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Differential expression of heat shock proteins in fish hepatocytes under hypoxic condition

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Abstract
Hypoxia in estuarine ecosystems is a problem of growing concern worldwide. Induction of signaling molecules (HSPs) is a crucial step in the cellular response to stress. Altered expression of such proteins in response to stress conditions is a key factor for the maintenance of cellular integrity and survival. This is the first study that attempts to confirm the role of HSPs in fish hepatocytes using *in-vitro* (induced) and *in-vivo* (natural) hypoxic conditions. The level of HNE, GRD, HIF1α, HSP70, HO-1, CYP1A2 and ASK1 were measured in the hepatocytes of *Mugil cephalus* inhabiting control and test sites with and without *in-vitro* hypoxic incubation. The oxidant-antioxidant status, heat shock proteins and its associated signaling molecules were differentially expressed in fish hepatocytes inhabiting pollutants induced hypoxic condition and *in-vitro* hypoxic incubation. The present *in-vitro* hypoxic incubation studies suggest that HSPs may play a protective role in the *Mugil cephalus* surviving in polluted waters.

Keywords: Fish, Heat shock proteins (HSPs), Hepatocytes, Hypoxia, Stress

1. Introduction
Oxygen, in addition to being an essential substrate of energy production, is a critical regulator of cellular function in all organisms. Fish are exposed to large oxygen fluctuations in their aquatic environment since the inherent properties of water can result in marked spatial and temporal differences in the concentration of oxygen. The biggest challenge fish face when exposed to low oxygen conditions is maintaining metabolic energy balance, as 95% of the oxygen consumed by fish is used for ATP production through the electron transport chain [1]. Therefore, hypoxia survival requires a coordinated response to secure more oxygen from the depleted environment and counteract the metabolic consequences of decreased ATP production in the mitochondria.
Hypoxia is generally considered a condition in which a water column contains less than 2.0 mg L^{-1} dissolved oxygen (DO), the point where the majority of aquatic organisms can no longer survive [2]. Pollock *et al.* [3] have suggested that hypoxia as any level of DO low enough to negatively impact the behavior and physiology of an organism. Some fish species have evolved the ability to survive low oxygen exposure. However extent of tolerance varies among species, depending on severity and duration of hypoxia. Under hypoxic conditions animals adopt different mechanism to tolerate hypoxia. Many of these responses are behavioral, including surface breathing, reduced activity, and/or increased ventilation rate [4]. Thus, the present study aims to study the impact of hypoxia in polluted fish hepatocytes by subjecting them to *in vitro* hypoxic condition. For many decades, animal cells have been cultured in air supplemented with carbon dioxide, but new applications for cell therapies require conditions mimicking those *in vivo*. Hypoxic environment may be established by using incubators with simultaneous flow of air, CO_2 and N_2. Hypoxia causes the formation of free radicals inducing severe alterations in cellular activities; prevention of such damages is made through defense strategies (small antioxidant molecules, enzymes) [5].
Fish are considered to be the bio-indicators of marine pollution because of their ability to respond to pollutants [6] and extensively used in pollution monitoring programs [7]. The liver hepatocytes are regarded as the major detoxification site whose detoxification functions can be directly related to survival mechanisms. It plays a major role against pollutants induced free radical damage by virtue of having a variety of antioxidants. The main cellular defense system against toxicity originating from active oxygen forms includes induction of antioxidant

enzymes that possess the property of scavenging and eliminating free radicals during oxidative stress [8]. The use of fish hepatocytes to reduce the necessity of whole animal models in pharmacological and toxicological studies is particularly promising. The fish hepatocyte preparation represents an important tool to understanding the role of the liver in the biology of fish species [9].

Hypoxia induces a series of adaptive cellular responses including generation of ATP through the glycolytic pathway involving increases in glycogen phosphorylase and aldolase as well as increased production of stress-related proteins [1]. At the molecular level, the adaptation involves increases in mRNA transcription of genes encoding for proteins involved in anaerobic and fat metabolism [10]. Many of these cellular and molecular responses to hypoxia are controlled by hypoxia-inducible factor (HIF), a transcription factor which regulates the expression of numerous genes during exposure to hypoxia [11]. HIF-1 consists of two subunits namely HIF-1α and HIF-1β. These two subunits are both basic helix-loop-helix (bHLH) proteins of the PAS family (PER, AHR, ARNT and SIM family); however, they display different responses to O_2 concentrations: HIF-1β is a non-oxygen-responsive nuclear protein and is constitutively expressed; HIF-1α is an essential transcription factor which mediates the adaptation of cells to low oxygen tensions, is regulated precisely by hypoxia [12].

