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Role of Hsp70 and the Associated Signaling Molecules in Fish Adipocytes during Pollutants Induced Hypoxia

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Abstract: *Adipose tissue is highly specialized to store lipid and/or release energy from lipid stores in response to a variety of signals. Adipose remodeling is the critical physiological process which involves lipogenesis and lipolysis to exhibit energy homeostasis. Recently, interest in the study of fish adipocytes has burgeoned due to the rising incidence of polluted environment. Determination of changes in adipocyte during stress provides the underlying mechanism involved in the survival process and adipose remodeling. Hence the present study was undertaken to evaluate the stress markers such as LHP and PC along with FA in fish adipocytes. Redox status was assessed by GPX, GST, GR, GRR and Trx. Alterations in structural contour and functional groups were identified by SEM and FTIR respectively. Expression of apoptotic and antiapoptotic proteins were monitored by analyzing HIF1α, TNFα, HSP70 and ASK1. Significance of HSP70 was studied with the novel HSP70 inhibitor Pifithrin-μ. Result of current research work depicts increased oxidative damage as assessed by elevated LHP, PC, HIF1α, TNFα, ASK1 and FA along with the reduction in level of GPX, GST, GR, GRR. However increased Trx expression was noted in stressed fish adipocyte. SEM and FTIR spectroscopy results demonstrated variations in structural morphology and thiol status. However adipocytes are functional which may be due to increased HSP70 in stressed fish adipocyte which was substantiated by its inhibition and its mediated alteration of stress markers, along with induction of ASK1. Present results proclaim the importance of HSP70 in adipocytes survival and maturation during pollutants induced hypoxia.*

Keywords: *Adipocytes, Antiapoptotic, Hypoxia, Heat shock protein 70, Lipogenesis*

I. Introduction

Adipocytes are energy reservoirs functioning by hoarding excess energy in the form of TG which are mobilized during periods of nutrient deprivation (Albalat et al. 2007). Adipogenesis is a multi-step process involving regulation of gene expression by the cascade of transcription factors and signaling proteins which leads to adipocyte development. Proliferation and differentiation (maturation) are key process of adipogenesis. Adipocyte maturation is a complex developmental process of converting preadipocytes into mature adipocytes which involves a highly orchestrated program of gene expression. Hence understanding the molecular mechanisms underlying adipocyte maturation is important for the analysis of adaptive strategy. Current research work deals with the analysis of grey mullet's (Scientific name: *Mugil cephalus*/ *M.cephalus*) adipocytes from unpolluted and polluted origin which is a valuable indicator of pollutants mediated OS damage in estuarine systems (Mansour and Sidky 2003).

Oxidant stress in adipocyte was considered to be key mediator of adipocyte dysfunction. Lipid peroxidation is the chief consequence of OS which resulted in LHP, a prominent non-radical intermediate. LHP regulates enzymes and redox-sensitive genes in normal pattern (Sen and Packer 1996). However, its uncontrolled production may result in the disruption of cellular metabolism (Halliwell and Chirico 1993). PC is also a widely accepted OS biomarker, which involves the formation of carbonyl moieties (-C=O) at amino acid side chains (Grune 2000) and its increase in the number correlates well to protein damage (Shacter 1994). Radical species are kept in check by a system of enzymatic antioxidants such as GPX, GST, GR and non enzymatic antioxidants such as GRR, Trx. GPX catalyzes the reduction of H_2O_2 to water with simultaneous conversion of GSH to GSSG (Meister 1994). GSSG is restored to a reduced form by GR and NADPH (Salinas and Wong 1999). GST is involved in the cellular detoxification and its activity is dependent upon GSH. Redox state plays a critical protective and adaptive role (Aispuro-Hernandez et al. 2008). Of that GSH plays a vital role in adipocyte formation which is required for maintenance of adipose mass (Schneider and Chan 2013) whereas Trx is crucial for the regulation of ASK1 (Holmgren 1985; Berk 2008). OS may produce disparate effects on adipocyte metabolism depending upon the intensity of ROS generation and antioxidant requirement. However its effect on fish adipocyte during pollutants induced hypoxia is investigated for the first time which will provide a novel insight into the impression of ROS on adipocyte homeostasis.

As the quantitative analysis of stress induced changes were executed in one way qualitative analysis was done by FTIR Spectroscopy. It measures the transitions in vibrational modes (mainly stretching and bending) of the functional groups of biomolecular constituents within cells, as a result of absorption and subsequent excitation by infrared (IR) radiation in the IR region between 4,000 and 400 cm-1 where each functional group in a molecule has characteristic absorption frequencies in the IR spectrum (Wei 2013; Gazi etal. 2007). FTIR Spectroscopy is used to analyze relevant amount of compositional and structural information (Grube 2008) to examine the initial response to environmental stress (Padmini and Tharani 2014 a, b).

