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Toxicity of Arsenic (Sodium Arsenite) to Fresh Water Spotted Snakehead *Channa punctatus* (Bloch) on Cellular Death and DNA Content

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Abstract: Arsenic is a devastating environmental pollutant that has caused severe pollution of ground water in Bangladesh. In this study, Spotted Snakehead (*Channa punctatus*, Bloch), a common freshwater fish of Bangladesh was exposed to sodium arsenite ($NaAsO_2$) to evaluate its toxic effects on that fish. Different concentrations of $NaAsO_2$ ranging from 0.5 mM-2 mM were used in this investigation. When the fishes were exposed to high concentration (2 mM) of $NaAsO_2$, they died within 2 and half hour. However, as the concentrations decreased, the survival period increased gradually. For 1mM and 0.5 mM of $NaAsO_2$, the survival time was over 5 hours and 18 hours, respectively. Survival durations of the exposed fishes did not vary according to different water sources such as distilled water, pond water and tap water as used in this study. Trypan blue dye exclusion test revealed that $NaAsO_2$ reduced liver cell viability in a concentration-dependent manner. 1 mM and 2 mM of $NaAsO_2$ decreased cell viability to 68% and 38%, respectively. However, 0.5 mM did not show any significant effect. Later, the molecular mechanism of the liver cell death was examined. It was found that the chromosomal DNA of liver cells were fragmented which suggest that $NaAsO_2$ might induce death of those cells through apoptosis, a process that involve fragmentation of DNA.

Key words: *Channa punctatus* · arsenic toxicity · cellular death · DNA fragmentation

INTRODUCTION

Arsenic, a sulfhydryl-reactive metalloid, is one of the most important and concerned global environmental toxicants. It is widespread in the aquatic environment as a result of both geogenic processes and anthropogenic disturbances [1, 2]. High concentration of arsenic in groundwater have been reported from several countries, including Argentina, Bangladesh, Chile, China, India, Japan, Mexico, Mongolia, Nepal, Poland, Taiwan, Veitnam and some parts of the United States [3-7]. The arsenic calamity of Bangladesh can be described as the largest known mass poisoning in the history, with an estimated 35-77 million people exposed to arsenic-contaminated drinking water [8].

Chronic arsenic poisoning can cause serious health effects including cancers, melanosis (hyperpigmentation or hypopigmentation or white spots), hyperkeratosis (harden skin), restrictive lung disease, peripheral vascular disease (blackfoot disease), gangrene, diabetes mellitus, hypertension and ischaemic heart disease [9]. *In vitro* experiments have shown multiple effects at the molecular level following arsenic exposure including differential expression of genes involved in cell cycle regulation, signal transduction, stress response, apoptosis, cytokine production, growth-factor and hormone-receptor production [10-12].

In aquatic environments, several species of microorganisms make arsenic biologically available to organisms including fish [2, 13]. Fish appear to be particularly susceptible to arsenic toxicity as they are continually exposed to it through gills and intake of arsenic-contaminated food. Embryos of medaka (*Oryzias latipes*) exposed to arsenic had a reduction in hatching success as well as reduction in time to hatching [14]. Arsenic can increase morphological abnormalities in the offspring of mummichogs whose parents were exposed to arsenic associated with alterations in gene expression of myosin light chain 2, tropomyosin, parvalbumin and type II keratin [2]. Arsenic exposure can interfere with the normal expression of GR (Glucocorticoid receptor)-mediated gene in the common killi fish [1].

The source of arsenic in the ground water of Bangladesh remains obscure, however, high arsenic

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concentrations have been found in the range of 1 to 1500 µg/L in ground water of Bangladesh [15]. In fisheries sector, ground water is readily used in various stages such as in hatchery operation and in brackish water aquaculture. Furthermore surface water reserves are also getting polluted due to unmanaged industrial effluents and urban waste water. So this sector is in a potential risk of being affected by arsenic toxicity but no proper attention has yet been paid to understand the toxic effects of arsenic especially at the molecular level on fish and other aquatic organisms in Bangladesh.

In the present study, we focused on arsenic toxicity on fishes at the molecular level and probably the first of this kind in Bangladesh, in which we demonstrated the direct toxicological effect of arsenic on fishes. Fresh water spotted snakeheads (*Channa punctatus*) were exposed to different concentrations of $NaAsO_2$ dissolved in different water types and its effects were examined for induction of death of the fishes followed by molecular changes in DNA of liver cell. We chose to focus on the liver because it is a significant site of arsenic accumulation and bio-transformation [16].

MATERIALS AND METHODS

Collection and acclimatization of fish: Live fishes were carried to the wet laboratory of the Department of Fisheries, University of Dhaka to acclimatize them for conducting the experiment. Five fishes of the same sizes were kept in each aquarium in 30 litre of water. Distilled water was kept in the aquarium for one day to confirm that there was no trace of any contaminants or foreign residue in the aquarium. Scoop net was used for handling of the fishes. Tap water was used by filtering with cloth and keeping two days for settling. Pond water was used as clear and fresh as possible. In this way three cycles was undertaken to carry out the whole experiment. All the fishes were acclimatized for a week in tap water and some were acclimatized in pond water. Proper aeration was done during these periods. No foods were given and the water was changed once in every day. Sexually immature healthy *Channa straitus*, as indicated by their activity and external appearance, were used for the experiments. Mainly the fishes having weight of 50~100 g and length between 15 ~ 21 cm were selected.