Heat shock proteins (HSPs) also known as stress proteins consist of a family of molecules that play a pivotal role in the cellular stress response [13]. Synthesis of HSP increases in response to heat shock [14] and to a variety of stressors including hyperosmolarity [15], ischemia [16], as well as superoxide radicals that are also formed during hypoxia and deoxygenation [17]. As such, heat shock proteins are potential biomarkers for environmental stress in fish [14]. HSP70 can be induced by a variety of stresses, including heat shock, oxidative stress, and mechanical stress [18]. HSP70 also appears to play a crucial role in the protection of cells from environmental stresses [19]. Many studies have reported that exposure of organisms to such diverse stressors as temperature extremes, pollutants, anoxia, parasitism, predation, or competition; all elicit reversible increases in HSP70 expression that serve to protect the organism against cellular damage [20, 21]. The chaperone functions of HSP70 appear to be closely related to stress tolerance in animal cells and overexpression of HSP70 enhances anti-apoptotic activity against cellular stress [22]. Resistance to apoptosis is associated with the overexpression of HSPs. The HSP70 may, therefore, provide a biomarker to identify stressful effects of environmental factors and to demonstrate a link between such factors and observed negative changes in life history traits of natural population.

Apart from the induction of high molecular weight HSPs, stress also induces low molecular weight HSPs like HSP32 (HO-1). Heme oxygenase (HO) is the first and the rate limiting enzyme in the catabolism of heme [23] to yield equimolar amounts of biliverdin, carbon monoxide (CO) and free iron. There are three forms of HO, the inducible HO-1 and the constitutively expressed HO-2 and HO-3 [24, 25]. However only HO-1 responds to xenobiotic induction [26]. HO-1 is induced in most cell types by many forms of environmental stress to play a protective role in cells exposed to oxidative stress [27]. HO-1 can be induced in a wide range of animal tissues, particularly liver following a number of stressful stimuli including heavy metals [28]. Heme may be an important inducer of both HSP70 and HO-1 via the heat shock element and metal regulatory element respectively [29]. Both HSP70 and HO-1 are members of the stress protein superfamily of multifunctional proteins that are induced by a variety of stresses and injuries that denature proteins [30].

Mugil cephalus elicits an adaptive defense mechanism in response to pollution stress in order to prolong the cell survival through detoxification of the pollutants. Cytochrome P450 family members participate in xenobiotic transformation as a detoxification mechanism [31]. CYPs have been found in nearly all vertebrate tissues examined but are generally most prevalent in the digestive tract, specifically the liver [32]. CYP1A is found in the liver, heart, gill, kidney, and intestinal tract of fish [33]. Fish cytochrome P450 plays a central role in the biotransformation of xenobiotics prior to their excretion [34].

Apoptosis signal regulating kinase 1 (ASK1) also known as mitogen-activated protein kinase kinase kinase 5 (MAP3K5) is a 155-kDa ubiquitously expressed protein belonging to the member of MAPKKK, a serine-threonine protein kinase. It is activated by various types of stress such as reactive oxygen species (ROS), hydrogen peroxide, tumor necrosis factor (TNF) α, lipopolysaccharide (LPS), endoplasmic reticulum (ER) stress and calcium influx [35] playing a key role in the regulation of oxidative stress response [36]. Oxidative stress is one of the most potent activators of ASK1, which is essential for oxidative stress induced cell death [37]. In this context, the present study aims to analyze the cytoprotective role of heat shock proteins in polluted fish hepatocytes under *in vitro* hypoxic condition. Thus the study may provide useful inputs for the understanding of the *in-vitro* hypoxic stimulation verses the natural pollutants induced hypoxia in fish.

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 control 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 test 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, iron etc to a significant extent compared to unpolluted estuary has also been confirmed by previous studies [38, 39]. It has also been reported that Ennore estuary significantly differs from Kovalam estuary in its physical, chemical and biological factors [40], thus it has been chosen as the test site. Water quality was assessed by analyzing dissolved oxygen level of both control and test sites. Dissolved oxygen level was estimated by CHEMLINE portable dissolved oxygen meter CL-930 and it is expressed as ppm.