Any alteration in the OS mediated thiol status imbalance results in the development of hypoxic environment which in-turn may promote cell survival or cell death. HIF1α is one of the key regulators of cellular and systemic oxygen homeostasis and plays a crucial role in switching on the hypoxia responsive genes at the transcriptional level to facilitate oxygen uptake or delivery. HIF1α is a constitutively expressed gene under normoxic conditions whereas it is upregulated during hypoxic conditions (Semenza 2000; Semenza 2012). It's alteration further initiates expression of a wide variety of genes, some of which are involved in apoptosis and cell cycle arrest. HIF1α is a candidate biomarker for deciphering the development of cellular adaptation to low oxygen stress (Annelies et al. 2007).

Hypoxia due to increased ROS products leads to the induction of several signaling molecules such as TNFα, ASK1 and HSPs (Chandel et al. 2000). TNFα, a proinflammatory cytokine (multifunctional signaling protein) exerts its biological activity by signaling via its receptors, which activates NFκB or MAPK pathway to induce cell survival/ cell death (Kawasaki et al. 2002). Apart from its role in oxidative state, TNFα has been demonstrated to regulate or interfere with adipocyte metabolism at numerous sites including transcriptional regulation, glucose, FA metabolism and hormone receptor signaling tissue (Sethi and Hotamisligil 1999). Hence it serves to alter almost every aspect of adipose system biology.

Apoptosis signaling kinase1 (ASK1) is the most extensively studied signaling molecule of apoptosis regulating pathway which is controlling a variety of cellular functions, including cell proliferation, survival, differentiation and inflammatory response. ASK1 is also known as mitogen activated protein kinase kinase kinase (MAPKKKs) is activated in response to proinflammatory and various other stress signals. Under nonstressed condition, ASK1 is sequestered by Trx in an inactive form (Saitoh et al. 1998) whereas its dissociation leads to apoptotic changes through the downstream signaling mechanism (Nadeau et al. 2007).

In contrast to oxidative state induction, environmental stressors also induce the cytoprotective function relayed by heat shock proteins, which protects the cell from FRIOS and further cytotoxicity (Guo et al. 2007). Heat shock protein 70 (HSP70) is often used as an early cytoprotective protein marker. Acting as molecular chaperones, it plays a vital role in mediating protein folding, assembly, transport, and degradation (Balch et al. 2008). It is often described as stress inducible HSP70 due to its differential expression (Morimoto 1993). Further the study determines the cytoprotective function of HSP70 by its inhibition with "Pifithrin-μ" a novel HSP70 inhibitor which induces apoptosis through the inhibition of P53 and also it acts as a direct inhibitor of the stress-inducible HSP70 (Leu et al. 2011). Present study is an effort to reveal a proficient aspect of HSP70 in adipocyte survival by pifithrin-μ incubation as adipocytes are crucial for control of metabolic homeostasis.

In this regard, the present study in our laboratory examined cytotoxic effect of environmental pollutants by analyzing cell viability and detection of structural contour by scanning electron microscopy along with the examination of its ultimate effect on cell signaling proteins.

II. Materials And Methods

1.1. Study site and study animal sampling

Kovalam and Ennore estuaries are situated on the east coast of India; chosen as the study sites. Kovalam estuary has been chosen as the control (unpolluted) site as it is surrounded by high vegetation and is free from industrial or urban pollution. Ennore estuary it has been chosen as the test (polluted) site, which receives untreated sewage from untreated/treated industrial effluents. Contamination of this estuary by heavy metals has already been confirmed by previous studies and the physical, chemical, biological characteristics were found to be differing among both estuaries (Padmini and Vijayageetha 2007a,b; Shirlin et al. 2014). Grey mullet with an average length of 30-32 cm were collected from control and test 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).

1.2. Isolation of adipocytes

Adipose tissue (subcutaneous) was carefully removed then it was washed with distilled water and the adipocytes were prepared by the method of Rodbell (1964). Adipose tissue was cut into small pieces and incubated in polypropylene tubes with isosmotic Krebs's buffer (pH 7.4, 280 mM) containing collagenase type II (0.3 mg/ml) and 1% BSA without glucose for 60 min in a water bath under gentle shaking at 15°C. The cell suspension was filtered through a double layer of nylon cloth and then washed three times by flotation. Finally,

floating cells (mature adipocytes) in the supernatant were carefully removed as it contains mature adipocytes and used. Pellet contains the pre-adipocytes (immature), fibroblast cells and cell debris were discarded. Following the adipocytes isolation, cell viability was assessed by Trypan blue staining (Strober 2001).

1.3. Protein and FA estimation

Protein concentration was determined by the Bradford method (1976). The protein concentration was expressed as mg protein/g of adipocytes. FA was estimated in both adipocytes by enzymatic colorimetric method and expressed as µg/g tissue.

