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HSP70 Downregulation by Pifithrin-μ Upregulates Pro-Apoptotic Changes in Stressed Fish Hepatocytes

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Abstract: ***Estuarine grey mullet is very sensitive to several environmental factors. Apoptosis signal regulating kinase-1 (ASK1) activity is regulated at multiple levels, one of which is through inhibition by heat shock protein (HSP70). In this study, we investigated the inhibitory effect of pifithrin-μ in the hepatocytes of Mugil cephalus inhabiting Kovalam and Ennore estuaries. The level of total antioxidant capacity (TAC) and the expression of 4-hydroxy-2-nonenal (HNE), HSP70, HO-1, phospho-ERK and ASK1 were measured in hepatocytes of M. cephalus inhabiting Kovalam and Ennore estuaries with and without pifithrin-μ incubation. A significant increase in oxidative stress along with decrease in antioxidant status in hepatocytes of M. cephalus inhabiting Ennore estuary was observed, whereas pifithrin-μ incubation moderately alters the stress and antioxidant status. The level of HSP70 was inhibited by 39% at 10 μM of pifithrin-μ for 60 minutes. Pifithrin-μ enhances the generation of ROS, possibly by inhibiting the anti-oxidant actions of HSP70. An alteration in the expression of HSP70 by pifithrin-μ inhibitor causes downregulation of phospho-ERK and upregulation of ASK1 expression. These proapoptotic changes in the stressed fish hepatocytes indicate that HSP70 threshold is a key factor in fish survival under polluted environmental stress.***

Keywords: ASK1; ERK; Estuary; Inhibitor; *Mugil cephalus;* Survival

1. Introduction

Growth, proliferation and differentiation of cells are processes continuously regulated by a strict control of the intracellular protein homeostasis, and deregulation of such processes can have severe consequences on cell survival. The problems of protein folding continuously challenge the cells, and these problems become more critical when cells are subjected to extended levels of stress. However, if the level of stress is sufficiently low cells may attempt to survive by producing a set of highly conserved protecting proteins, namely heat shock proteins (HSPs), which assist folding of damaged proteins, preserve them from degradation, and impede toxic intracellular aggregates from threatening cell survival (1).

Heat shock proteins are molecular chaperone that assist protein folding and are potential biomarkers for environmental stress in fish (2). Constitutive heat shock protein isoforms are synthesized under normal cellular conditions, whereas inducible isoforms can be synthesized in response to both biotic and abiotic stressors (3). HSP70 are constitutively expressed in cells under normal (non stressful) conditions and function as molecular chaperones, to keep other proteins from inappropriate aggregations. Its expression is markedly induced in response to environmental stresses, such as heat shock, UV and γ-irradiation, and chemical exposure (4,5). Apart from the induction of high molecular weight HSPs, stress also induces low molecular weight HSPs like HO-1. HO-1 is a 32-kDa protein also known as heat shock and stress response protein HSP32. HO-1 is highly inducible by hemin and other non-heme agents such as ultraviolet (UV), hydrogen peroxides, heavy metals, hypoxia, and nitric oxide (6).

ERK1/2 is a serine/threonine kinase of the MAPK superfamily that mediates intracellular signal transduction in response to a variety of stimuli (7). The ERK module is activated by MAPK/ERK kinase (MEK) 1/2, which phosphorylate at the Thr-Glu-Tyr motif (8). The Ras/Raf/MEK (mitogen-activated protein kinase/ERK kinase)/ERK (extracellular-signal-regulated kinase) pathway is at the heart of signalling networks that govern proliferation, differentiation and cell survival (9).

Apoptosis, molecularly regulated cell death, can be induced by a range of environmental, physical or chemical stresses, and is characterized by a sequence of precisely regulated events that culminate in the self-destruction of a cell. Many studies in recent years have shown that the heat shock proteins play critical roles in modulating the apoptotic cascades (10,11). Apoptosis signal regulating kinase 1 (ASK1) is a 155-kDa ubiquitously expressed protein belonging to the member of MAPKKK, a serine-threonine protein kinase. It is activated in response to reactive oxygen species (ROS), hydrogen peroxide, tumor necrosis factor (TNF) and other stress stimuli (12) playing a key role in the regulation of oxidative stress response (13).

HSP70 is an ATP-dependent chaperone that is induced by cellular stress and protects cells against various apoptotic stimuli. HSP70 mainly acts as stabilizer of multiprotein complexes and prevents the intracellular accumulation of misfolded or damaged proteins (14). In stress condition, HSP70 has an important role in cell cycle control, survival and inhibition of caspase-dependent and -independent

apoptosis. Recently, the small molecule pifithrin-μ was identified as a specific inhibitor of inducible HSP70 (15). Pifithrin-μ interferes with the carboxyterminal substrate-binding domain of inducible HSP70 and disrupts its association with client proteins (16).

Fish are an excellent vertebrate model to investigate the physiology, function and regulation of HSPs, because they are exposed to thermal and other stressors in their natural environment. The relationship between HSP synthesis and the development of thermotolerance has been studied by some investigators (17,18). The effects of daily and seasonal temperature fluctuations as well as acclimation temperature have also been examined, especially in fish species (19,20).