2.2. Study Animal and Sampling
The species *Mugil cephalus* was identified by the use of Food and Agriculture Organization (FAO) species identification sheets [41]. *M. cephalus* with an average length of 30-32 cm were collected from unpolluted and polluted estuaries using baited minnow traps. Collected fish were 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 [42, 43] with slight modification as described by Padmini and Usha Rani [21].

2.4. Hypoxic Incubation
For *in-vitro* hypoxic incubation, cells were placed for 1 hour, 2 hours and 3 hours into a sealed chamber (18 °C) which was gassed with a humidified gas mixture containing 1% O_2 and 5% CO_2, balanced with nitrogen in the Forma water jacketed CO_2/O_2 incubator (Model: 3131, Thermo fisher scientific, USA).

2.5. Cell Viability Assay
The cell viability of hepatocyte preparations was assessed using trypan blue staining [44].

2.6. Protein Preparation
The protein concentration was determined by the classical method of Bradford [45] with coomassie brilliant blue G-250, using bovine serum albumin as a standard.

2.7. Assay of Glutathione Reductase (GRD)
The activity of glutathione reductase was assayed by the method of Acedo *et al.* [46]. The enzyme activity was expressed as nanomoles of NADPH oxidized/minute/mg protein.

2.8. Quantification of HNE, HSP70, HO-1, CYP1A2 and ASK1 using ELISA
The inducible form of HNE, HSP70, HO-1, CYP1A2 and ASK1 in hepatocytes of *M. cephalus* inhabiting Kovalam and Ennore estuaries with and without *in vitro* hypoxic incubation 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), CYP1A2 ELISA kit (E93294Hu, Uscn Life Science Inc, China) and ASK1 (E91358Hu 96T, Uscn Life Science, Inc, USA) according to the manufacturer's instructions.

2.9. 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 fish hepatocytes inhabiting control and test sites. All data were presented as mean ± SD of 18 fish per site. Differences were considered significant at $p<0.05$, $p<0.01$ and $p<0.001$.

3. Results
3.1. Dissolved Oxygen (DO)
Figure 1 describes the dissolved oxygen (DO) level of control and test site water. DO level was decreased by 29% ($p<0.01$) in test site water when compared to control site water.

3.2. Cell Viability
The liver cells of *M. cephalus* collected from test site showed decrease in their viability (77%) compared with hepatocytes isolated from control site (91%) (Figure 2). Hypoxic incubation for 1 hour, 2 hours and 3 hours, cell viability was decreased by 14%, 26% and 36% in the hepatocytes isolated from control site and 21%, 35% and 47% in the hepatocytes isolated from test site when compared to hepatocytes isolated from control and test site without *in-vitro* hypoxic incubation. Of the varying time intervals, 1 hour hypoxic incubation was found to be effective by cell viability differences (Figure 2).

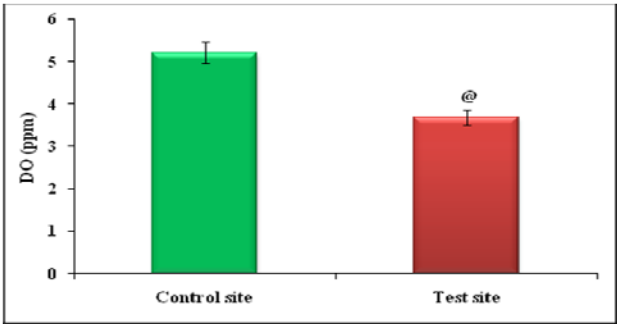

Fig 1: Level of DO in the water of control and test sites. Values are an average of five samples from each site.

@p<0.05 When compared to control site water

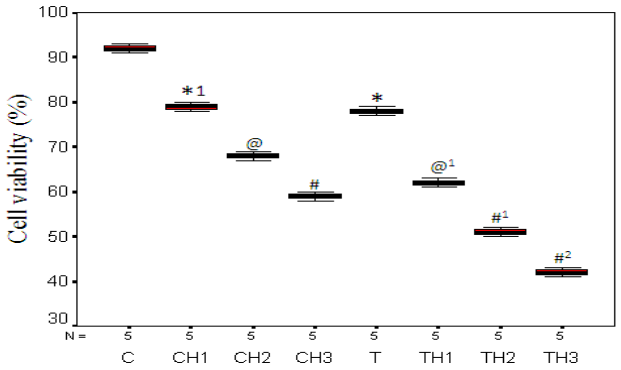

Fig 2: Cell viability of *M. cephalus* inhabiting pollutants induced hypoxic and *in-vitro* hypoxic (1 hour, 2 hours and 3 hours) condition. Values are expressed as mean ± SD (n=18 fish per site).

