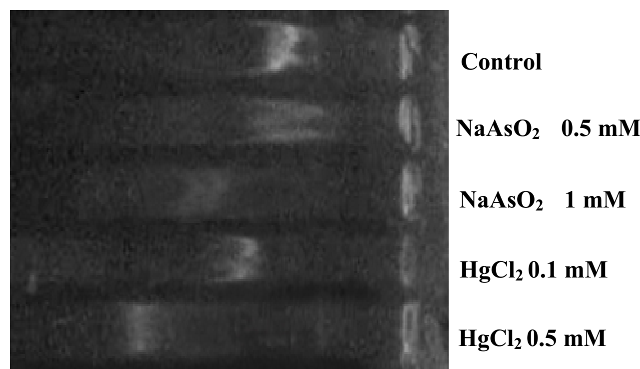

Fig. 7 Visualization of $NaAsO_2$- and $HgCl_2$-induced chromosomal DNA fragmentation by agaroge gel electrophoresis. Fishes were exposed to either $HgCl_2$ or $NaAsO_2$ with indicated concentrations. Liver cells collected after the death of the fishes (for Arsenic 12-18 h and for mercury 4-8 h) and DNA was isolated and resolved on 1% agarose gel. A representative of three experiments with consistent results is shown.

ing a ladder (Akhand *et al.* 1998; Hossain *et al.* 2000). In this study we did not observe any such ladder; however, fragmentation was obviously occurred as the DNA was detected at a lower position.

It is known that both $HgCl_2$ and $NaAsO_2$ induce apoptotic cell death in mammalian cells (Akhand *et al.* 1998; Hossain *et al.* 2000, 2003; Tabellini *et al.* 2005). We observed that both $HgCl_2$ and $NaAsO_2$ induced the death of liver cells involving DNA fragmentation. This result indicated the death of the cells through apoptosis. It has been shown that cadmium exposure induced apoptosis in oyster hemocytes (Sokolova *et al.* 2004). A recent report by Wang *et al.* (2004) also demonstrated arsenic-mediated DNA-fragmentation and cell cycle arrest in two fish cell lines (JF and TO-2) that might involve oxidative stress as a causative factor.

Induction of higher expression of certain protein

Later it was examined whether $HgCl_2$ or $NaAsO_2$-induced death could have any effect on cellular protein profile. In control fish sample, we observed a clear protein band on the upper portion of the gel as indicated by an arrow mark (**Fig. 8**). We could not detect any other clear protein band on the gel. It was also observed that both $HgCl_2$ and $NaAsO_2$ intensified the protein band detected on untreated control sample. This result may suggest higher expression of this particular protein by $HgCl_2$ and $NaAsO_2$. It was also observed that the protein band was more intensified by the $HgCl_2$ treatment compare to $NaAsO_2$ treatment. The intensification of the band was increased in a concentration dependent manner. It might be due to the fact that both $HgCl_2$ and $NaAsO_2$ induce expression of some particular protein by the fish to overcome the adverse or toxic effect created by the heavy metals. A recent report revealed that arsenic can induce the synthesis of specific stress proteins in fish (Roy and Bhattacharya 2006). It was reported that heavy metals induce the synthesis of metal binding protein metallothionein in *Tilapia* (Cheung *et al.* 2004). This finding should not be taken as contradictory with our finding of heavy metal-mediated reduction of total muscle protein content. This reduction was due to high energy demand to overcome the stress created by the toxic effect of the heavy metal. On the other hand, the expression of a particular protein might be due to detoxify the heavy metal in the liver cell.

The findings from the present investigation can be summarized as both of the metals are reported to decrease the total muscle protein content, liver cell were forced to death by $HgCl_2$ and $NaAsO_2$ and these deaths of the liver cells were accompanied with DNA fragmentation indicating cell death through a mechanism, termed programmed cell death

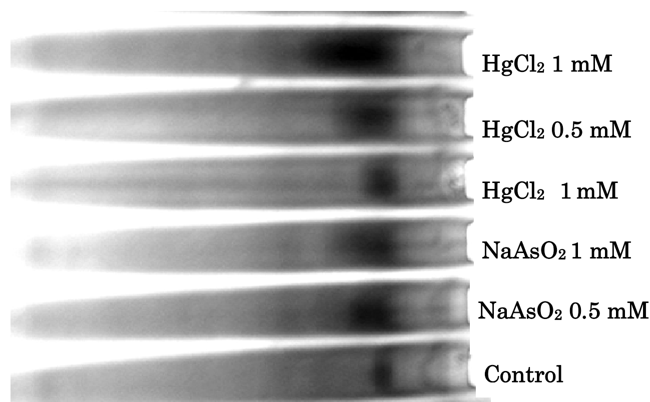

Fig. 8 Observation of toxic effect of $HgCl_2$ and $NaAsO_2$ on cellular protein profile by polyacrylamide gel electrophoresis. Fishes were exposed to either $HgCl_2$ or $NaAsO_2$ with indicated concentrations. Liver cells collected after the death of the fishes (for arsenic 12-18 h and for mercury 4-8 h) and cellular protein was isolated as described in the methods and materials. Cellular protein was resolved on 10% polyacrylamide gel.

or apoptosis. It was also observed that both metals might induce the expression of some particular proteins.

Liver cell death might involve a cascade of signal transduction that might be common to different cell types. Therefore, this study could be extended to find out the whole signal cascades for $HgCl_2$ and $NaAsO_2$ mediated fish cell death. If we know the exact molecular mechanism of heavy metal mediated fish cell death we can later get an idea of designing drugs for their remedy. Moreover, the interpreted pathway could be used as a model for other toxic elements and a guide to study signaling pathways.

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