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ACCUMULATION OF A 70 KDA STRESS PROTEIN IN THE NILE TILAPIA, *OREOCHROMIS NILOTICUS*, AND ITS USE AS A BIOMARKER OF CU EXPOSURE

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ABSTRACT

The induction of 70 kDa heat - shock protein (hsp70) in the Nile tilapia, *Oreochromis niloticus*, was investigated. The accumulation of hsp70 was determined in tissues of the liver, gills, spleen and heart of heat shocked fish. Other specimens were also exposed for 96 hr to four concentrations (1.0, 0.5, 0.1 and 0.001 mg/l) of Cu as copper sulphate and the accumulation of hsp70 was determined in the liver and gills. In both experiments, antibodies raised against hsp70 were employed as probes for dot-blot analysis and immunobinding assay of tilapia tissues. Decreasingly, hsp70 was abundant in the spleen, gills, heart and liver of heat shocked fish. Also, data showed greater hsp70 level in the liver and gills of fish exposed to elevated Cu concentrations than the that of controls.

These data suggest that hsp70 accumulation may hold a promise as a molecular indicator of contaminant exposure and may give an early warning of adverse biological effects. The present study indicates that the biomarker method is very easy to use, practical, rapid, and sensitive for assessing water quality in tropical water and is recommended for their incorporation into the future monitoring program.

INTRODUCTION

In order to achieve a more substantial appraisal of water quality, the assessment must not be based only on chemical measurements and analyses of the water itself, but even more so, on the impact of existing conditions on aquatic biota. This is possible with the use of biotests or biomarkers, e.g. investigations of the induction of heat shock proteins (proteotoxicity evaluate) (Hallare *et al.*, 2005).

Biological and ecological responses to contaminant stressors may range from changes at the molecular level to population and community

levels (Fairbairn *et al.*, 1995). These indicators or biological markers that are measured in animals can contribute to detecting, quantifying, and understanding the significance of exposure to chemicals in the environment. One of these biomarkers is a suite of proteins, called heat-shock proteins (hsp) or stress proteins (Atkinson and Walden, 1985). Some evidence indicates that two major stress proteins, hsp60 and hsp70, are present at low concentrations in non-stressed cells. Fish have been extensively studied using both whole organisms and cells in culture. Induction of stress protein synthesis is highly specific in fish (Dyer *et al.*, 1993). The concentrations of stress-70 appear to depend on tissue type. This can help in identifying tissues that are most vulnerable to damage caused by particular environmental stressors. These tissues may represent the "weakest line" or rate-limiting physiological process responsible for lethality (Sanders, 1993). In the teleosts, *Fundulus heteroclitus,* two members of stress-70 family, hsp 76 and 74, are expressed in gills and heart tissues upon heat shock, whereas only hsp76 is detected in the liver, skeletal muscle and brain (Dyer *et al.*, 1993). In response to a wide variety of environmental stressors, including elevated temperature, trace elements, amino acid analogs, cells from all organisms examined increase the synthesis of hsp70 and other stress proteins (Sanders, 1993). Copper sulphate is one of the pesticides that can be troublesome. The most copper sources in the environment are the agricultural fertilizers and pesticides, and these represent the frequent cause of poisoning in aquatic ecosystems (Dueck *et al.*, 1987). Copper compounds are also found in preservatives, additives and colouring agents used in the food industry, and in medical products (Clark *et al.*, 1981). Under adverse environmental conditions, the synthesis of hsp70 increases and it takes on new but related roles to protect the cell from proteotoxicity (Gething and Sambrook, 1992). Member of Hsp70 were significantly overexpressed after *in vivo* exposure to metal concentration as low as 0.1μM of cadmium(II), lead(II) or chromium(VI) (Fulladosa *et al.*, 2005). Sanders *et al.* (1995) characterized hsp70 induction by Cu as copper sulphate in the fathead minnow *Pimephales promelas.*

The stress protein 70 was detected and quantitated in the present study for two reasons. Firstly, to identify the most expressive tissue of the four examined ones. Secondly, to evaluate the changes in stress protein levels in liver and gills as a biomarker of pollution stress on fish.

MATERIALS AND METHODS

Animal:

Oreochromis niloticus (Nile Tilapia) was studied in the laboratory tanks that have water of constant temperature, 28°C. Fish were fed once daily on Tilapia Grower Diet (Star Milling Co., Perris, CA, USA).