The grey mullet (*Mugil cephalus*) have several characteristics required in a sentinel species, such as wide geographic distribution; great abundance, salinity and temperature tolerance; they are common in coastal waters and enter lagoons, estuaries and rivers that are frequently subjected to pollution. This species were selected due to their different characteristics in terms of ecology, biology, trophic position in the food chain and feeding behaviour. Fish liver plays a major role in the uptake, biotransformation and excretion of pollutants (21). However, some metabolites produced are highly reactive and ultimately more toxic, affecting the structural integrity of DNA (22). Earlier we have demonstrated that there is a significant increase in the expression of HSP70 during pollution stress in mullet (2). We also hypothesised that enhancement of HSP70 may be the reason for increased viability and decreased ASK1 expression in hepatocytes under polluted condition. In this context, the present study aims to identifying the pro-apoptotic changes in terms of p-ERK and ASK1 expression when HSP70 is downregulated by the inhibitior pifithrin-μ.

2. Materials and Methods

2.1. Study Site

Kovalam and Ennore estuaries were chosen as the two study sites for the current research work. Kovalam estuary (12°47′16 N, 80°14′58 E) is situated on the east coast of India and is about 35 km south of Chennai. It runs parallel to the sea coast and extends to a distance of 20 km. It was chosen as the unpolluted site for the present investigation as it is surrounded by high vegetation and it is free from industrial or urban pollution. Ennore estuary (13°14′51 N, 80°19′31 E) also situated on the east coast of India, is about 15 km north of Chennai. It runs parallel to the sea coast and extends over a distance of 36 km. This estuary was chosen as the polluted site as in its immediate coastal neighbourhood are situated, a number of industries which include petrochemicals, fertilizers, pesticides, oil refineries, rubber factory and thermal power stations that discharge their effluents directly into this estuary. Contamination of this estuary by heavy metals like lead, cadmium, mercury, zinc and iron to a significant extent compared to unpolluted estuary has also been confirmed by previous studies (23,24). It has also been reported that Ennore estuary significantly differs from Kovalam estuary in its physical, chemical and biological factors (25), thus it has been chosen as the polluted site.

2.2 Study animal and sampling

M. cephalus, a natural inhabitant of the estuaries, identified by the use of Food and Agriculture Organization (FAO) species identification sheets (26). *M. cephalus* with an average length of 30-32 cm were collected from unpolluted and polluted estuaries using baited minnow traps. Collected fish was placed immediately into insulated containers filled with aerated estuarine water at ambient temperature (25-30°C) and salinity (24-29 ppt). Fish were maintained in the above specified conditions until the start of the experimental procedures. Fish were killed by severing the spinal cord, and the liver was removed immediately.

2.3 Isolation of hepatocytes

The isolation of hepatocytes was carried out according to established protocols (27,28) with slight modification as described by Padmini and Usha Rani (20). In brief, after the fish was anesthetized with a solution of ethyl m-aminobenzoate (MS-222; 0.5g/l of water), a midventral incision was made to expose the liver and the portal vein was cannulated in the direction of the liver. The liver was perfused with a perfusion buffer containing 290 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 10 mmol l^{-1} N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), 0.5 mmol l^{-1} ethylene glycol-bis (2-aminoethyl)-tetraacetic acid (EGTA), 25 mmol l^{-1} tricine, (pH 7.8), to remove red blood cells. Liver was then removed, perfused initially and then incubated in a cell suspension buffer (SB) (292.5 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 2.5 mmol l^{-1} $MgCl_2$, 3 mmol^{-1} $CaCl_2$, 2 mmol l^{-1} $NaHCO_3$, 2 mmol l^{-1} $NaHPO_4$, 5 mmol l^{-1} glucose and 50 mmol l^{-1} HEPES, pH 7.8) that contained 5 units ml^{-1} type IV collagenase (Sigma, USA) for 1 hour, to separate cells. Cells were sieved through 60 and 200 mm mesh screens and were then pelleted via centrifugation at 100 g for 10 minutes. The cells were then suspended in SB and allowed to recover for 1 hour. The cells were stored at -20°C prior to assay.

2.4 Cell viability assay

The cell viability of hepatocyte preparations was assessed using trypan blue staining (29). This dye exclusion polluted is used to determine the number of viable cells present in a cell suspension and is based on the principle that live cells possess intact cell membrane that exclude dyes such as trypan blue, whereas dead cells do not. In brief, suspension cells were harvested by centrifugation. An equal volume of 0.4% (w/v) trypan blue was added to a cell suspension at a concentration of approximately $1x10^6$ per ml. The cells were then incubated for 3 minutes and loaded onto a hemocytometer. Nonviable, deep blue cells as well as viable, clear cells were counted in three separate fields using bright field optics and the viability percentage was calculated by dividing the number of viable cells by the number of total cells and multiplying it by 100.