*p<0.05, *1p<0.05, @p<0.01, #p<0.001 When compared with control hepatocytes without *in-vitro* hypoxic condition
@1p<0.01, #1p<0.001, #2p<0.001 When compared with test hepatocytes without *in-vitro* hypoxic condition

3.3. Hydroxynonenal (HNE)
The level of HNE was described in the hepatocytes of *M. cephalus* inhabiting control and test sites with and without *in-vitro* hypoxic incubation (Figure 3a). The level of HNE was significantly increased by 42% in test hepatocytes compared with control hepatocytes without *in-vitro* hypoxic incubation. When incubated with *in-vitro* hypoxia, the level of HNE was significantly increased by 39% in the control hepatocytes and 33% in the test hepatocytes compared to control and test hepatocytes respectively without *in-vitro* hypoxic incubation.

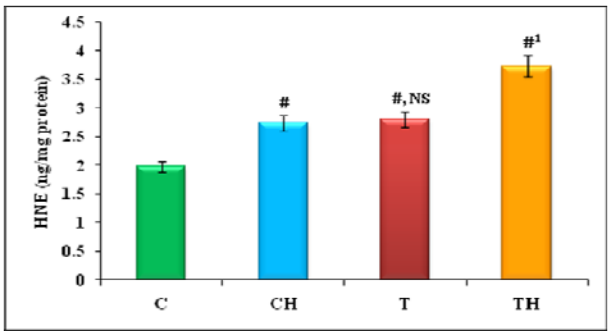

Fig 3a: Level of HNE in the hepatocytes of *M. cephalus* inhabiting pollutants induced hypoxic and *in-vitro* hypoxic condition. Values are expressed as mean ± SD (n=18 fish per site).

#p<0.001 When compared with control hepatocytes without *in-vitro* hypoxic condition
#1p<0.001 When compared with test hepatocytes without *in-vitro* hypoxic condition
NS- not significant when compared to control hepatocytes with *in-vitro* hypoxic condition

3.4. Glutathione Reductase (GRD)

The level of GRD was presented in fish hepatocytes inhabiting control and test sites with and without *in-vitro* hypoxic incubation (Figure 3b). A significant decrease in the level of GRD ($p<0.01$) was observed in test hepatocytes (21%) compared with control hepatocytes without *in-vitro* hypoxic incubation. On hypoxic incubation, the level of GRD was decreased by 19% in the control hepatocytes and 17% in the test hepatocytes compared with control and test hepatocytes respectively without *in-vitro* hypoxic incubation.

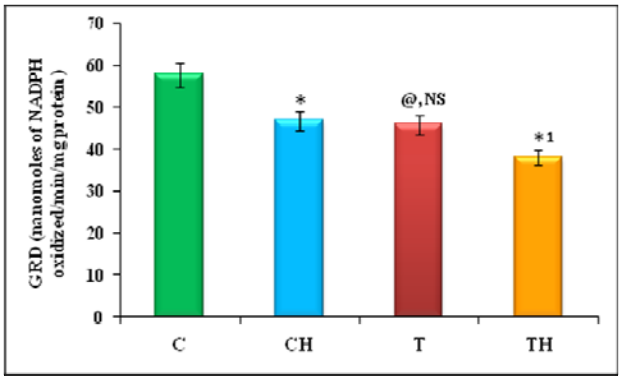

Fig 3b: Level of GRD in the hepatocytes of *M. cephalus* inhabiting pollutants induced hypoxic and *in-vitro* hypoxic condition. Values are expressed as mean ± SD (n=18 fish per site).