Arsenic treatment: The acclimatized fishes were selected by appearance and movement as well as fin condition and then transferred by a scoop net into glass jar of 10 litre capacities with 0.5 mM, 1 mM and 2 mM sodium arsenite ($NaAsO_2$). Glass jar was properly cleaned with distilled water and kept in room temperature. Uptake of arsenic by fish was done mainly by intake of water. For each dose three fishes were examined in three different jars. Survival durations of different fishes in different concentrations were recorded.

Preparation of single cell suspensions of liver: Fish liver was isolated by cutting the ventral aorta after being killed by exposure to different concentrations of sodium arsenite for five hours. The liver was aseptically removed and pushed through a nylon mesh (100 nm) to make single cell suspension with normal saline.

Liver cell viability assay (Trypan blue dye exclusion method): Equal volume of 0.2% (w/v in sterile isotonic saline) Trypan blue was added to liver cell suspension and mixed well. The resultant cell suspension (about 10 µl) was added into the counting chamber of a haemocytometer (Neubauer®) covered with a cover slip. Cells were counted under a light microscope. Dead cells take up the blue stain of trypan blue, whereas the live cell have yellow nuclei.The counting chamber, with a cover slip in place, represents a total volume of 0.1 mm. The subsequent cell concentration per ml was determined.

Sample preparation for DNA analysis: 100 µl of liver cell suspension (from 1×10^8 cells/ml) was added with hypotonic lysing buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% SDS) followed by the addition of 2 µl of proteinase K (20 mg/ml) and 6 µl of RNAse (10 mg/ml). The resultant mixture was incubated at 55ºC for 1 hour. 10 µl of the DNA sample mixed with 1 µl dye and was loaded on 1% agarose gel (with 0.1µg/ml ethidium bromide). The sample was run for about 1 hour at 50 mV. Gel was observed under UV light for viewing DNA bands and photographs were taken.

RESULTS AND DISCUSSION

Spotted snakehead fishes of the same size were exposed to $NaAsO_2$ dissolved in different types of water such as distilled water, pond water and tap water to determine their survival period. It was observed that 2 mM of $NaAsO_2$ (in distilled water) induced death of the exposed fishes within 2 and half hour as shown in Table 1. However, as the concentration of $NaAsO_2$ was decreased the survival periods of fishes increased. When the concentration was 1 mM the survival time was more than 5 hour and for 0.5 mM in between 17-20 hours.

Table 1: Survival period of fish after exposure to $NaAsO_2$ dissolved in distilled water

$NaAsO_2$ concentration	Sample no.	Length & weight of fish	Survival period
0.5 mM	1	L: 20 cm	
		W: 58.4 gm	18 h. & 30 min
	2	L: 20 cm	
		W: 58.9 gm	16 h. & 50 min.
	3	L: 19.5 cm	
		W: 58 gm	20 h.
1 mM	1	L: 21cm	
		W: 65 gm	4 h. & 50 min.
	2	L: 21cm	
		W: 65.2 gm	5 h. & 10 min.
	3	L: 21 cm	
		W: 64.9 gm	5 h. & 25 min.
2 mM	1	L: 16 cm	
		W: 39.2 gm	2 h. & 25 min.
	2	L: 15.5 cm	
		W: 39 gm	2 h. & 10 min.
	3	L: 16.2 cm	
		W: 39.6 gm	2 h. & 35 min.

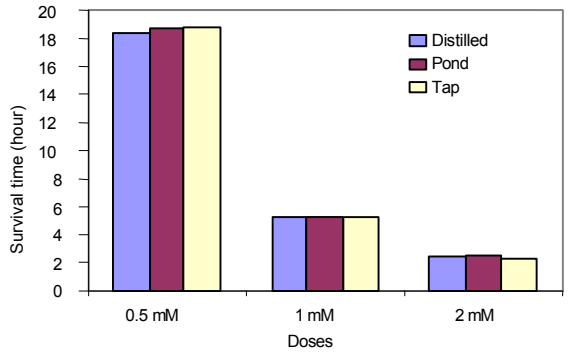

Fig. 1: Comparison of survival period of fish after exposure to different concentrations of $NaAsO_2$ in different types of water

Almost similar results were found when the fishes were exposed to $NaAsO_2$ dissolved in pond water and tap water (Fig. 1). Therefore we found that the toxic effects of arsenic is mainly dependent on its concentration but not on the type of water where it is dissolved and water source is not important in $NaAsO_2$-mediated fish death. The reason for examining different water types was because the tap water of aquatic lab of Fisheries Department contains a large amount of iron, which might interfere with the arsenic toxicity. However the results of this study ruled out that possibility. During exposure a large amount of slime was produced by the fishes due to