2.5 Protein preparation

Hepatocytes were harvested in cell suspension buffer, centrifuged, and resuspended in cell lysis buffer (20 mM Tris pH 7.5, 1% Triton X-100, 1 mM ethylenediamine

tetraacetic acid (EDTA), 1 mM ethylene glycol-bis (2-aminoethyl)-tetraacetic acid (EGTA), 1 mM phenylmethyl sulphonylfluoride (PMSF), 5 mM sodium pyrophosphate, 2 mM sodium orthovanadate and protease inhibitor). The cell suspension was incubated for 30 minutes at 4˚C, with occasional shaking or it was sonicated and centrifuged at 16,000xg for 10 minutes in a 4˚C refrigerated microfuge to remove the cellular debris. The supernatant was the cell lysate, whose protein concentration was determined by the classical method of Bradford (30) with coomassie brilliant blue G-250, using bovine serum albumin as a standard.

2.6 Estimation of total antioxidant capacity (TAC)

Total antioxidant capacity of hepatocytes of *M. cephalus* inhabiting Kovalam and Ennore estuaries was evaluated by the method described by Prieto *et al.* (31). 200µl of fish hepatocytes sample was treated with 3 ml of reagent mixture (0.6M H_2SO_4, 28mM NaH_2PO_4 and 4mM ammonium molybdate) and incubated at 95^{o}C for 90 minutes and then cooled. Absorbance was measured at 695 nm against blank. Methanol was used as blank. The total antioxidant activity was expressed as Trolox equivalent in mmol /L.

2.7 Quantification of HNE, HSP70, HO-1 and ASK1 using ELISA

The inducible form of 4-hydroxy-2-nonenal (HNE), HSP70, HO-1 and ASK1 in hepatocytes of *M. cephalus* inhabiting Kovalam and Ennore estuaries were quantified using HNE (MBS161454 96T, My Biosource, USA), HSP70 (MBS706016 96T, My Biosource, USA), HO-1 ELISA kit (ADI-EKS-800, Enzo Life Sciences, New York, USA) and ASK1 (E91358Hu 96T, Uscn Life Science, Inc, USA) according to the manufacturer's instructions.

2.8 Immunoblot analysis of phospho-ERK

Hepatocytes protein aliquots containing 50 µg proteins were ran on 10% SDS-polyacrylamide gels simultaneously. The gels were then blotted on to PVDF membranes (BioTrace PVDF 0.4 lm, Pall Corporation, Germany) according to the method of Towbin *et al.* (32). The antibodies used were anti-ERK1/2 (KAP-MA001) and anti β-actin (CSA-400), and followed by goat antimouse IgG secondary antibody treatment and color development was done using BCIP-NBT substrate system. The band intensities were scanned with the Hp Scan Imager and quantified using the TotalLab software, gels, USA. The results were confirmed by individually performing the blotting studies of these proteins.

2.9 Pifithrin-µ incubation study

Pifithrin-µ solution was prepared by dissolving pifithrin-µ (Sigma- Aldrich) in water. Then 200 µL of pifithrin-µ solution (10µM, 20µM and 30µM) was added to the respective sample tubes (containing 100µg of protein / 200µL of fish hepatocytes) and the tubes were incubated for 60 minutes, 90 minutes and 120 minutes at 18˚C with 5% CO_2 in the incubator. Following the incubation, cell viability was assessed in all the tubes and the effective concentration at the time having effective efficiency was utilized for the quantification of HSP70. Along with the analysis of HSP70 after inhibition with pifithrin-µ, cell viability, HNE, TAC, HO-1, phospho-ERK and ASK1 were assessed to study the cytoprotective function of HSP70.

2.10 Statistical analysis

Data were analyzed using statistical software package version (SPSS) 7.0. Student's t-test was used to ascertain the significance of variations between Kovalam and Ennore estuary fish hepatocytes. All data were presented as mean ± SD of 20 fish per estuary. Differences were considered significant at $p<0.05$, $p<0.01$ and $p<0.001$.

3. Results

3.1. Cell viability

The liver cells of *M. cephalus* collected from Ennore estuary showed decrease in their viability (75%) compared with hepatocytes isolated from Kovalam estuary (87%) **(Figure 1).**

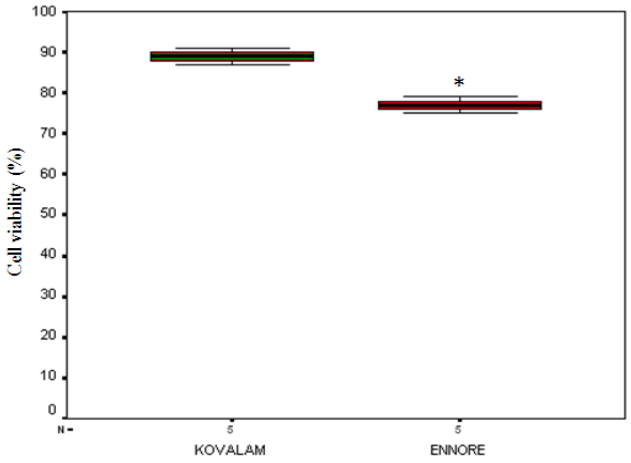

Figure 1: Cell viability of hepatocytes prepared from the livers of *M. cephalus* inhabiting Kovalam and Ennore estuaries. Values are expressed as mean ± SD (n=20 fish per estuary)
*$p<0.05$ When compared with hepatocytes of *M. cephalus* inhabiting Kovalam estuary

