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# Pollution induced nitrative stress and heat shock protein 70 overexpression in fish liver mitochondria

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ABSTRACT

Exposure to heavy metals and organic pollutants in natural water bodies can have detrimental effects on fish health. A combination of biochemical and energy studies were used to observe the changes in fish liver mitochondria in response to environmental pollutant induced nitrative stress in natural field conditions. The fish samples *Mugil cephalus* were collected from polluted (Ennore) and unpolluted (Kovalam) estuaries for a period of two years. The results revealed elevated nitrite ($NO_2^-$) and nitrate ($NO_3^-$) levels, increased nitric oxide (NO) synthesis and 3-nitrotyrosine expression, decreased respiratory chain enzyme activities and ATP/ADP ratio, reduced mitochondrial superoxide dismutase (MnSOD), glutathione peroxidase (Gpx) levels, diminished thiol status that leads to alterations in the mitochondrial function and elevated mitochondrial heat shock protein 70 (mtHSP70) expression (30%) to a significant extent in fish from the polluted estuary than in the unpolluted estuary. The overexpression of HSP70 under stress may aid mitochondrial survival by protecting against nitrative stress induced damage. The results also reveal the percentage increase in fish liver mitochondrial HSP70 in response to cumulative effect of environmental pollutants.



## 1. Introduction

Chemical composition or quantity of pollution in the environment prevents the functioning of natural processes and produces undesirable environmental and health effects with consequent accumulation and effects on living organisms. Water pollutants which originate from both point and non-point sources exert toxic effects on aquatic organisms that ranges from biochemical alterations in single cell to the changes in whole population (Bernet et al., 1999). The study area, Ennore estuary, located in the North Chennai of Tamilnadu, India is one of the water bodies that is under great threat due to pollution (Padmini et al., 2004). As the fish are considered as one of the bioindicators of the water pollution, the extent and effect of pollution in them can be monitored by examining the sublethal indices or biomarkers (Padmini et al., in press-a).

Liver performs a number of important and complex biological functions like energy metabolism that are essential for survival. It serves as an appropriate organ for the study of pollutant effects due to its high metal accumulating capacity and susceptibility to histopathological damage by metals (Bernet et al., 1999). Mitochondria are the "energy factory" of cells and the maintenance of their activity by preserving protein content and function is a key aspect at cellular level (Janowsky et al., 2006). The mitochondria which occupy a greater part of the liver cell (Sylviane and Phillipe, 1992) undergo a number of cytological alterations and apparent changes in response to toxic environmental chemicals (Kohler, 1999). It also constitutes the greatest source of

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oxidants and acts as the primary locus for the intracellular formation and reactions of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Radi et al., 2002) Reactive species are the by products of the mitochondrial respiratory chain that are physiologically counteracted by the intracellular antioxidant systems (Green et al., 2004). In a normal cell, there is always a balance between pro-oxidant and antioxidant pathways. Upon stress stimuli, an imbalance of the redox mileu develops and leads to the accumulation of ROS (Gutterman, 2005).

Heat shock proteins (HSPs) or stress proteins are a conserved group of inducible proteins that are thought to protect cells from stresses such as hypoxia, ischemia, reactive oxygen species, heavy metals etc. that typically result in protein malfolding (Mestril and Dillman, 1995). HSP function as molecular chaperones by regulating cellular homeostasis and promoting cell survival (Bukau and Horwich, 1998). HSP70 is a stress induced protein that plays important role in defense mechanisms against agents that promote oxidative injury and has been proposed as a significant biochemical biomarker of environmental stress caused during toxicant exposure (Tiano et al., 2004). MtHSP70 is essential for driving preproteins across the membrane in to the matrix and constitutes the motor unit of mitochondrial protein import machinery (Kang et al., 1990; Schatz and Dobberstein, 1996).