@p<0.01, *p<0.05When compared to control hepatocytes without *in-vitro* hypoxic condition
*1p<0.05 When compared to test hepatocytes without *in-vitro* hypoxic condition
NS- not significant when compared to control hepatocytes with *in-vitro* hypoxic condition

3.5. Hypoxia Inducible Factor (HIF-1α)

The expression of HIF-1α in the control and test hepatocytes with and without *in-vitro* hypoxic incubation was noticed in Figure 4. The expression of HIF-1α was significantly increased by 39% in the test hepatocytes compared to control hepatocytes without *in-vitro* hypoxic incubation. When incubated with *in-vitro* hypoxia, the expression of HIF-1α was significantly increased by 27% in the control hepatocytes and 29% in the test hepatocytes when compared to control and test hepatocytes respectively without *in-vitro* hypoxic incubation.

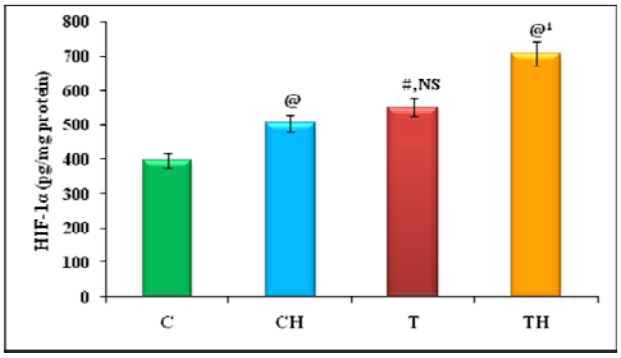

Fig 4: Level of HIF-1α in the hepatocytes of *M. cephalus* inhabiting pollutants induced hypoxic and *in-vitro* hypoxic condition. Values are expressed as mean ± SD (n=18 fish per site).

#p<0.001, @p<0.01 When compared to control hepatocytes without *in-vitro* hypoxic condition
@1p<0.01 When compared to test hepatocytes without *in-vitro* hypoxic condition
NS- not significant When compared to control hepatocytes with *in-vitro* hypoxic condition

3.6. Heat Shock Protein 70 (HSP70)

The expression of HSP70 in the control and test hepatocytes with and without *in-vitro* hypoxic incubation was given in Figure 5. The expression of HSP70 was significantly enhanced by 52% in the test hepatocytes compared to control hepatocytes without *in-vitro* hypoxic incubation. On hypoxic incubation, the expression of HSP70 was significantly increased by 40% in the control hepatocytes and the expression of HSP70 was decreased by 15% in the test hepatocytes when compared to control and test hepatocytes respectively without *in-vitro* hypoxic incubation.

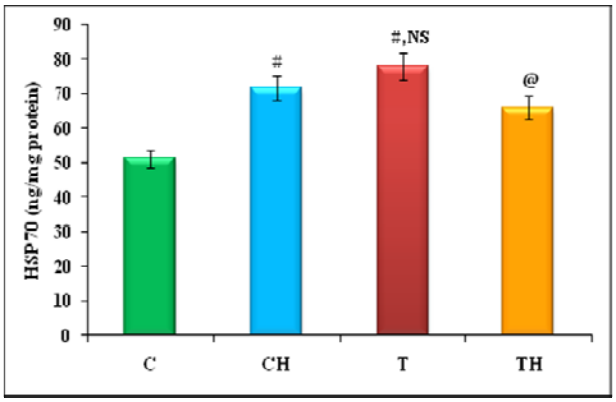

Fig 5: Level of HSP70 in the hepatocytes of *M. cephalus* inhabiting pollutants induced hypoxic and *in-vitro* hypoxic condition. Values are expressed as mean ± SD (n=18 fish per site).

#p<0.001 When compared to control hepatocytes without *in-vitro* hypoxic condition
@p<0.01 When compared to test hepatocytes without *in-vitro* hypoxic condition
NS- not significant when compared to control hepatocytes with *in-vitro* hypoxic condition

3.7. Heme Oxygenase-1 (HO-1)

Figure 6 denotes the expression of HO-1 in the control and test hepatocytes with and without *in-vitro* hypoxic

incubation. HO-1 was significantly increased by 95% in the test hepatocytes compared to control hepatocytes without *in-vitro* hypoxic incubation. On hypoxic incubation, the expression of HO-1 was significantly increased by 79% in the control hepatocytes and the expression of HO-1 was decreased by 14% in the test hepatocytes when compared to control and test hepatocytes respectively without *in-vitro* hypoxic incubation.