1.4. Scanning electron microscopy

The control and test adipocytes were diluted to a concentration of 1:100 with PBS. The cells were then fixed with 4% glutaraldehyde overnight at 4°C followed by centrifugation at 100 g for 5 min. The supernatant was discarded and the pellet was resuspended in 1% osmium tetroxide prepared in 0.1M PBS for 2 h at room temperature. The centrifugation process was repeated and the samples were dehydrated with an ascending ethanol series (10–100%). The absolute ethanol was finally displaced by liquid carbon dioxide which served as the transitional fluid for critical point drying. Dried samples were mounted on aluminium stubs and sputter coated with gold for 60 seconds (Hitachi, E1010, Europe). Electron accelerators for SEM were operated at 15 kV and the samples were viewed with a field emission scanning electron microscope. Electron accelerators for field emission SEM were operated at 10 kV.

1.5. Estimation of LHP and PC

LHP was estimated by the method of Jiang et al. 1992. The values were expressed as µm of H_2O_2/mg protein. PC content of adipocytes was estimated according to the method of Levine et al. 1990. The results were expressed in terms of picomoles/mg protein

1.6. Assay of GPX, GST, GR and GRR

Activity of GPX was estimated according to the method of Rotruck et al. 1973. The activity of GPX was expressed as GSH consumed in nmoles / minute/ mg protein. The activity of GST was assayed by the method of Habig et al. 1974. The activity of GST was expressed as nmoles of CDNB conjugates formed/ minute/ mg protein. GR activity was assessed by the method of Aceto et al. 1990. The activity of GR was expressed as nmoles of NADPH oxidised/ minute/ mg protein. The thiol status (GRR) was assessed by spectrofluorimetry using the method of Hissin and Hilf 1976.

1.7. Western immunoblotting analysis

Aliquots of the sample containing optimal protein concentration were run on 10% SDS-polyacrylamide gel. Gel was run on a electrophoretic system and was then be blotted on to PVDF membrane (Biotrace, Germany) according to the method of Towbin et al. 1979 after which the blotted membrane was blocked specifically. The blot was then be washed thoroughly and cut into two equal halves. One part of the blotted membrane was incubated with antibody against Trx (mouse monoclonal (MSA-150); 1:1000 dilution), (Stressgen Bioreagents, Victoria, BC, Canada) and TNFα (mouse monoclonal antibody; MAB-4F11) (1:2000 dilution; BPD-HYB-141-08) along with an anti-β-actin antibody (rabbit polyclonal (CSA- 400); 1:1000 dilution) being used to assess equal loading for overnight at 4°C for optimal time duration. The membrane was incubated with appropriately diluted alkaline phosphatase labeled IgG secondary antibody prepared in TBS-Tween buffer. The 3-bromo-4-chloro-indolylphosphate- nitroblue tetrazolium (BCIP-NBT) substrate system was used to detect the alkaline phosphatase conjugate of all the blots separately. The band intensities was quantified using TOTAL-LAB software.

1.8. FTIR spectroscopic analysis

The isolated adipocyte and potassium bromide (all dry solid state) were lyophilized in order to remove most bound water, which might interfere with the measurement of amide I, band. Sample was mixed with dried Kbr and subjected to a pressure of 5×106 Pa and made into a clear pellet of 13 mm diameter and 1 mm thickness. The spectrometer was continuously purged with dry nitrogen. The absorption intensity of the peak was calculated using the base line method. Each observation was confirmed by taking at least three replicates. The spectra were recorded in the range of 4000-400 cm-1 using FTIR (PerkinElmer FTIR Spectrometer RX I).

1.9. Quantification of HIF1α, ASK1 and HSP70 using ELISA kit

HIF1α, ASK1 and HSP70 in adipocytes was quantified using ELISA kit (ABIN366532, 96 T, Antibody online and USA), (E91358Hu 96 T, Uscn Life Science Inc, USA), (EKS-700B, Stressgen, Canada) according to the manufacturer's instruction.

1.10. Pifithrin-μ incubation studies

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 sample (both control and test)) and the tubes were incubated for 60 min, 90 min and 120 min in the incubator at temperature 18^{o} C with 5% CO_2. Following the incubation cell viability was assessed in all the tubes. The effective concentration at the time having effective efficiency in altering cell viability was utilized for the quantification of HSP70 to demonstrate the effect of pifithrin-μ on HSP70 inhibition. Simultaneously effect of HSP70 inhibition mediated changes in LHP, GRR and ASK1 was monitored to study the protective function of HSP70.

