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Toxicological Effects and Molecular Changes Due to Mercury Toxicity in Freshwater Snakehead (*Channa punctatus* Bloch, 1973)

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ABSTRACT: The objectives of this work was to examine the toxicological effect and molecular changes caused by a heavy metal pollutant mercuric chloride ($HgCl_2$), in fresh water snakehead *Channa punctatus*, locally known as Taki fish in Bangladesh. When fishes were exposed to $HgCl_2$, it induced death of the fishes in a concentration-dependent manner. 1 mM, the highest concentration tested in this study, was found to be the deadliest and it induced death of the fishes within 35 to 40 minutes. As the concentration lowered (0.5 mM – 0.1 mM) the survival time increased dose-dependently to near about five hours. $HgCl_2$ dissolved in different types of water (such as distilled water, tap water and pond water) was found to have no effects in changing the required time of fish death. We later investigated the effect of $HgCl_2$ on fish liver cells as liver cells are known to be mostly affected by toxic metals. $HgCl_2$ found to decrease liver cell viability to 32% by the higher concentration (1 mM) tested. Liver cell viability increased up to 82% with decreasing concentration of $HgCl_2$ from 0.5 mM to 0.1 mM. To characterize the mechanism of cell death induced by $HgCl_2$, the changes in molecular level was then examined. It was found that the heavy metal induced chromosomal DNA fragmentation and expression of certain proteins.

Key words: Mercuric chloride, Freshwater Snakehead, DNA fragmentation

INTRODUCTION

Toxic metals, such as mercury, arsenic, lead, cadmium, aluminum, and so on appear to cause serious toxic effects on aquatic animals especially fishes. Mercury, lead and arsenic are the top three toxic elements having the most adverse health effects on the public based on toxicity and current exposure levels (ATSDR/EPA, 2001), followed by cadmium, chromium and nickel. Mercury is considered a devastating environmental pollutant, mainly after the environmental disaster at Minamata and several other poisoning accidents due to the use of mercury pesticides in agriculture. Mercury is one of the most toxic metals and it grouped under European Economic Community's black list pollutants because of its high toxicity, persistence and bioaccumulation in the ecosystem (Moore & Ramamoorth, 1984). Mercury, a sulfhydryl-reactive heavy metal, exists in very small amounts in the nature. Bangladesh, as a developing country, is at a high risk of environmental pollution, especially the inland water bodies faces the highest pollution problems because of uncontrolled industrial effluents, inorganic fertilizers and chemicals used in agriculture (Khaleque & Elias, 1995; D'monte, 1996). These lead to high level of heavy metals in water bodies. Moreover, fishes are also exposed to mercury due to contaminated inland water bodies that definitely is causing adverse fish health leading to decrease fish quality as well as fish production. To deal with this adverse situation properly by an appropriate diagnosis, a detailed investigation is needed to elucidate the molecular mechanisms underlying the pathogenesis of the mercury-mediated complications. As a potent sulfhydryl-reactive compound (Winski & Carter, 1995), mercury has been shown to affect