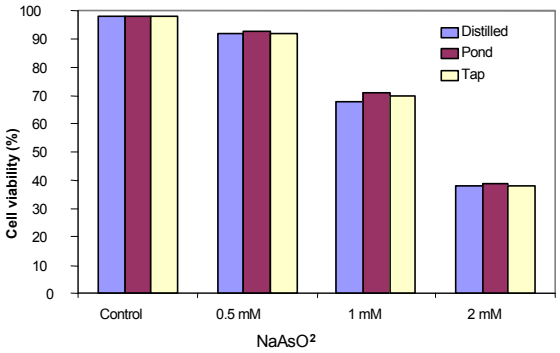

Fig. 2: Comparison of liver cell viability by $NaAsO_2$ dissolved in different water types

toxic effects of arsenic and compared with the control fishes the exposed fishes were found to suffer from suffocation and respiratory problems that might cause them to die.

As toxic materials are generally deposited in liver; we also examined whether liver cells were affected by arsenic. Fishes were exposed to different concentrations of $NaAsO_2$ for five hours and the single cell suspension of the liver was prepared. We found that high concentrations of arsenic caused cell death in large numbers, whereas low concentrations induced a lower rate of cell death. As shown in Fig. 2, the control liver cells were almost all live (98% viable cells). However, viable cell number decreased drastically (38%) after exposure of the fishes to 2 mM of $NaAsO_2$. Viable cell number gradually increased with decreasing concentrations of $NaAsO_2$ (for 1 mM= 68% viable cells and for 0.5 mM=92% viable cell). Arsenic is known as a potential sulfhydryl-reactive compound that aggregates a number of cell surface proteins [10]. Aggregation of cellular proteins, production of reactive oxygen species and activation of protein tyrosine kinases by arsenic might be together or individually involved in the process of cell death [10].

Finally, we tried to characterize the death of liver cells by arsenic. It has been reported that $NaAsO_2$ induces apoptotic death of murine T lymphocytes through activation of c-Jun amino terminal kinase [10]. In this study, it was also investigated whether $NaAsO_2$-induced liver cell death was accompanied by DNA fragmentation. After exposure of fishes with different concentrations of $NaAsO_2$, liver cell DNA was isolated. This isolated DNA was run on 1% agarose gel and the photograph was taken under UV light. As shown in Fig. 3, chromosomal DNA was detected on the upper portion of the gel in control fish sample. When the fish was exposed to 0.5 mM

Fig. 3: $NaAsO_2$ induced DNA fragmentation of fish liver cells

$NaAsO_2$ for 8 hours, the liver cell DNA was not observed at the position where chromosomal DNA was detected in $NaAsO_2$-untreated control fish. This indicated that the liver chromosomal DNA in 1 mM $NaAsO_2$-treated fish was probably fragmented. However, the fragmented DNA was not observed on the lower portions of the gel. This might be due to the formation of very small fragment that were run out of the gel. On the other hand, no fragmentation of liver DNA was observed in 2 mM $NaAsO_2$-treated fish sample. The reason behind this was probably due to the necrotic death of the cells by severe toxic effects of $NaAsO_2$ with higher concentrations. These results suggested that the lower concentrations of $NaAsO_2$ induced apoptotic cell death; however, higher concentrations induced necrotic cell death. Arsenic induced apoptosis of murine cells or cell lines reported by different investigators [10-12] supports our finding.

A recent report by Wang *et al*. [17] also demonstrated arsenic-mediated DNA-fragmentation and cell cycle arrest in two fish lines (JF and TO-2) that might involve oxidative stress as a causative factor. Many investigators have already reported different signal cascades for apoptotic death of different animals' cells. As a potent sulfhydryl-reactive compound [18], arsenic has been shown to affect numerous intracellular signal transduction pathways causing many alterations in cellular functions [10-12, 19]. These actions of arsenic may result in the induction of apoptotic and necrotic death for normal [10, 20] and malignant cells [21], the inhibition of growth and angiogenesis [22] and the promotion of cellular differentiation [23]. Such effects have been observed in cultured cell lines and in animal models, as well as in clinical studies. On the other hand, the signaling effects of arsenic in fishes are still largely unknown but different tissues were examined for changes in cellular death, protein expression and DNA content. These changes may link to the arsenic-mediated intracellular signal cascades that are involved in various complications. The elucidation of arsenic-induced signal transduction pathways that lead to aberrant gene expression could help to identify novel molecular targets that may be useful in getting remedy of arsenic-mediated complications

Here, we have shown the death of spotted snakehead and its liver cells by arsenic and the liver cell death was accompanied by DNA fragmentation. However, this type of liver cell death might involve a cascade of signal transduction that might probably be common to different cell types. Therefore, this study could be extended to find out the whole signal cascades for arsenic-mediated fish cell death. Moreover, the found pathway could be used as a model for other toxic elements and a guide to study signaling pathways.

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