1.11. Statistical Analysis

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

III. Results

Table 1

It depicts decreased ($p<0.05$) cell viability (17%), significantly decreased ($p<0.001$) protein concentration (71%) and increased ($p<0.001$) FA level (1.8 fold) in test adipocytes when compared to control adipocytes. It also exhibits OS markers such as increased LHP (45%) ($p<0.001$), PC (25%) ($p<0.01$) and level of antioxidants such as decreased GPX ($p<0.001$) (40%), GST ($p<0.01$) (25%), GR ($p<0.001$) (36%) and GRR ($p<0.001$) (23%) in test fish adipocytes.

Scanning electron microscopy

Fig 1: presents the scanning electron microscopic image of control and test fish adipocytes. In this figure, panels A and B demonstrate spherical shape of adipocytes (PanelA) with regular surfaces, smooth membranes, characteristics arrangement of some areas (Panel B). Panels C and D demonstrate the signs of swelling, the surface lesions, alteration in membrane structures, shape of the cells and liquefaction (Panel C and D), disturbance in adipocyte cytoskeleton and surface bleb formation.

Figure 2: Fig 2: depicts the increased expression of Trx in test fish adipocytes as compared to control.

FTIR spectroscopic analysis

Comparative FTIR spectra of control and test adipocytes of M. cephalus inhabiting Kovalam and Ennore estuaries were noted in **Fig 3** and the assignment of absorption band of FTIR spectral wave number of test adipocytes of M. cephalus were presented in **table 2**. The spectrum of test adipocytes vary from the control adipocytes in varying functional groups of protein, lipids and other biomolecules

Expression of HIF1α: Fig 4 shows the expression of HIF1α ($p<0.001$) (38%) in test fish adipocytes.

Immunohistochemical expression: Fig 5 shows the increased expression of TNFα in test fish adipocytes when compared to control fish adipocytes.

Signaling proteins: Fig 6 & 7 shows differential expression of ASK1 ($p<0.05$) (20%) and HSP70 ($p<0.001$) (46%) in test fish adipocytes.

Pifithrin-μ incubation studies

Fig 8 exhibits the effect of pifithrin-μ on cell viability. Of the varying concentrations (10μM, 20μM and 30μM) at different time incubation (60 min, 90 min and 120 min) 10μM for 60 min was found to be effectively decreased the cell viability. **Fig 9** represents the % difference of 10μM pifithrin-μ activity for 60 min on HSP70, cell viability, LHP, GRR and ASK1. Level of HSP70 after incubation with pifithrin-μ was decreased in control adipocytes by 11 % ($p<0.05$) and test adipocytes by 30% ($p<0.001$). Inhibition of HSP70 by incubation with pifithrin-μ decreased the cell viability in control (14%) ($p<0.05$) and test adipocytes (22%) ($p<0.01$). Whereas it increased LHP level in control (19.8%) ($p<0.05$) and test adipocytes (35%) ($p<0.001$) with decreased GRR level in control (23%) ($p<0.01$) and test adipocytes (41%) ($p<0.001$). ASK1 level was increased in control (17%) ($p<0.05$) and test adipocytes (40%) ($p<0.001$) after HSP70 inhibition. Further the results showed pifithrin-μ inhibits HSP70 by 31%, decreases cell viability by 5%, GRR by 21.5%, increases LHP by 18% and ASK1 by 23%.

IV. Discussion

Adipogenesis is a multistep process which involves the regulation of diverge signaling proteins. Current study renders the better understanding of signaling proteins role and its effect on adipocyte maturation during pollution induced hypoxic condition. Decreased cell viability in test fish adipocytes when compared to control adipocytes exhibits the cytotoxic effects of environmental pollutants on grey mullet's adipocytes. In the present work, mature adipocytes of both estuarine fish were analyzed which depicts the effect of pollution induced stress on adipocyte maturation. Significantly decreased protein concentration and increased FA level was observed in test adipocytes than control adipocytes. It suggests the occurrence of diversification of energy to accomplish the impending energy demands during exposure to xenobiotics. Depleted FA demonstrated the suppression of lipogenesis and its effect on the adipocyte metabolism. During adipogenesis, adipocytes acquire maximum FA for TG synthesis and further lead to lipogenesis (Jennifer et al. 2011). However in the present work incoherently FA level was increased depicting that it might be due to degradation of TG as demonstrated in the previous study (Padmini and Parimala 2014) which also clearly demonstrated the effect of pollution on adipocyte maturation.

Evaluation of toxicant-induced changes in cells by SEM revealed the cell structural disturbance in test fish adipocytes. Regular surfaces with grain like processes, smooth membranes and characteristic arrangement were observed in control fish adipocytes (A & B). However, the signs of swelling, surface lesions, alteration in membrane structures and shape of the cells, disturbance in adipocytes cytoskeleton along with the lipofection were demonstrated in test fish adipocytes (C & D), indicating the impact of pollutant stress on the morphology of the adipocytes. Consistent with the present findings, cytoskeletal disruption and surface blebs formation have been demonstrated under various stress conditions (Neira et al. 2002). Results provide direct evidence that pollutants such as existence of heavy metals and polycyclic aromatic hydrocarbons (PAH) in Ennore estuary may be the causative factor for the distruption in lipogenesis (Bennion and Grundy 1975; Vijayavel et al. 2006; Shirlin et al. 2014).