Heat shock exposure:

Ten fish were acclimatized for 72 hr at the normal temperature (28°C). Fish were moved to a similar tank where the water was previously heated up to 34°C, and were left for two hours. Then, the fish were moved back to the tank of normal temperature and were left for two more hours. Fish were anaesthetized by putting on the dry ice; they were then dissected to obtain liver, heart, spleen and gills. These body organs were kept directly with dry ice and then kept in -70°C for the next analysis.

Copper sulphate exposure:

Fourty fish were exposed to four concentrations of Copper sulphate as a fungicide (1.0, 0.5, 0.1 and 0.001 ppm) ten fish each. Other ten fish were kept in an extra tank as control. The experiment was continued for 96 hr. Water in all tanks was replaced daily. Fish were anaesthetized by putting on the dry ice; they were then dissected to obtain liver and gills. These organs were kept directly with dry ice and then kept in -70°C for the following assays.

Homogenization:

The selected body organs from control specimens, heat shocked or copper sulphate exposed fish were homogenized in tris-buffered saline (1% SDS) by glass polytone. The homogenate was heated in water bath at 90°C for 5 minutes, and then centrifuged at 14,000 rpm for 10 minutes. The supernatant was obtained and kept in refrigeration until use.

BCA protein assay:

Total protein content of the supernatant was quantified using a BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Ten µl of the supernatant was diluted in 200 µl BCA reagent (BCA detection reagent A : 4% $CuSO_4.5H_2O$ reagent B = 50:1) and incubated at 37°C for 30 min. Photometric absorption was recorded at 562 nm against a suitable blank.

Dot-blotting:

Fifty µl of each sample and hsp70 standard were directly applied to nitrocellulose membrane (0.22 Nm) (Schliecher and Schuell, NH, USA) that was placed in Bio-Dot microfiltration apparatus (Bio-Rad labs,

CA, USA). The entire samples were allowed to filter through the membrane by gentle vacuum. The membrane was removed and applied by the immunoassay.

Immunoassay:

The nitrocellulose membrane was placed in non-fat dry milk (5%) as a blocking solution. Then it was immersed overnight in the primary monoclonal antibody SPA-802 solution (StressGen Biotechnologies Corp., Canada) of mouse anti-human hsp70; dilution of 1: 5000 in non-fat dry milk (10%) and washed in TBS. The secondary antibody (peroxidase-conjugated goat anti-mouse IgG; dilution 1: 1000 in 10% non-fat dry milk) was added for 2 hr. After subsequent TBS washing the antibody complex was detected by an enhanced chemiluminescent substrate kit of immunoblot utilizing a horseradish peroxidase lable (Pierce, Rockford, IL, USA). The chemiluminescent detection system was visualized by exposure the membrane to Kodak BioMax light film (Kodak, Japan) in a dark room. After developing the film, the protein bands were accomplished by the use of densitometric image analysis system Image-Pro Plus (Media Cybernetics, L.P., Silver Spring, MD, USA) after background substraction. Heat shock protein 70 was calculated as referred to the weight of total protein (picogram hsp70/microgram protein; pg/μg) estimated in 0.2 g of the examined tissues.

Statistical analysis:

The data were statistically analyzed using the student's "*t*" test (Snedecor & Cochran, 1969) and also by using one-way analysis of variance, ANOVA. Data were expressed as Mean ± SE for all experiments and the levels of significant were only considered when $P \leq 0.05$.

RESULTS

Liver of control fish showed heat shock proten 70 (hsp70) level of 33±7 pg/μg protein. The amount of hsp70 was also observed as a normal concentration, in the tissue of gills, spleen and heart (90±2, 218±45 and 80±4 pg/μg protein) (Table 1, Fig. 1). By the exposure of tilapia fish to heat-shock for two hours, the amount of hsp70 was significantly increased ($p<0.05$) in liver from 33±7 (at normal) to 107±25 pg/μg protein. A highly significant increase of hsp70 level was observed in gills tissue from 90±2 (in normal) to 214±8 pg/μg protein. Spleen tissue recorded the highest level of induced hsp70 that elevated from 218±45 (in normal) to 706±11 pg/μg protein (Table 1). While, in the heart tissue, hsp70

increased in a highly significant value ($p<0.01$) from 80±4 (in normal) to 145±4 pg/μg protein.