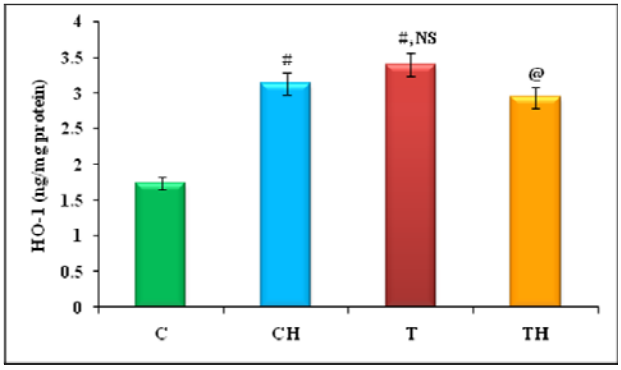

Fig 6: Level of HO-1 in the hepatocytes of *M. cephalus* inhabiting pollutants induced hypoxic and *in-vitro* hypoxic condition. Values are expressed as mean ± SD (n=18 fish per site).

#p<0.001 When compared to control hepatocytes without *in-vitro* hypoxic condition
@p<0.01 When compared to test hepatocytes without *in-vitro* hypoxic condition
NS- not significant when compared to control hepatocytes with *in-vitro* hypoxic condition

3.8. Cytochrome P450 1A2 (CYP1A2)

The expression of CYP1A2 was depicted in fish hepatocytes inhabiting control and test sites with and without *in-vitro* hypoxic incubation (Figure 7). A significant decrease in the expression of CYP1A2 was observed in test hepatocytes (35%) compared with control hepatocytes without *in-vitro* hypoxic incubation. When incubated with *in-vitro* hypoxia, the expression of CYP1A2 was significantly decreased by 28% in the control hepatocytes and 26% in the test hepatocytes compared with control and test hepatocytes respectively without *in-vitro* hypoxic incubation.

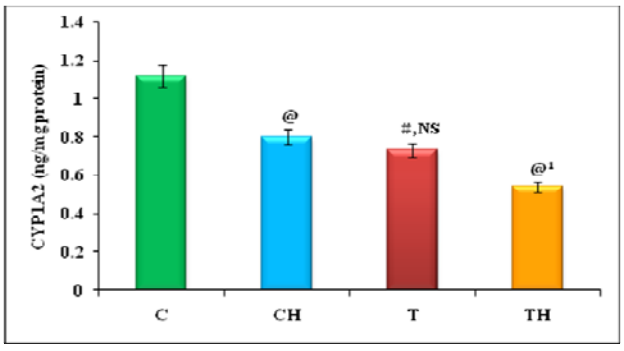

Fig 7: Level of CYP1A2 in the hepatocytes of *M. cephalus* inhabiting pollutants induced hypoxic and *in-vitro* hypoxic condition. Values are expressed as mean ± SD (n=18 fish per site).

#p<0.001, @p<0.01 When compared to control hepatocytes without *in-vitro* hypoxic condition
@¹p<0.01 When compared to test hepatocytes without *in-vitro* hypoxic condition
NS- not significant when compared to control hepatocytes with *in-vitro* hypoxic condition

3.9. Apoptosis Signal Regulating Kinase 1 (ASK1)

The expression of ASK1 in the control and test hepatocytes with and without *in-vitro* hypoxic incubation was mentioned in Figure 8. The expression of ASK1 (17%) was increased to a small extent in the test hepatocytes compared to control hepatocytes without *in-vitro* hypoxic incubation. On hypoxic incubation, the expression of ASK1 was increased by 13% in the control hepatocytes and 24% in the test hepatocytes when compared to control and test hepatocytes respectively without *in-vitro* hypoxic incubation.