Previously we have demonstrated oxidative stress in liver and liver mitochondria of the *M. cephalus* collected from polluted Ennore estuary (Padmini et al., in press-b; Padmini and Vijaya Geetha, 2007a). In spite of heavy pollution, the fish inhabiting in Ennore estuary are able to survive combating the stressed situation. Hence it is important to understand the various damages that occur in these fish during stress conditions and mechanism that are involved in providing resistance to such damages which allow the fish to survive. Moreover the studies on fish liver mitochondrial nitrative stress related to HSP70 is not reported so far. Therefore, in the present study the impact of nitrative stress on respiratory enzyme activities, ATP levels, antioxidant levels and thiol status were investigated in mitochondria to gain further insight into the oxidant/antioxidant balance as they are the critical factors operating in the fish that are exposed to high contaminants. In addition, mtHSP70 levels were studied to assess its expression during oxidative and nitrative stress in polluted field condition.

## 2. Materials and methods

### 2.1. Chemicals

Sulphanilamide, *N*-1-naphthyl ethylene diamine dihydrochloride, NADH, cytochrome c, Superoxide dismutase (SOD), catalase and NG-methyl-L-arginine (L-NMMA) were purchased from Sigma-Aldrich, USA. Ammonium dihydrogen phosphate, 2,4-dinitrophenyl hydrazine, *O*-phthalaldehyde, *N*-ethylmaleimide were obtained from Sisco research laboratories, Mumbai, India. 3-nitrotyrosine antibody (SC-32731) was obtained from Santacruz Biotechnology, California and mouse monoclonal HSP70 antibody conjugated with alkaline phosphatase (SPA-810) from Stressgen Bioreagents, Columbia, Canada.

### 2.2. Animals and study site

*M. cephalus* (Grey mullets), a natural inhabitant of the estuaries, identified by the use of Food and Agriculture Organisation's (FAO) species identification sheets was chosen as the experimental animal for the study (Fischer and Bianchi, 1984).

Two estuaries were chosen as the experimental sites for the study: (1) Kovalam estuary (12°49′N and 80°5′E), an unpolluted site situated about 35 km south of Chennai is far away from the influence of industrial pollution (2) Ennore estuary (13°14′N and 80°20′E) situated about 15 km north of Chennai is a highly polluted estuary due to the discharge of effluents from industries like oil refineries, fertilizer company, thermal power stations etc. surrounding this site. Contamination of the Ennore estuary by heavy metals like Fe, Pb, Cd, Mg, Zn etc. to a significant extent has been confirmed by a previous study (Padmini and Vijaya Geetha, 2007b).

### 2.3. Fish sampling

Grey mullets (*M. cephalus*) with average length of 30 cm were collected freshly twice in a month for two years from April 2006–March 2008 from both the estuaries to study the effect of pollution. Sampling fish was collected with baited minnow traps in a shallow estuary and were placed immediately into insulated containers filled with aerated estuarine water at ambient temperature. Fish were sacrificed by severing their spinal cord, and the liver was removed immediately for the isolation of mitochondria.

### 2.4. Isolation of fish liver mitochondria

The procedure of Johnson and Lardy (1967) with slight modifications was employed to isolate mitochondria. About one gram of tissue was weighed, washed twice with ice cold buffer, and 5 ml of Kohler's homogenizing buffer. The homogenate was centrifuged in the refrigerated centrifuge at 500 ×*g* for 10 min. The supernatant was recentrifuged at 8000 ×*g* for 30 min. The resulting pellet containing the mitochondrial fraction was resuspended and recentrifuged under same experimental conditions, to obtain the pure fraction of mitochondria. we purified crude mitochondrial fraction by resuspending such fraction in three packed cell volumes of mitochondrial suspension buffer (10 mM Tris HCl pH 6.7, 0.15 mM $MgCl_2$, 0.25 M Sucrose, 1 mM PMSF, 1 mM DTT) and centrifuging at 9500 ×*g* for 5 min for repelleting the pure mitochondria. It was then suspended in 0.25 M sucrose solution (pH 7.4) and homogenized for one minute, which was then used for further studies. The presence of mitochondria in the pellet fraction was confirmed by the assay of succinate dehydrogenase enzyme (marker enzyme). Proteins were estimated by the method of Bradford (1976) with the use of bovine serum albumin as the standard.

### 2.5. Detection of nitric oxide: total nitrite and oxidation of oxyhemoglobin

Total nitrite concentration in fresh mitochondria was used as an indicator of nitric oxide (NO) synthesis. Fish liver mitochondrial