Increased level of OS markers such as LHP, PC and decreased level of antioxidants such as GPX, GST, GR and GRR were observed in test fish adipocytes. Increased LHP and PC in test adipocytes suggests the prevalence in oxidation of FA and protein (Bagnyukova et al. 2006; Craig et al. 2007) due to the diminution of lipogenic process (Huypens et al. 2005). Normally, these radical species are kept in check by a system of antioxidants. However in the present study suppression of GPX, GR, GST and GRR was observed suggesting the loss of antioxidant status (Mattia et al. 1994) due to the elevated OS (Lee et al. 2008). However increased expression of Trx was observed in test fish adipocytes and demonstrating its role in regulating ASK1 which further suggests the independent role of GSH and Trx during pollution induced hypoxic stress condition.

FTIR spectra of control and test adipocytes of M. cephalus inhabiting Kovalam and Ennore estuaries were noted and the assignment of absorption band of FTIR spectral wave number of test adipocytes of M. cephalus were presented in table 2. Qualitative analysis of stress induced changes using FTIR spectroscopic analysis (Fig 3 and Table 2) showed altered stretching in OH, N-H, C=O and C-C bond in test fish adipocytes. The C-H stretching in the region of ~2950cm-1 depicts the disturbance of lipid molecules along with increased glycerol absorption which was observed in the region below ~1500cm-1 in test adipocytes. The C=O (N-H) amide stretching in the region of ~1636, ~1646, ~1653 depicts the proteins disturbance. Increased LHP was observed in test adipocytes based on the absorption in the region of ~3458cm^{-1} (Fig 3 and Table 2). FTIR spectra showed increased S-H (thiol absorption) in the region of ~2569cm-1 in control fish adipocytes whereas increased disulfide was observed in test adipocytes which is depicted by the peak at the region of ~529cm-1. Overall, the spectrum of control and test fish adipocytes differ in the shape of absorbance curve, indicating obvious changes in structure and contents of biological components due to stress.

Prevalence of adipocyte hypoxia due to pollution induced stress was depicted by elevated HIF1α in stressed fish adipocytes. Similarly, Gracey et al. (2011) has reported the up-regulated HIF1α expression in Gillichthys mirabilis during hypoxia and an induction of HIF1α mRNA has been also reported in a hypoxia-challenged species of bream fish (Shen et al. 2010) suggesting HIF1α serves as the efficient biomarker of hypoxia. It activates a broad range of genes to protect cells against hypoxia (Miyata et al. 2011; Alvarez-Ordonez et al. 2010) which was proved by upregulated HSP70 as observed in the present work. Apart from its regular role in stimulating survival proteins, it also mediates repression of lipogenic proteins which often results in limitation of adipogenesis (Hemmrich and Thomas 2007). It was well substantiated in the present work where enhanced expression of HIF1α and its subsequent effect on adipocyte maturation was noted; which was elucidated clear from the decreased mature adipocytes in stressed condition.

Increased expression of TNFα in test fish adipocytes when compared to control fish adipocytes confirms the anti-lipogenic effect of TNFα. HIF1α is known to induce TNFα (Grosfeld et al. 2002) and its enhancement inhibits adipocytes maturation by decreasing lipid accumulation (Hemmrich and Thomas 2007). Similarly in the present work HIF1α and TNFα were increased suggesting distruption in adipocyte maturation.

However there is an existence of possible adipogenesis process in stressed fish adipocytes which might be due to the potential role of HSP70 in regulating TNFα (Gao et al. 2010).

Comparatively increased ASK1 level was encountered in test fish adipocytes suggesting that ASK1 is regulated through HIF1α and TNFα during pollution induced hypoxic stress. However it was insignificant suggesting decisive role of Trx in regulating ASK1 expression (Liu et al. 2000; Padmini and Usha Rani 2009). It is ultimately depicting that Trx is under the control of HSP70 (Padmini and Vijaya Geetha 2009).