3.2. 4-Hydroxynonenal (HNE)

The level of oxidative stress was assessed by the measurement of HNE in hepatocytes. The expression of HNE (40%) was significantly increased in hepatocytes of *M. cephalus* inhabiting Ennore estuary compared to hepatocytes of *M. cephalus* inhabiting Kovalam estuary **(Figure 2a).**

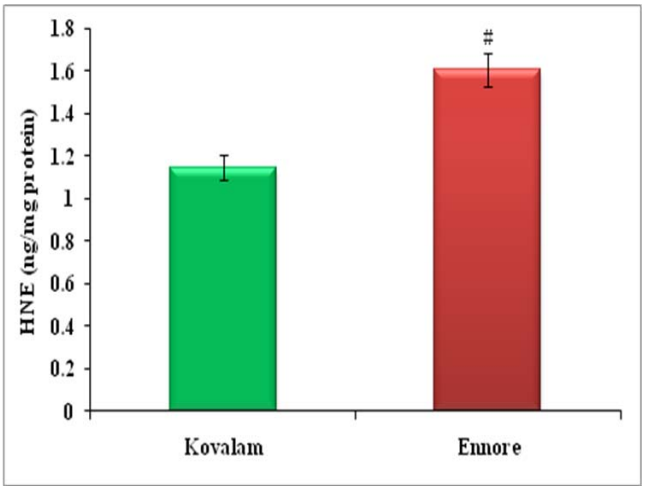

Figure 2a: 4-hydroxy-2-nonenal (HNE) level of hepatocytes prepared from the livers of *M. cephalus* inhabiting Kovalam and Ennore estuaries. Values are expressed as mean ± SD (n=20 fish per estuary).
#p<0.001 When compared with hepatocytes of *M. cephalus* inhabiting Kovalam estuary

3.3. Total antioxidant capacity (TAC)

Total antioxidant capacity was presented in fish hepatocytes inhabiting Kovalam and Ennore estuaries **(Figure 2b).** A significant decrease in the level of TAC (p<0.001) was observed in hepatocytes of *M. cephalus* inhabiting Ennore estuary (36%) compared with hepatocytes of *M. cephalus* inhabiting Kovalam estuary.

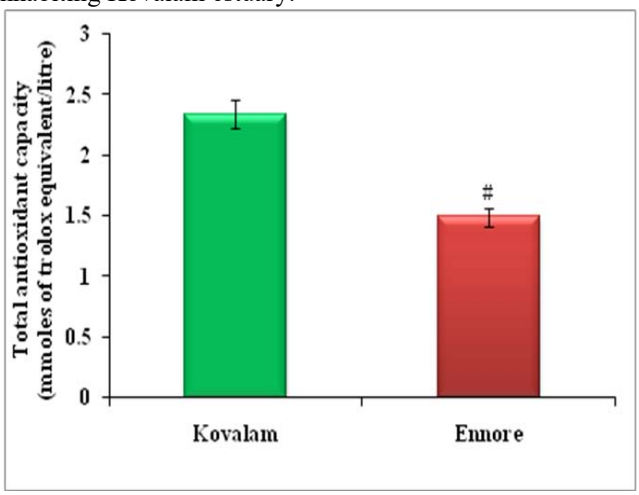

Figure 2b: Total antioxidant capacity in hepatocytes of *M. cephalus* inhabiting Kovalam and Ennore estuaries. Values are expressed as mean ± SD (n=20 fish per estuary)
#p<0.001 When compared with hepatocytes of *M. cephalus* inhabiting Kovalam estuary

3.4. Heat shock protein 70 (HSP70)

The expression of HSP70 (55%) was significantly enhanced in hepatocytes of *M. cephalus* inhabiting Ennore estuary compared to Kovalam estuary **(Figure 3a).**

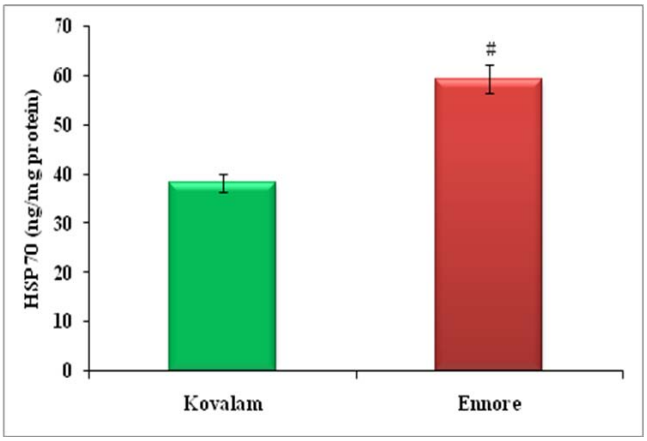

Figure 3a: Level of HSP70 in hepatocytes of *M. cephalus* inhabiting Kovalam and Ennore estuaries. Values are expressed as mean ± SD (n=20 fish per estuary)
#p<0.001 When compared with hepatocytes of *M. cephalus* inhabiting Kovalam estuary