Copper exposure:

Fish were exposed to sublethal concentrations of Cu as $CuSO_4$, (0.001, 0.1, 0.5 and 1.0 ppm). By the comparison to the control fish, liver showed a gradual significant ($p<0.001$) increase of hsp70 amount as a result of exposure to the increased concentrations of Cu (Table 2, Fig. 2). The amount of hsp70 revealed the highest value significantly ($p<0.001$) when fish exposed to 1.0 mg/l $CuSO_4$. As regard to fish gills, hsp70 recorded a non-significant decrease than in control gill tissue, then revealed two sharp significant increases ($p<0.001$) at 0.1 and/or 0.5 and 1.0 mg/l $CuSO_4$ (Table 2).

DISCUSSION

The stress response results in the rapid and coordinated induction of a group of proteins referred to as the stress proteins and in the concomitant reduction of normal cellular proteins (Kong *et al.*, 1996). One group of the stress proteins is the heat shock proteins (hsps) that are mainly induced by exposure of cells to elevated growth temperatures, amino acid analogs, or various heavy metals (Klann and Shelton 1989; Schlesinger, 1990; Welch *et al.*, 1991). Heat shock proteins family included hsp60, hsp70 & hsp90 that are present in the cytosol and/or nuclei. The heat shock response is highly conserved during evolution and most of hsps are also constitutively synthesized in considerable amounts even in the unstressed normal cells (Hunt and Morimoto, 1985). These facts lead to a suggestion that the expression of hsps must be essential for the survival of the unstressed cells as well as of the stressed cells.

Statistical analysis revealed that hsp70 concentrations were significantly higher than the control concentrations in liver, gills, spleen and heart tissues of *O. niloticus*, after sudden heat of 34°C for 2 hrs. The accumulation of hsp70 in the different examined tissues was 2 to 3-fold greater than in the control fish. The spleen was recorded as the most sensitive of the studied organs where it had the highest response of hsp70 accumulation. Gills were the second target of heat shock protein, then heart and lastly the liver tissue (liver, may be because of its normal high growth temperature). Induction of stress protein or heat-shock protein synthesis is highly tissue specific both in vertebrates and invertebrates. The specific stress proteins induced, the temperature range of induction, and the concentration of hsp70 appear to depend on tissue type (Sanders,

1993). The author mentioned that the intensity of stress response and relative concentrations of stress-70 (hsp70) and chaperonin among the various tissues can help to identify tissues that are most vulnerable to damage caused by a particular environmental stressor.

The heat shock response has been reported in poikilothermic animals which may differently adapt to heat shock or regulate the hsp expression (Misra *et al.*, 1989). The specific stress proteins induced vary with different tissues, probably this in part may be due to different tissue sensitivities to particular toxicants. Within a tissue, the stress protein induction process appears to be non-specific (i.e., all stressors cause essentially the same response although the kinetics may differ) (Sanders, 1993). One of the major effects of heat shock is an unfolding or incomplete folding of proteins appeared early in heat-shocked cells (Hightower, 1980).

Concerning the chemical stressors as $CuSO_4$, the presented data showed a concentration-dependent accumulation of hsp70 in liver and gill tissues. The concentration of hsp70 was increased in both liver and gill cells when fish were exposed to higher concentrations of Cu. Hsp70 was increased to 11-fold in liver tissue than the control, while increased to 6-fold in gill tissue. Levinson *et al.* (1980) showed that transition series metals induce a specific group of heat-shock proteins in cultured chick embryo cells and in cultured human foreskin cells. Also, Brown and Rush (1984) showed that injections of arsenite into rabbits lead to the product of a 74-kDa hsp in the rabbits' kidneys, heart and liver. Arsenite was the most effective metal with respect to producing elevation in hsp70. Cultured hepatocytes exposed to zinc had a 3-fold elevation in hsp70 (Bauman *et al.*, 1993). Similarly to the current data, Cu^{2+} and Cu-EDTA complex can induce significantly the expression of hsp70 (Shen *et al.*, 2004). It was found that the fish exposed to the concentration of Cu^{2+} below the national fishery water quality standard of 0.01 mg/l, also had a significant increase in the expression level of hsp70. The mechanism underlying copper hepatotoxicity was investigated in primary cultures of rainbow trout hepatocytes. $CuSO_4$ treatment (0, 25, 50, 100 and 200 μM) resulted in a dose-dependent elevation in heat shock protein 70 (hsp70) expression at 24 and 48hr post-exposure (Feng *et al.*, 2003). Also, induction of hsp70 was observed upon copper (10-30 μM) exposure in liver and gonads, but not in gills of zebrafish, *Danio rerio* (Airaksinen *et al.*, 2003). DeBoeck *et al.* (2003) revealed that exposure to Cu (1.9 μM,) increased hsp70 levels in gills, erythrocytes, and liver and

decreased its levels in the brain and kidney. Moreland *et al.* (2000) also revealed that both hsp 60 and 70 were induced two fold in culture of PLHC-1 (Pociliopsis lucida hepatoma cell line) cells when treated with 16 ppm copper.