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numerous intracellular signal transduction pathways causing many alterations in cellular functions (Hossain *et al*., 2000; Yang & Frenkel, 2002; Hossain *et al*., 2003; Tabellini *et al*., 2005). Mercury occurs naturally in the environment (Church & Scudlark, 1998) but is primarily introduced into aquatic systems through anthropogenic pathways, including effluent containing dyes, fungicides used in agriculture, mining and smelting, chlorine alkali production facilities, industrial waste and emission, and ultimately through atmospheric deposition caused by the combustion of fossil fuels and municipal and medical wastes (Clarkson, 1994; Porcella, 1994). Once in the aquatic environment, metallic mercury is readily transformed to organic methylmercury through biological processes that include sulfate reducing bacteria and some fungi (Church & Scudlark, 1998; Porcella, 1994), greatly increasing its bioavailability to aquatic biota. Fish mainly accumulate mercury through dietary pathways (Jernelov & Lann, 1971; Phillips & Buhler, 1978; Rodgers & Beamish, 1981; Harris & Snodgrass, 1993; Hall *et al.,* 1997). Piscivorous fish species normally accumulate mercury at faster rates than similarly sized omnivorous, planktivorous, or benthivorous species (Phillips *et al.*, 1980; Brouard *et al.*, 1994; Olivero *et al.*, 1998; Neumann & Ward, 1999; Mueller & Serdar, 2002). The 96-h LC_{50} vary between 33 and 400 µg/L for freshwater fish and are higher for sea-water fish. Carnivorous species present higher mercury contents than no carnivorous species since they occupy the highest tropic level of the food chain (Palheta & Taylor, 1995; Maurice-Bourgoin *et al.*, 2000). Taki (*Channa punctatus*) is an important fresh water fish species in Bangladesh. As it is a predator, it is particularly vulnerable to the impact of chemical agents, namely heavy metals, which have the capacity to accumulate in aquatic tropic chains. Toxicological effects and molecular changes caused by mercury in fish are still unclear and therefore needed in order to understand their effects in cellular dysfunction. Impairment of protein and gene function may have deleterious effects on the individual fishes. This work aimed to study the effects of mercury chloride on the function and integrity of protein and to evaluate the response of $HgCl_2$-exposed cells. These actions of mercury may result in the induction of apoptotic and necrotic death for normal (Hossain *et al*., 2000; Bustamante *et al*., 1997) and malignant cells (Li & Broome, 1999), the inhibition of growth and angiogenesis and the promotion of cellular differentiation (Miller *et al*., 2002). 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 mercury in fishes are still largely unknown. The present study focuses on investigating the effect of mercury in spotted snakehead (*Channa punctatus*, Bloch), a commonly available Bangladeshi fish popularly known as Taki. Spotted snakehead fishes were exposed to mercury and changes in different physical parameters were noted. Also different tissues were examined for changes in cellular death, protein expression and DNA content. These changes may link to the mercury-mediated intracellular signal cascades that are involved in various complications. The elucidation of mercury-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 mercury-mediated complications.

MATERIALS & METHODS

Sexually immature healthy Taki (*Channa punctatus*), as indicated by their activity and external appearance, were purchased from local markets (Polashi bazaar) of Dhaka. Most of the fishes were acclimatized for a week in tap water and some were acclimatized in pond water. Proper aeration was done during those periods but no foods were given. The water was changed once in every day. Mainly the fishes having weight of 50~100 g and length between 15 ~ 21 cm were selected for mercury treatment.

The acclimatized fishes were selected by appearance and movement as well as fin condition and then transferred into glass jar of 10L capacity with 0.1 mM, 0.5 mM and 1 mM mercuric chloride ($HgCl_2$). Uptake of mercury by fishes was done mainly by intake of water. For each dose three fishes were examined in three different jars. Survival time of different fishes in different concentration was recorded. Length and weight of the fishes were also noted (Balance: XB-120A Precisa, Swissmade; BJ-610C Precisa).

Production of a large amount of slime was observed after mercury treatment.

Fish liver was isolated after being killed by exposure to mercuric chloride. The animals were dissected by cutting the ventral aorta. The liver was aseptically removed and pushed through a nylon mesh (100 nm) to make single cell suspension with normal saline.

Equal volume of 0.2% (w/v in sterile isotonic saline) Trypan blue was added to 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 has 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 and percentage of viability were determined.

Liver was collected after different intervals of mercury treatment. Liver cell suspension was taken in an eppendorf tube and washed twice with PBS through centrifugation (Model: Mikro 22R; Hettich Zentrifugen) at 4ºC for 5 min at 1500 rpm. Then the cells were counted using haemocytometer and 1×10^8 cells/ml was made with PBS. Dead cells were counted by trypan blue dye exclusion method. 100 µl of the cell suspension was added with 2× concentrated sample buffer (125mM Tris-HCl, pH 6.8; 20% v/v Glycerol; 4% w/v SDS; 0.02% Bromophenol blue; 10% 2-Mercepto Ethanol) to lyse the cells and dissolve the cellular proteins. Thereafter the dissolved proteins were denatured by heating for 3 minutes in boiling water for bath. Samples were then analyzed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) or kept at -30ºC for further use.

Liver protein samples were run at 30 mA on 10% polyacrylamide gel using PAGE buffer. After around 2 hours when the blue line reached the bottom of the gel, the power switch was turned off. The gel was carefully removed from the gel slab and the separating gel was stained with Coomassie Brilliant Blue followed by destaining with Destaining buffer. Photographs were taken thereafter.

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 & DISCUSSION

To determine the survival time, taki (*Channa punctatus)* fishes of same size in terms of length and weight were exposed to mercuric chloride ($HgCl_2$) in different types of water such as distilled water, Curzon Hall pond water and tap water. As shown in Fig. 1. it was observed that 1 mM of $HgCl_2$ (in distilled water) induced death of the exposed fishes within 35-40 minutes. During this time vigorous slime production was also observed. Compared to the control fishes (without $HgCl_2$ exposure) the $HgCl_2$–exposed fishes were found to suffer from suffocation and respiratory problems that might cause them to die quickly. However as the concentration of $HgCl_2$ decreased the survival time increased. When the concentration was 0.5 mM the survival time was about one hour and more than four hours for 0.1 mM. Almost similar results were found when the fishes were exposed to $HgCl_2$ dissolved in Curzon Hall pond water and tap water.