3.5. Heme oxygenase-1 (HO-1)

The expression of HO-1 (98%) was significantly increased in hepatocytes of *M. cephalus* inhabiting Ennore estuary compared to Kovalam estuary **(Figure 3b).**

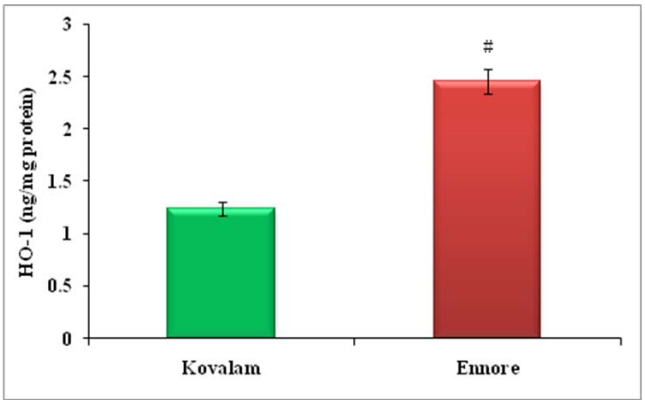

Figure 3b: Level of HO-1 in hepatocytes of *M. cephalus* inhabiting Kovalam and Ennore estuaries. Values are expressed as mean ± SD (n=20 fish per estuary)
#p<0.001 When compared with hepatocytes of *M. cephalus* inhabiting Kovalam estuary

3.6. Immunoblot analysis of phospho-ERK

Blotting analysis showed significant increase in the expression of p-ERK (p<0.01) in hepatocytes of *M. cephalus* inhabiting Ennore estuary compared with hepatocytes of *M. cephalus* inhabiting Kovalam estuary. The representative blots for proteins are given in **Figure 3c.**

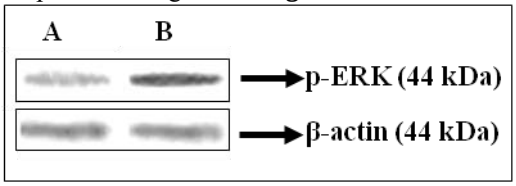

Figure 3c: Immunoblot analysis of phospho-ERK in hepatocytes of *M. cephalus* inhabiting Kovalam and Ennore estuaries. β-actin has been used as the loading control
A- Hepatocytes of *M. cephalus* inhabiting Kovalam estuary
B- Hepatocytes of *M. cephalus* inhabiting Ennore estuary

3.7. Apoptosis signal regulating kinase 1 (ASK1)

The expression of ASK1 (15%) was increased to a small extent in hepatocytes of *M. cephalus* inhabiting Ennore estuary compared to Kovalam estuary **(Figure 3d).**

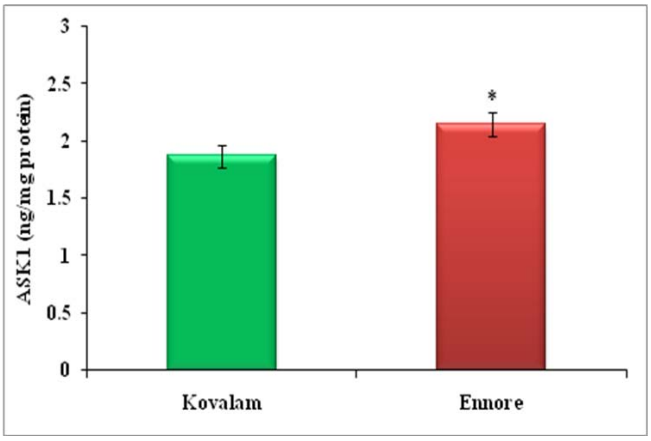

Figure 3d: Level of ASK1 in hepatocytes of *M. cephalus* inhabiting Kovalam and Ennore estuaries. Values are expressed as mean ± SD (n=20 fish per estuary)
*p<0.05 When compared with hepatocytes of *M. cephalus* inhabiting Kovalam estuary