In response to a wide variety of environmental stressors, including elevated temperatures, trace metals, thiol reactive agents and amino acid analogs, cells from many organisms increase the synthesis of hsp70 and other stress proteins (Mizzen *et al.*, 1989; Sanders, 1993). This expression increase of stress proteins is thought to play related protective and reparative functions to reduce protein aggregation and non-native conformations caused by environmental perturbations (Welch, 1991). Specifically, increased levels of stress proteins may facilitate the repair and recovery of metabolic pathways compromised as a consequence of the stress event (Rothman, 1989; Welch 1990). Hsp70 can stabilize certain proteins so that they will be functional following severe stresses (Lindquist and Craig, 1988). Hsp70 is also thought to play a role in the transport of proteins into the mitochondria and endoplasmic reticulum (Schlesinger, 1990).

Data from the current experiment suggest that the ideal stress marker may be met by examining the accumulation of hsp70 in tissues. This indication is presented after exposure to environmentally physical and chemical contaminants. The present study also indicates that the biomarker method is very easy to use, practical, rapid, and sensitive for assessing water quality in tropical water.

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Table 1. The amount of hsp70 in different tissues of both normal and heat shocked fish, *O. niloticus*.

Treatment / Tissue	Control (M±SD)		Heat shock (M±SD)	
	Total protein (mg/l)	HSP70 (pg/µg)	Total protein (mg/l)	HSP70 (pg/µg)
LIVER	11.7±2	033±7	10.7±0.5	107±25*
GILLS	14.5±0.6	90±2	15.1±0.9	214±8**
SPLEEN	13.5±1	218±45	17.5±2	706±11**
HEART	9.7±0.7	80±4	8.1±0.6	145±4**

** $P < 0.01$

Table 2. The amount of hsp70 in the liver and gills of *O. niloticus* exposed to four $Cuso_4$ concentrations.

Tissue / Treatment		LIVER		GILL	
		Total protein (mg/l)	HSP70 (pg/µg)	Total protein (mg/l)	HSP70 (pg/µg)
Control (M±SD)		11.7±2	033±7	14.5±0.6	080±4
$CuSO_4$ (mg/l)	0.001 (M±SD)	8.5±0.7	145±7**	10.9±0.6	058±5
	0.1 (M±SD)	5.8±0.5	173±8**	6.2±0.2	262±3**
	0.5 (M±SD)	5.7±0.3	184±1**	7.1±0.5	267±2**
	1.0 (M±SD)	5.5±0.3	383±2**	6.6±0.4	423±2**