HSP70 is known for its cytoprotective role and it is well confirmed by our previous studies on fish hepatocytes during environmental pollution stress (Zeng 2014). The role of HSP70 in adipocytes during stress has seldom been demonstrated. Expression of HSP70 is crucial in the regulation of adipocytes metabolism (Gomez et al. 2007). Present work for the first time describes the role of HSP70 in adipocytes which was achieved by its increased expression ($p<0.001$) (46%) (Fig 7) observed in stressed fish adipocytes. The results predict the cytoprotective role of HSP70 in adipocyte, which was due to its role in modulating lipolytic proteins. The cytoprotective function of HSP70 was further substantiated with the pifithrin-μ incubation studies exhibiting the effect of different concentration of pifithrin-μ on cell viability at different time. Pifithrin-μ was known to enhance the generation of ROS, possibly by inhibiting the anti-oxidant actions (Zeng et al. 2014). It is coherent to the results ascertained in the present study where decreased cell viability, GRR, HSP70 along with increased LHP and ASK1 level during HSP70 inhibition influenced by pifithrin-μ was noted. Results observed on HSP70 inhibition by pifithrin-μ emphasize that HSP70 is crucial in the process of adipocytes survival during pollution induced hypoxic condition. In stressed fish adipocyte, before incubation with pifithrin-μ HSP70 was upregulated along with HIF1α, TNFα and ASK1 at moderately significant level. However pifithrin-μ incubation increases the expression of ASK1 in drastic manner, with severe OS enhancement and loss of thiol status. Pifithrin-μ incubation also decreased the cell viability of both control and test adipocytes but its effect was extreme in test fish adipocyte demonstrating the crucial role of HSP70 in cytoprotective mechanism.

Elevated OS, declined antioxidant status, upregulated HIF1α, TNFα and ASK1 were observed in stressed fish adipocyte, which is demonstrating the cytotoxic effect evoked by the environmental pollutants. It indirectly describes the effect on adipocytes maturation as mature adipocytes were analyzed in the present work. Disrupted adipocyte homeostasis was observed by elevated lipolytic mechanism as assessed through increased FA and TNFα. However, the fish are surviving in the polluted estuary which might be due to the adaptive strategy acquired by the fish. In the present study we confirm that augmentation of HSP70 may contribute to the adipocytes survival and adipocytes maturation by altering anti adipogenic proteins such as HIF1α, TNFα and ASK1. Further the cytoprotective role of HSP70 was substantiated by pifithrin-μ incubation. From this, our work for the first time demonstrates the HSP70 role in imparting adipocytes survival and adipose remodeling during pollutants induced hypoxia.

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Conflict of Interest

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

Abbreviations:

AO - Antioxidant
ASK1 - Apoptosis signal regulating kinase 1
FA - Fatty acid
FRIOS - Free radical induced oxidative stress
FTIR - Fourier transform infrared spectroscopy
GPX - Glutathione peroxidase
GR - Glutathione reductase
GRR - Glutathione redox ratio
GST - Glutathione S transferase
HIF1α - Hypoxia inducible factor α
HSP70 - Heat shock protein 70
IHC - Immunohistochemistry
LHP - Lipid hydroperoxide
OS - oxidative stress
PC - Protein carbonyl
ROS - Reactive oxygen species

SEM - Scanning electron microscopy
TNF α - Tumor necrosis factor α
Trx - Thioredoxin

Reference

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Table 1: Cell viability, level of protein, FA, oxidants and antioxidants in control and test adipocytes of *M. cephalus*. Values are expressed as means ± SD (n=20 fish per estuary)

S.No	Parameters	Control adipocytes	Test adipocytes
1	Cell viability (%)	95±0.76	79±0.57*
2	Level of Protein (mg/g tissue)	17±0.09	5±0.02$^{@}$
3	Level of FA (µg/g tissue)	151.3±0.87	435±0.98$^{@}$
4	LHP level (µm of H_2O_2/mg protein)	43.4±0.53	63.1±0.61$^{@}$
5	Level of Protein carbonyls (picomoles/mg protein)	102±0.92	128±0.99$^{\#}$
6	GPX activity (nmoles of GSH consumed/ min/ mg protein)	25±0.22	15±0.09$^{@}$
7	GST activity (nmoles of CDNB conjugates formed/ min/ mg protein)	800±0.95	600±0.57$^{\#}$
8	GR activity (nmoles of NADPH oxidised/ min/ mg protein)	58±0.69	37±0.23$^{@}$
9	GRR (%)	3.5±0.05	2.70±0.03$^{\#}$

*$p<0.05$ decreased when compared to control fish adipocytes
$^{@}p<0.001$ highly significant when compared to control fish adipocytes
$^{\#}p<0.01$ significant when compared to control fish adipocytes

Table 2: Assignment of absorption band of FTIR spectral wave number of test *M. cephalus* adipocytes

S.No	Wave number (cm^{-1})	Functional Group	Component
1	~3322, ~3333, ~3367, ~3458	OH (stretch)	Alcohols or phenols
2	~2950	C-H (stretch)	Lipids
3	~2569	S-H	Thiols
4	~2091	C=O	Esters (Lipids)
5	~2355, ~2343, ~2357	C≡N	Nitriles
6	~1646, ~1636, ~ 1653	C=O (N-H)	Amide I and II
7	~1404	O-H (C-H bending)	Carboxylic acid
8	~1239	C-N (stretch)	Amine
9	~1085	C-O	Ester
10	~529	S-S	Disulfide

Fig 1: Scanning electron micrographs of adipocytes of *M. cephalus* inhabiting control (A, B) and test (C, D) estuaries.