3.8. HSP70 inhibition studies by pifithrin-μ

Treatment with pifithrin-μ decreased cell viability through induction of apoptosis in the hepatocytes of *M. cephalus.* The dose-effect curves showed a near demise of viable cells at concentrations between 10, 20 and 30 μM for 60 minutes, 90 minutes and 120 minutes **(Figure 4)**. Of the varying concentration at different time 10μM for 60mts was found to be effective by cell viability differences. Hence the effect of pifithrin-μ was further elucidated with the analysis of cell viability, HNE, TAC, HO-1, phospho-ERK and ASK1 after HSP70 inhibition. Level of HSP70 after inhibition with pifithrin-μ (HSP70 inhibitor) was decreased in the fish hepatocytes inhabiting Kovalam estuary by 16% (*p<0.05) and fish hepatocytes inhabiting Ennore estuary by 37% ($^{\#}$p<0.001). Inhibition of HSP70 with pifithrin-μ decreased the viability of fish hepatocytes inhabiting Kovalam estuary by 14% (*p<0.05) and fish hepatocytes inhabiting Ennore estuary by 21% ($^{@}$p<0.01). HSP70 inhibition mediated stress was assessed by increased HNE level in the fish hepatocytes inhabiting Kovalam estuary by 16% (*p<0.05) and fish hepatocytes inhabiting Ennore estuary by 53% ($^{\#}$p<0.001) with decreased TAC in the fish hepatocytes inhabiting Kovalam estuary by 18% (*p<0.05) and fish hepatocytes inhabiting Ennore estuary by 48% ($^{\#}$p<0.001). After HSP70 inhibition, the expression of HO-1 was decreased in the fish hepatocytes inhabiting Kovalam estuary by 18% (*p<0.05) and fish hepatocytes inhabiting Ennore estuary by 41% ($^{\#}$p<0.001). After HSP70 inhibition, the level of ASK1 was increased in the fish hepatocytes inhabiting Kovalam estuary by 16% (*p<0.05) and fish hepatocytes inhabiting Ennore estuary by 48% ($^{\#}$p<0.001). The present results demonstrate that the level of HSP70 was inhibited by 39% and this inhibition further decreases the cell viability by 7%, TAC by 23%, HO-1 by 56%, phospho-ERK and increases the level of HNE by 45% and ASK1 by 32% **(Figure 5a & 5b)**. These results indicate that the inhibition of HSP70 (39%) leads to cell death by increased expression of ASK1 (32%).

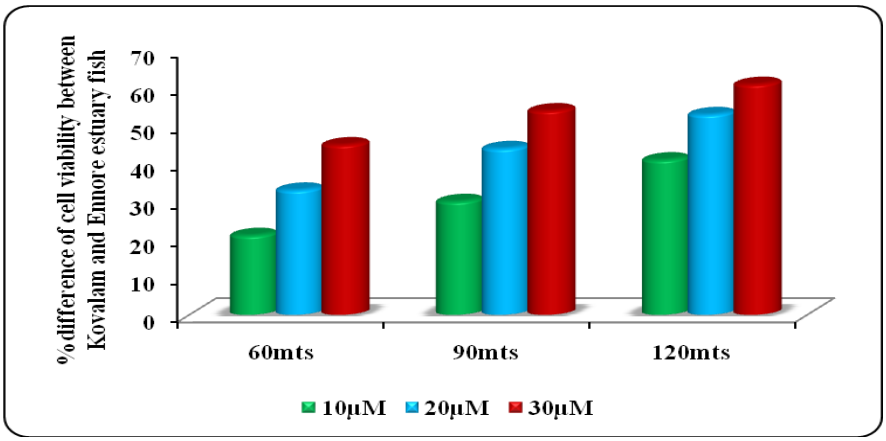

Figure 4: Effect of pifithrin-μ on cell viability at different time with varying concentrations in the hepatocytes of *M. cephalus* inhabiting Kovalam and Ennore estuaries. Values are expressed as % difference (n=20 fish per estuary)

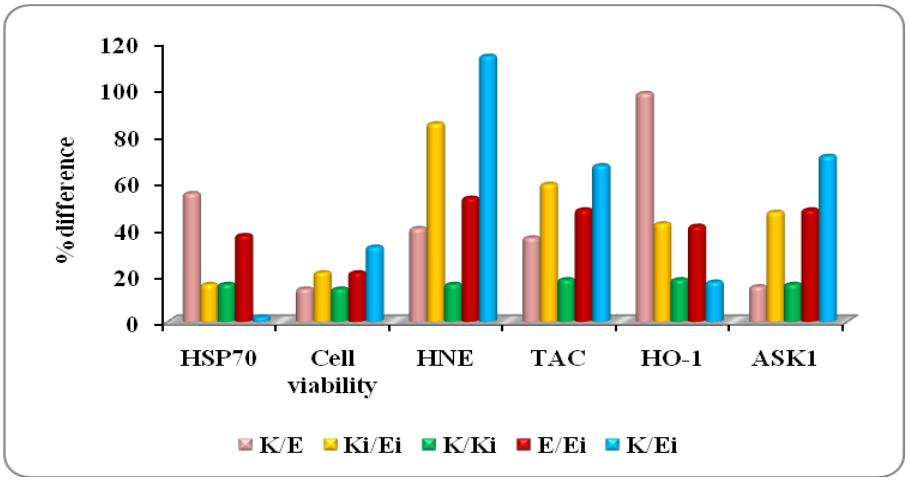

Figure 5a: HSP70 inhibition studies with pifithrin-μ and its effect on the level of cell viability, HNE, TAC, HSP70, HO-1 and ASK1 in the hepatocytes of *M. cephalus* inhabiting Kovalam and Ennore estuaries. Values are expressed as % difference (n=20 fish per estuary)

K- Hepatocytes of *Mugil cephalus* inhabiting Kovalam estuary
E- Hepatocytes of *Mugil cephalus* inhabiting Ennore estuary
Ki- Hepatocytes of *Mugil cephalus* inhabiting Kovalam estuary with pifithrin-μ
Ei- Hepatocytes of *Mugil cephalus* inhabiting Ennore estuary with pifithrin-μ