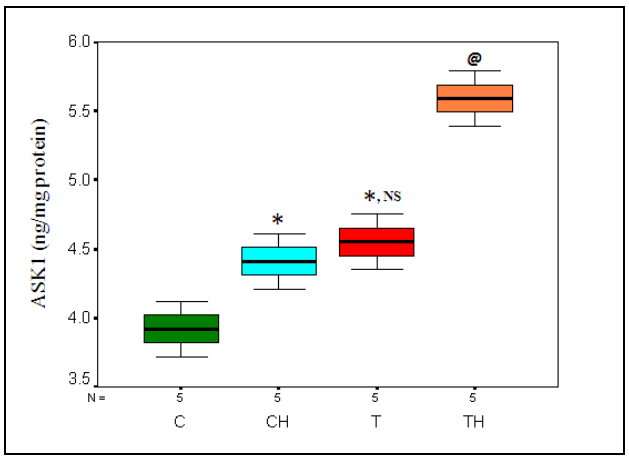

Fig 8: Level of ASK1 in the hepatocytes of *M. cephalus* inhabiting pollutants induced hypoxic and *in-vitro* hypoxic condition. Values are expressed as mean ± SD (n=18 fish per site).

*p<0.05 When compared to control hepatocytes without *in-vitro* hypoxic condition
@p<0.01 When compared to test hepatocytes without *in-vitro* hypoxic condition
NS- not significant when compared to control hepatocytes with *in-vitro* hypoxic condition

4. Discussion

Hepatocyte viability was assayed to evaluate the impact of hypoxic stress. The present results demonstrated that *in vitro* hypoxic incubation have toxic effects on fish, significantly decreasing viability of fish hepatocytes. Hypoxia is known to cause oxidative stress; oxidative stress has significant impact on cellular macromolecules and functions at all levels. Consistent with this, it has also been demonstrated in the following sections that there was a significant increase in oxidative stress status characterized by the elevation of the stress biomarkers in fish hepatocytes from Ennore estuary; the values being significantly correlated with cell viability results.

Organisms are challenged by a multitude of stresses throughout their lifespan which cause irreversible damage to proteins that in turn could impair cellular processes [47]. Under pathological or other stressful conditions where reactive species are produced, cellular macromolecules such as DNA, proteins, and lipids are modified into DNA adducts, oxidized proteins, and lipid peroxides, respectively. From the lipid peroxidation of plasma membrane, reactive lipid aldehydes such as HNE or hydroxyhexanal are produced via enzymatic and nonenzymatic reactions [48]. Newly synthesized HNE can readily move across cell membranes and can interrupt the normal functions of proteins as well as DNAs [49]. Numerous reports have demonstrated that HNE inhibits functions of various cellular proteins [48]. Soh *et al.* [50] demonstrated that HNE can also stimulate the ASK1-

SEK1-JNK pathway rapidly and selectively. In our study, the level of HNE was significantly increased in fish hepatocytes under *in vitro* hypoxic condition than pollutants induced hypoxic condition which may probably drive the cell towards apoptosis.

Glutathione reductase (GRD), an important enzyme plays a key role in GSH recycling and the maintenance of intracellular GSH concentrations [51]. The reduced activity of glutathione reductase may also be one of the reasons for the decreased GSH/GSSG ratio as it catalyzes the conversion of oxidized glutathione to reduced glutathione for the maintenance of the intracellular GSH level. The glutathione mediated detoxification process may also be affected by reduced glutathione levels. This might be a factor responsible for the lack of elimination of toxic compounds that enter the fish and thus result in their accumulation, aggravating oxidative stress. Detoxification enzyme variations also exert negative effects such as increased susceptibility to reactive oxygen species formation, increased energy demand, proliferation of cells, etc. [52]. The reduced activity of GRD in fish hepatocytes under *in vitro* hypoxic condition when compared with pollutants induced hypoxic condition was observed in the present study.

HIF-1α protein has been named the master regulator involved in the homeostasis of cells under hypoxic conditions and has been found to be up-regulated in fish exposed to hypoxia [53]. HIF-1α is a transcription factor which targets genes involved in three main groups of low oxygen homeostasis: vascular development, production of blood cells or altering energy metabolism [54]. HIF-1α protein has been shown to increase under constant hypoxic exposure in laboratory populations of Atlantic croaker (*Micropogonias undulatus*), supporting its use as a biomarker of hypoxia in estuarine fish [55]. Acute hypoxia can increase HIF-1α expression in brain and liver, whereas chronic hypoxia leads to a significant change in HIF-1α expression in muscle [56]. HIF-1 contributes to the tracking of the ASK1 activity preventing over-activation of the enzyme that would lead to quick and often irreversible activation of apoptotic signaling pathways inducing programmed cell death [57]. In our present report, HIF-1α was significantly increased in the fish hepatocytes under *in vitro* hypoxic condition when compared to pollutants induced hypoxic condition.