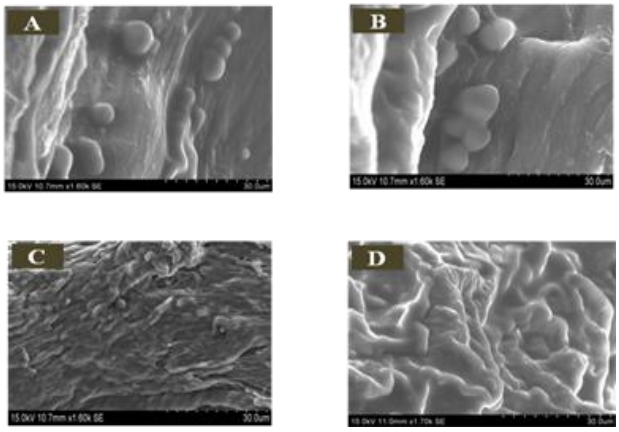


Fig 2: Expression pattern of Trx in control and test adipocytes of *M. cephalus*.

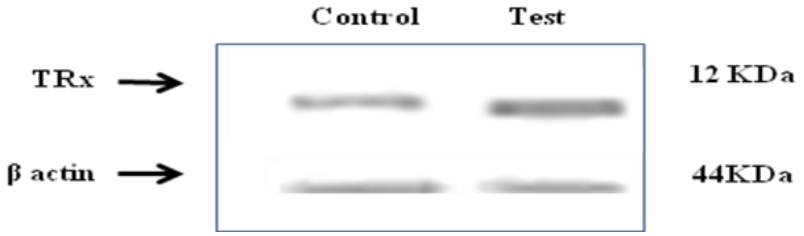
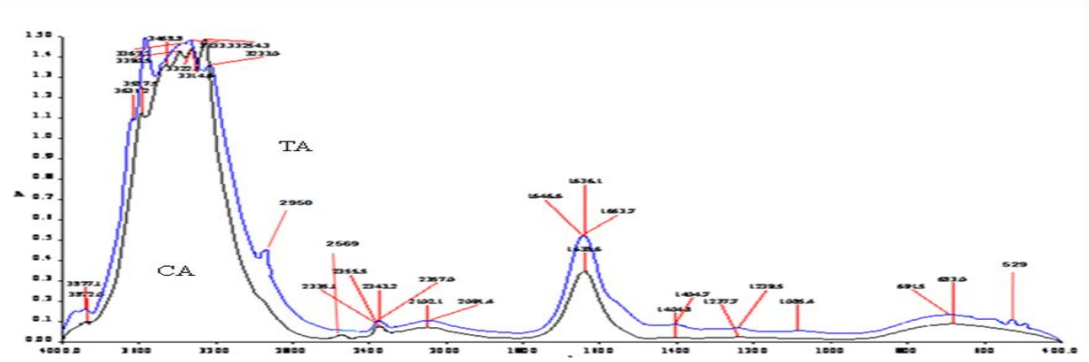
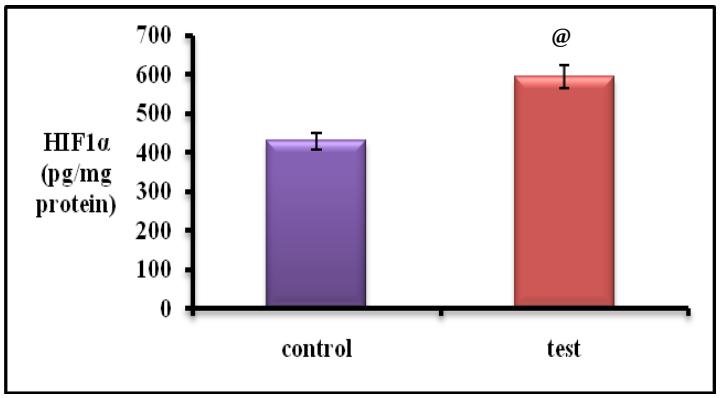


Fig 3: Comparative FTIR spectra of control and test adipocytes of *M.cephalus*.



CA- control adipocytes; TA- test adipocytes

Fig 4: Level of HIF1α in control and test adipocytes of *M. cephalus*. Values are expressed as means ± SD (n=20 fish per estuary)



[@]p<0.001 increased highly significant when compared to control fish adipocytes

Fig 5: Expression pattern of TNFα in control and test adipocytes of *M. cephalus*.