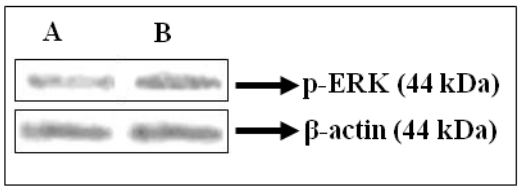

Figure 5b: Immunoblot analysis of phospho-ERK in hepatocytes of *M. cephalus* inhabiting Kovalam and Ennore estuaries with pifithrin-μ inhibitor. β-actin has been used as the loading control

A- Hepatocytes of *M. cephalus* inhabiting Kovalam estuary with pifithrin-μ inhibitor
B- Hepatocytes of *M. cephalus* inhabiting Ennore estuary with pifithrin-μ inhibitor

4. Discussion

Cells respond to stress in a variety of ways ranging from activation of pathways that promote survival to eliciting programmed cell death that eliminates damaged cells. The cell's initial response to a stressful stimulus is geared towards helping the cell to defend against and recover from the insult. The cell's survival critically depends on the ability to mount an appropriate response towards environmental or intracellular stress stimuli. Antioxidant defence mechanisms and stress proteins such as heat shock proteins play a vital role against such stress stimuli. There are many different types of stress and the response a cell deals with these conditions will depend on the type and level of the stress. Pollution and other stress response mediate an increase in chaperone protein activity, thus counteracting the stress and promoting cell survival.

The HSP70s are an important part of the cell's machinery for protein folding and help to protect cells from thermal or oxidative stress. HSP70 may act as survival protein under stress condition (20,33). It seems to act as an anti-apoptotic molecule at multiple points. HSP70 prevents apoptosis by inhibiting the JNK-signaling cascade (34,35,36). HSP70 has been established as a negative regulator of ASK1 (37). HSP70 inhibits apoptosis through either its chaperoning function or its binding activities to specific target molecules. Our previous study also demonstrated that the enhanced levels of HSP70 downregulates the expression of ASK1 in stressed fish hepatocytes (38).

MAPKs are typical signaling mediators that transmit intracellular signals initiated by extracellular stimuli to the nucleus. MAPK signaling regulates a variety of cellular activities, including cell growth, differentiation, survival, and cell death (39). The phosphorylation status of MAPK profoundly affects both cell fate and cellular function. ERK can be activated in response to oxidative stress and can play an important role in promoting cell survival (40). We have reported earlier the expression of ERK was increased in polluted site fish. It may protect cells against environmental pollutant related stress (41).

Cells that are constantly exposed to any stress results in the generation of reactive oxygen species (ROS) leading to oxidative stress (OS) which can compromise antioxidant defense, cellular function and survival. In case of moderate stress the inner balance is usually restored but under severe or prolonged stress conditions, the compensatory abilities of the organism may be exhausted which results in physiological disturbance or even death. The present result demonstrates that environmental pollutants have cytotoxic effects on aquatic organisms, significantly increasing stress, thereby decreasing cell viability by 14% in hepatocytes of fish from Ennore estuary (75%) compared to Kovalam estuary (87%).

Pifithrin-μ at the high concentration did not affect the intrinsic ATP hydrolysis rate of the chaperone. Leu *et al.* (15) demonstrated that pifithrin-μ interacts with HSP70 through the substrate binding domain. Schlecht *et al.* (42) showed that pifithrin-μ neither directly affects the affinity of HSP70 for peptide substrates, nor the kinetics of binding and

release, making an inhibitory mechanism mediated by the substrate binding domain. HSP70 is a critical co-chaperon for HSP90 and is involved in the delivery of client proteins to HSP90 (43) and HSP70 inhibition can induce tumor-specific apoptosis via HSP90 function (44). Recently, Pifithrin-μ, originally described as an inhibitor of p53-mediated apoptosis (45), was reported to promote cancer cell death by specifically inhibiting the heat-inducible HSP70 and its interactions with co-chaperones without affecting the constitutively expressed HSC70 (15). In our work, the expression of HSP70 was decreased significantly by 39% after pifithrin-μ incubation.

The enhanced cell survival is curtailed following HSP70 inhibition (46). Pifithrin-μ inhibits HSP70-induced stabilization of lysosomal membrane permeabilization, resulting in increased cell death (47). In our work, the cell viability was decreased after pifithrin-μ incubation. Decreased cell viability could be due to loss of membrane permeabilization as reprted by Sekihara *et al.* (47). Pifithrin-μ induces apoptosis through the direct inhibition of the stress-inducible HSP70 (48) and enhances the generation of ROS, possibly by inhibiting the anti-oxidant actions (49). In our study, ROS production peaked in one hour after the addition of pifithrin-μ, results indicates that pifithrin-μ leads to oxidative damage. The cellular stress in turn initiates the unfolded protein response, but this fails to restore cell homeostasis so proteins aggregate and cells die (49).

Cells respond to stressful stimuli inducing a set of heat shock (stress) proteins (HSPs) that play an important role in repair and protective mechanisms. HSP70 and HO-1 are well known markers of cell injury and oxidative stress. HO-1 is increased in response to a variety of cellular stresses and stimuli including ischemia, hypoxia, oxidative stress and inflammatory cytokines (50). Previous data suggested that the HO-1 mediated protective role might depend on the cellular milieu in terms of whether an increase of HO is beneficial or detrimental to the cell (51). Mautes and Noble (52) demonstrate that HSP70 is induced in microglia /macrophages that also express HO-1. In our study, the expression of HSP70 was inhibited by pifithrin-μ incubation and also the expression of HO-1 was decreased significantly by 56% due to the inhibition of HSP70 expression.