** $P < 0.01$

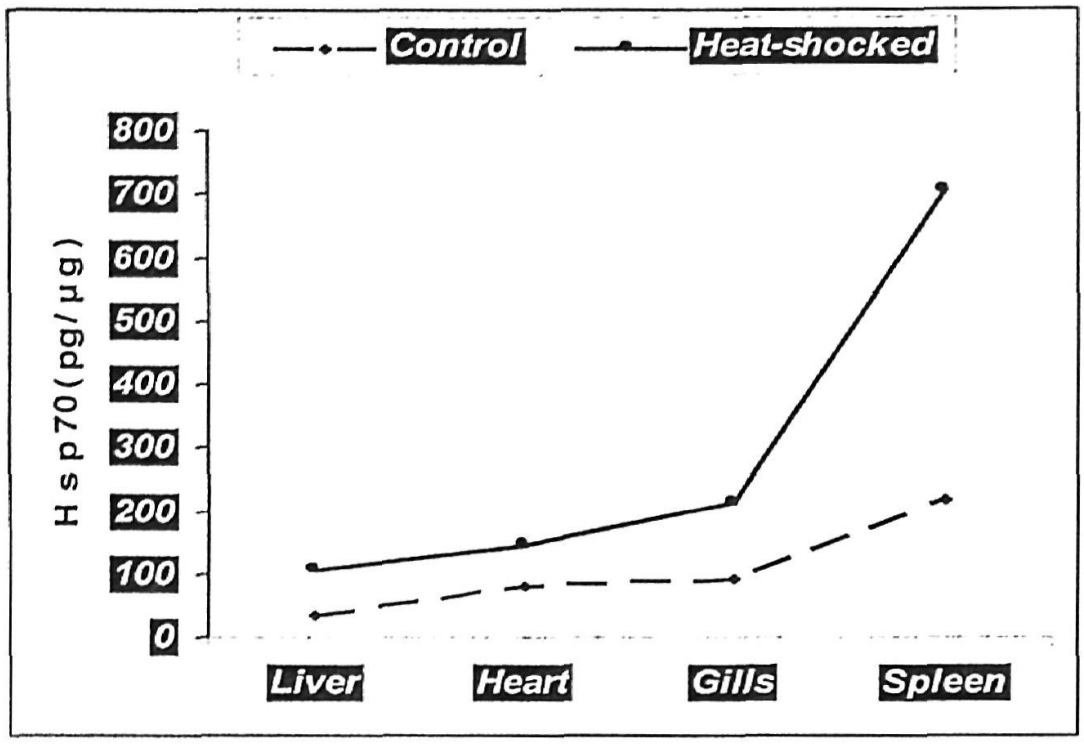

Figure 1. The amount of hsp70 in different tissues of both normal and heat shocked fish, *O. niloticus*.

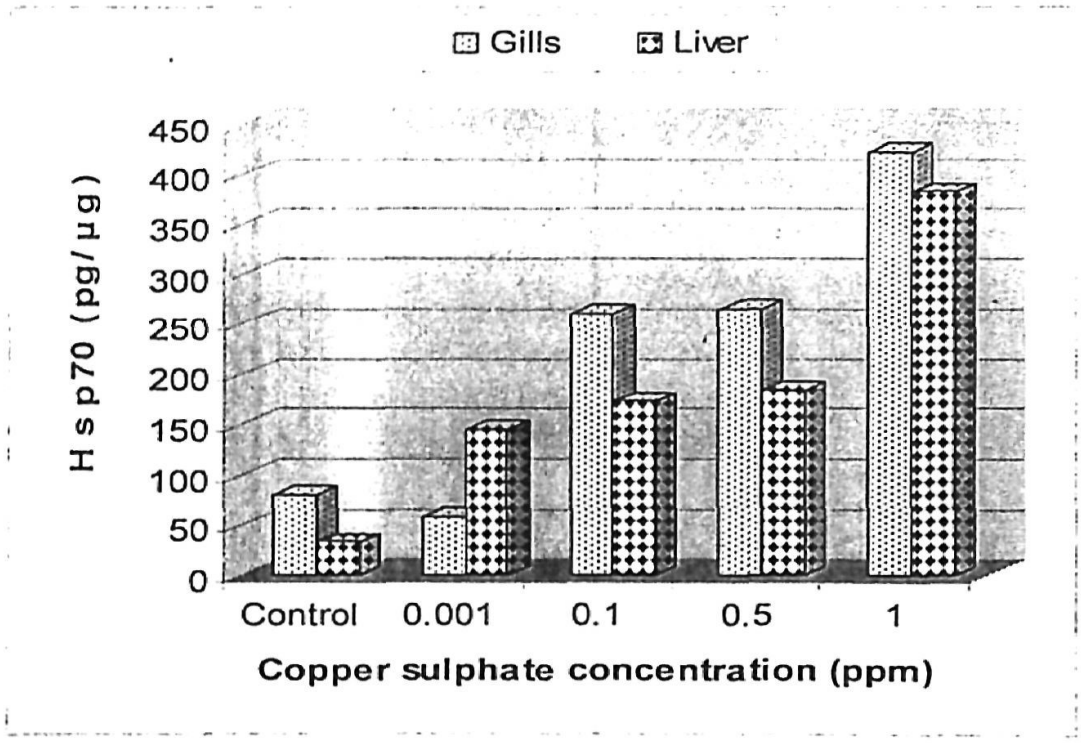

Figure 2. The amount of hsp70 in the liver and gills of *O. niloticus* exposed to four $CuSO_4$ concentrations.