When the fishes are exposed to toxic materials, fish liver is found to deposit most of them (Goldwater, 1971). In this study, it was also examined whether the fish livers were affected by $HgCl_2$ toxicity. Fishes were exposed to different concentrations of $HgCl_2$ for three hours and the single cell suspension of the liver was prepared as described in the materials and method. With higher concentrations the fishes were found dead earlier as previously observed, however, liver cell suspension were prepared after 3 hours of $HgCl_2$ exposure. Liver cell suspension was mixed with trypan blue and viable cell number was counted using haemocytometer. As shown in Table 1. all the control liver cells (collected from fishes that were not exposed to $HgCl_2$) were almost alive

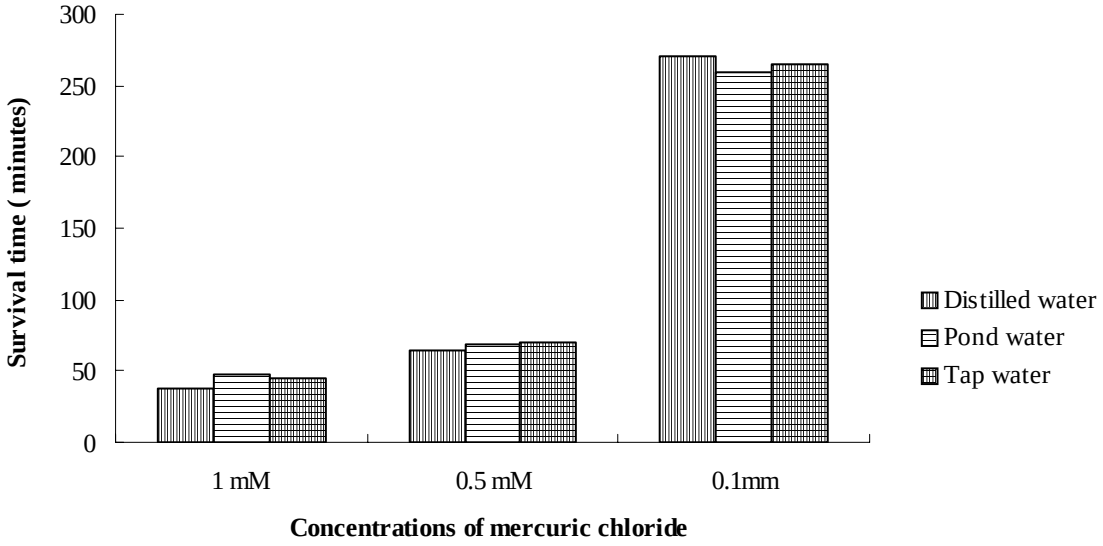

Fig. 1. Comparison of survival time of fish after exposure to different concentrations of $HgCl_2$ in different types of water

(98% viable cells). However, viable cell number decreased drastically (32%) after exposure of the fishes to 1 mM of $HgCl_2$. Viable cell number gradually increased with decreasing concentrations of $HgCl_2$ (for 0.5 mM =61% viable cells and for 0.1 mM =82% viable cells). The results shown here is the mean of 3 experiments. The effect of $HgCl_2$ dissolved in different water types on liver cell viability was also examined. The results shown in Fig. 2. demonstrated that water types did not show any significant variation in affecting liver cell viability.

Table 1. $HgCl_2$ induces death of liver cell (In distilled water)

Doses	No. of viable liver cells (%)
Control	98
1mM	32
0.5mM	61
0.1mM	82

It has been reported that $HgCl_2$ (depending on its concentration) induces death of murine T