Pifithrin-μ causes caspase-dependent and -independent cell death of human pancreatic cancer cells (53). Pifithrin-μ mediated inhibition of HSP70 family proteins in tumor cells results in impairment of the autophagy-lysosome system. Pifithrin-μ impaired the mitochondrial localization of p53 (15). Ma *et al.* (54) reported that pifithrin-μ markedly suppressed the survival protein activities. Pifithrin-μ led to a remarkable loss of viability and decrease of intracellular AKT and ERK1/2 levels within a very narrow concentration range in leukemia cells (16). In our work, the expression of ASK1 was significantly increased along with the expression of prosurvival kinase p-ERK was significantly decreased in the fish hepatocytes after pifithrin-μ incubation. The data suggests that pifithrin-μ leads to apoptosis by the inhibitory effect of HSP70. Pifithrin-μ can be useful for studies a cellular survival pathway mediated by HSP70 in fish. Results observed on HSP70 inhibition by pifithrin-μ emphasize that HSP70 is crucial for cell survival during environmental contaminants induced stress condition **(Figure 6)**.

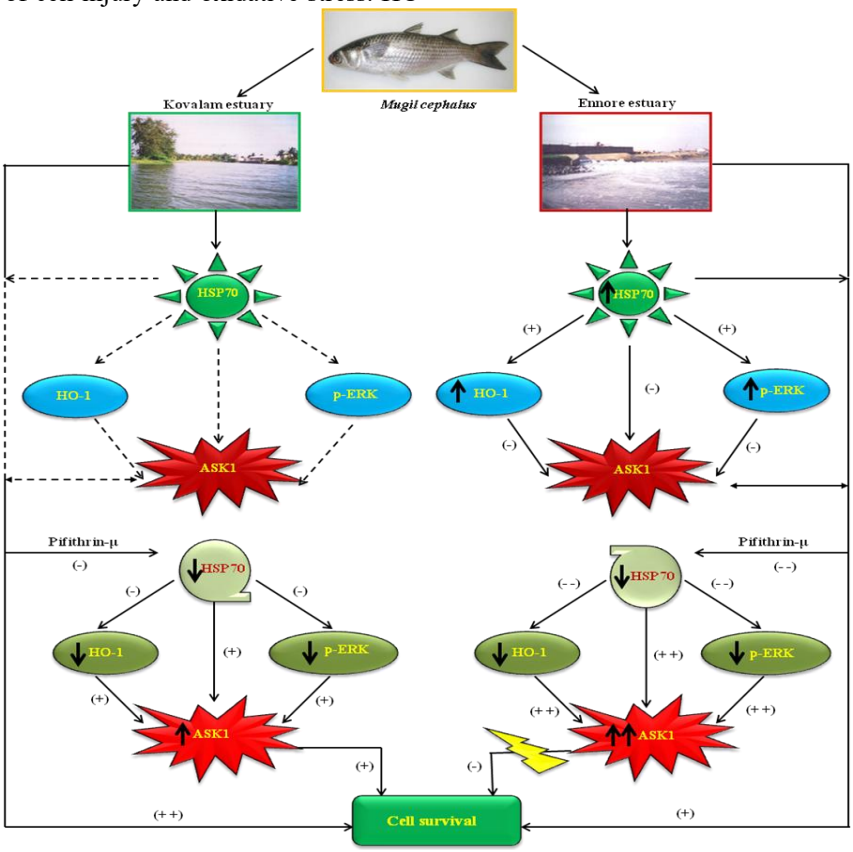

Figure 6: Schematic diagram illustrates the role of HSP70 in the mechanisms of cellular survival. HSP70 induction in response to environmental pollutant-mediated oxidative stress promotes the activation of p-ERK. p-ERK activation under similar conditions promotes cellular survival. HSP70 downregulates ASK1 expression allows cell survival under stress

condition. HSP70 downregulation by pifithrin-μ enhance the ASK1 expression allows cell death. Thus HSP70 plays important antiapoptotic role against ASK1 mediated apoptotic signaling.

5. Conclusion

In this study we investigated how hepatocytes respond to pollutants induced stress condition in terms of HSP70 expression and associated signaling molecules. HSP70 exhibits crucial functions in the maintenance of cell homeostasis. HSP70 is generally considered to be an anti-apoptotic molecule and modulation in its activity offers a great possibility to influence protein homeostasis and cell survival. In addition, we report for the first time that the inhibitory effect of pifithrin-μ in stressed fish hepatocytes. An alteration in the expression of HSP70 by pifithrin-μ causes downregulation of ERK and upregulation of ASK1 expression. These proapoptotic changes are the significant contributors to apoptosis. Therefore, the current results suggest that HSP70 threshold may exert anti-apoptotic effects in fish survival under pollutants induced stress condition.

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7. Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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