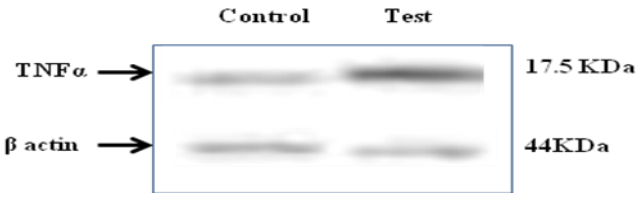
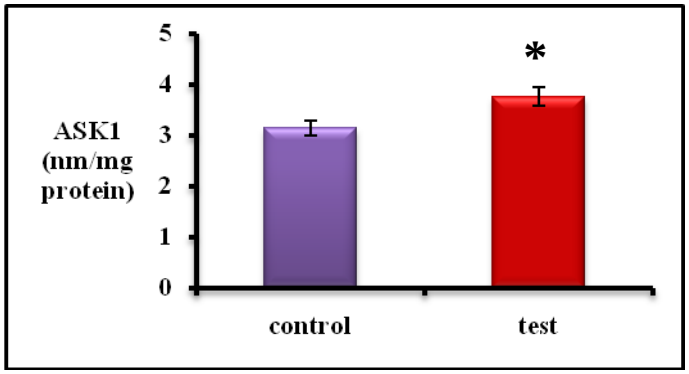
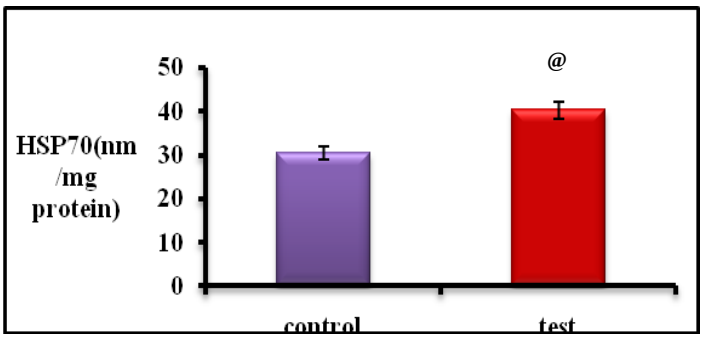


Fig 6: Level of ASK1 in control and test adipocytes of *M. cephalus*. Values are expressed as means ± SD (n=20 fish per estuary)



*p<0.05 increased when compared to control fish adipocytes

Fig 7: Level of HSP70 in control and test adipocytes of *M. cephalus*. Values are expressed as means ± SD (n=20 fish per estuary)



[@]p<0.001 increased highly significant when compared to control fish adipocytes

Fig 8: Effect of pifithrin- μ on cell viability at different time with varying concentrations in control and test adipocytes of *M. cephalus*.

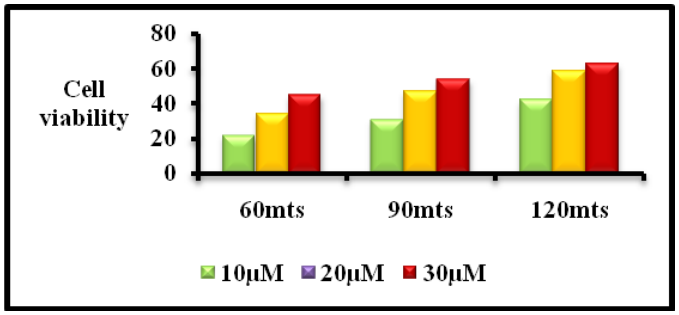
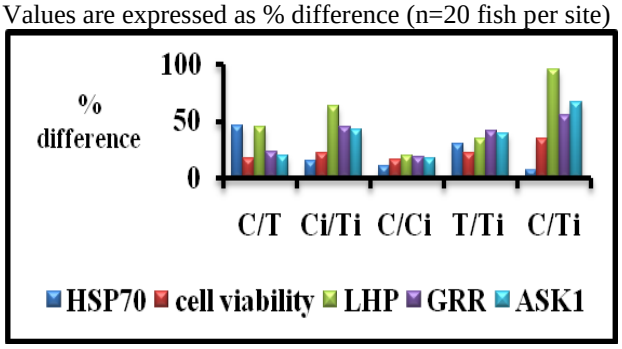


Fig 9: Effect of pifithrin-μ on the level of HSP70, Cell viability, LHP, GRR and ASK1 in control and test adipocytes of *M. cephalus*. Values are expressed as % difference (n=20 fish per site)



C/T- control adipocyte vs test adipocyte
Ci/Ti- control adipocyte with inhibitor vs test adipocyte with inhibitor
C/Ci- control adipocyte vs control adipocyte with inhibitor
T/Ti- test adipocyte vs test adipocyte with inhibitor
C/Ti- control adipocyte vs test adipocyte with inhibitor