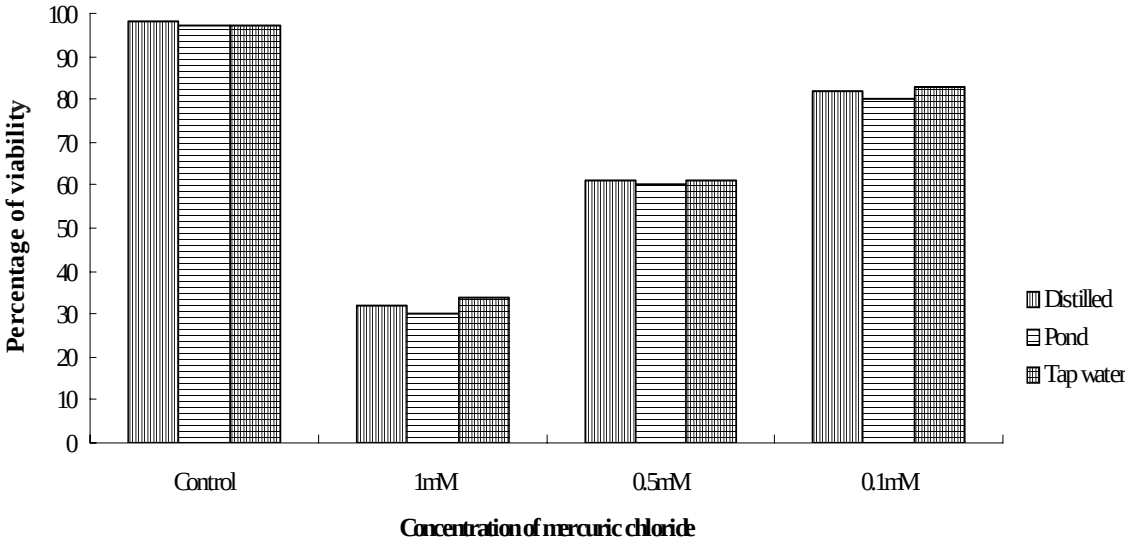

Fig. 2. Comparison of liver cell viability by $HgCl_2$ dissolved in different water types

lymphocytes associated with or without DNA fragmentation (Akhand *et al*., 1998). In this study, it was also investigated whether or not; $HgCl_2$-induced liver cell death was accompanied by DNA fragmentation. After exposure of fishes with different concentrations of $HgCl_2$, liver cell DNA was isolated as described in materials and methods. The isolated DNA was done 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 0.1 mM $HgCl_2$ for 8 hours, the liver cell DNA was not observed at the position where chromosomal DNA was detected in $HgCl_2$– untreated control fish. This indicated that the liver chromosomal DNA in 0.1 mM $HgCl_2$- 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 0.5 mM $HgCl_2$-treated fish sample. The reason behind this was probably due to the necrotic death of the cells by severe toxic effects of $HgCl_2$ with higher concentrations. These results suggested that the lower concentrations of $HgCl_2$ induced apoptotic cell death; however, higher concentrations induced necrotic cell death.

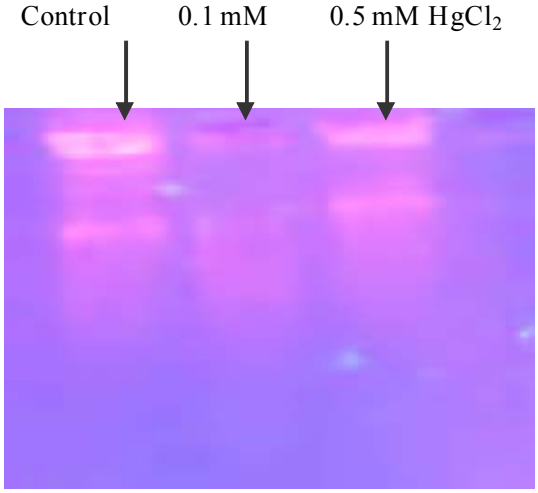

Fig. 3. Visualization of mercuric chloride induced DNA fragmentation by gel electrophoresis. Liver cells were incubated at 37^{0}C with or without 0.1 mM and 0.5 mM of $HgCl_2$ for 8 hours. These cells were lysed in hypotonic lysing buffer and DNAs were then analyzed by agarose gel electrophoresis

Later it was examined whether $HgCl_2$-induced all death could have any effect on cellular protein profile. Liver protein samples were prepared as described in materials and methods. Protein samples were run on 10% polyacrylamide gel. Compare to the control, the intensity of a protein band on the upper portion of the gel increased with increasing concentrations of $HgCl_2$. This result might suggest increasing expressions of some proteins by $HgCl_2$. Lower concentration (0.1 mM) of $HgCl_2$ showed no change in the protein profile.Rapid industrialization of developing countries is partly responsible for contaminating water bodies. Water inhabitants especially fishes are severely affected by toxic metals. In this study it was directly investigated the toxic effect of mercuric chloride ($HgCl_2$) in fishes. The aim of this study was to evaluate the toxicological effect and molecular changes caused by $HgCl_2$ in fresh water fish *Channa punctatus*.

The results showed that there was marked variation in the survival time of fish in different concentrations of $HgCl_2$; however, almost no variation in the survival time of fish in different types of water was observed (Fig. 4). It was found that survival time was 35 to 40 minutes for 1mM concentration of $HgCl_2$, and 55 to 70 minutes for 0.5mM concentration of $HgCl_2$, and 250 to 290 minutes for 0.1mM concentration of $HgCl_2$. As the tap water of aquatic lab of the Department of Fisheries of Dhaka University contains significant amount of iron which may interfere with the mercury toxicity, acclimatization of fish with pond water was also done to find out if there was any interference in the survival time of fish. Otherwise pond water is the natural habitat of *Channa punctatus*. We also used distilled water to find out the difference of the result because this is free from any type of impurities.

During acclimatization fishes were not fed so that their gut become empty and the effect of chemicals could be distinct. The result indicates that water quality is not important for $HgCl_2$ toxicity. It is the concentration that is solely responsible for the toxic effect of $HgCl_2$. Mercury–induced cytotoxicity was also evaluated by trypan blue dye exclusion, a method that differentiates necrotic cell death (with a disrupted plasma membrane). The cytotoxic effects of mercury

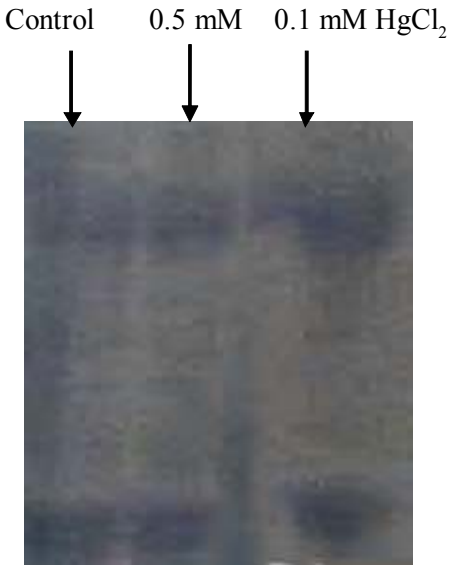

Fig. 4. Mercuric chloride induces tyrosine phosphorylation of cellular proteins of liver cell. Liver cells (10^7/100μL) were incubated at 37^{0}C with or without mercuric chloride for 6 hours.

concentrations depend upon exposure time, as described by others for lake trout thymocytes (Miller *et al.*, 2002).

As toxic elements are mainly deposited in liver cells, the effect of $HgCl_2$ in liver cell viability was investigated. We observed that viable cell numbers were counted after 6 hours incubation of fishes with different concentration (1mM, 0.5mM, 0.1mM). Approximately 32%, 61% and 82% of the cells were viable when incubated with 1mM, 0.5mM and 0.1mM concentration respectively. We found that high concentrations of $HgCl_2$ caused rapid cell death, whereas low concentrations induced a lower rate of cell death that revealed the result of Akhand *et al.* (1998) while studying the level of $HgCl_2$-mediated phosphorylation of intracellular proteins. They showed that high concentrations (10 to 100 μM) of $HgCl_2$ caused rapid cell death without DNA fragmentation, whereas a low concentration (1μM) induced typical apoptosis with DNA fragmentation.

From this study it was found that certain protein levels was increased that was supported by other reports (Simpson, 1961; Steer *et al.,* 1974; Utschig *et al.* 1995). Similar results were obtained by Shalaby (1997) who recorded a significant increase in liver total protein in common carp, *Cyprinus carpio*; exposed to metals such as Cu, Cd or Zn. However, Verma & Tonk (1983) observed a decrease in protein content of muscle in mercury-exposed fish, *Notopterus notopterus*.

Fish, as an entirely aquatic animal, face the greatest environmental hazard due to various environmental pollutants especially heavy metal pollution. A short-term exposure to high levels of heavy metals may induce stress reactions in fish which may bring adverse physiochemical changes.

CONCLUSION

This is probably the first study in Bangladesh in which we exposed fresh water fish to mercury and examined its toxic effects. Here we reported that the fish liver cells were forced to death by mercury effect. These deaths of liver cells were accompanied by DNA fragmentation indicating cell death through a mechanism involving apoptosis. The detailed molecular mechanism of fish cell death is still unclear. 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. Mercuric chloride is a sulfhydryl reactive compound. The effect of mercury can be neutralized by using di-thio-Threitol. The mechanism to detoxify the water can be improved by using this compound.

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