Activation of heat shock proteins (HSPs) is critical for adaptation to low oxygen levels (hypoxia) and for enduring the oxidative stress of reoxygenation. The induction of HSP70 in response to stressors is thought to be critical to prevent proteotoxicity and enhance cell survival [58]; it perhaps the reason for the preferential synthesis of HSPs even at the expense of other cellular proteins [59]. Environmental contaminants such as heavy metals and β-naphthoflavone (BNF) have been shown to induce HSP70 in fish tissues, including hepatocytes [59]. Liver HSP70 concentrations were higher in rainbow trout exposed to BNF [60]. Enhanced levels of HSP70 in polluted site fish may reflect a protective response against environmental pollutant-related stress. Overall, HSPs can be activated or induced by a number of stresses and they act to protect the cell by influencing a variety of cellular processes which determine cellular fate. HSP70 expression was significantly reduced by chronic hypoxia in bronchiolar epithelial cells [61]. In the present study, the level of HSP70 was significantly decreased in fish hepatocytes under *in vitro* hypoxic condition.

Heme oxygenase-1 (HO-1), the heat shock protein 32 (HSP32) family of proteins, is postulated to be a component of cellular defense mechanisms against oxidative stress-mediated injury [62]. HO system is one of the key regulators of cellular redox homeostasis, which responds to oxidative stress (ROS) via HO-1 induction. HO is implicated in protection against oxidative stress, proliferation and apoptosis in many cell types. HO-1 is an inducible gene whose transcription is increased in response to a variety of cellular stresses and stimuli including ischemia, hypoxia, oxidative stress and inflammatory cytokines [63]. Harbrecht *et al.* [64] described that oxidative/nitrative stress stimulates the induction of HO-1 and HSP70, which protect cells from apoptotic cell death induced by oxidative or nitrative stress. In our present report, HO-1 was significantly decreased in the fish hepatocytes under *in vitro* hypoxic condition when compared to pollutants induced hypoxic condition.

CYP1A responses in fish as a biomarker of aquatic pollution for understanding the influences of factors such as water temperature, season, sexual maturation, developmental status and diet [65]. Hypoxic conditions modulate the CYP1A signaling pathway. Hypoxia induces the production of reactive oxygen species that in turn activate NFκB, which represses AhR and downregulates CYP1A1 and CYP1A2 [66]. Cells adapt to hypoxia by upregulating the transcription of multiple genes, the majority of which are induced by HIF-1[67]. HIF-1α translocates to the nucleus, and dimerizes with HIF-1β or ARNT to form HIF-1. Since ARNT is also a heterodimerization partner of AhR, hypoxia decreases the availability of ARNT, thus causing a downregulation of CYP1A1 and CYP1A2 [68]. CYP1A protein activity and expression is affected by many environmental factors such as temperature, pH, size and age of the organism [69]. Work performed with rabbits and zebrafish has shown that several genes encoding Cytochrome P450 proteins are regulated at mRNA-level in hypoxia [70]. Kurdi *et al.* [71] proved that hypoxia reduces the hepatic expression of CYP1A1 and CYP1A2 in rabbits.

ASK1 acts as a critical mediator in ROS induced cell damage that leads to cell death as ASK1 is already proved to play a specific role in TNF along with ROS induced JNK/P38 activation and cell death [39]. ASK1 signaling cascades are regulated by molecular chaperones. The chaperones of the HSP70 family may inhibit the activity of ASK-1 by having the physical association with ASK1 thereby inhibiting the homo-oligomerisation of the kinase and hence acts as an endogenous inhibitor of ASK-1[72]. Our previous study also demonstrated that the enhanced levels of HSP70 downregulates the expression of ASK1 in stressed fish hepatocytes [73]. In the present study, the level of ASK1 was significantly increased in the fish hepatocytes under *in vitro* hypoxic condition when compared to pollutants induced hypoxic condition due to decreased expression of HSP70.

5. Conclusion

Environmental hypoxia is an important environmental stressor that has manifold effects on aquatic life. ROS can be up-regulated in hypoxic environments [74, 75]. The antioxidant enzymes that make up the antioxidant defense system are expected to increase under hypoxia in order to detoxify ROS. The present results suggest that *in-vitro* hypoxia closely mimics the pollutants induced hypoxic condition correlated in terms of HSPs expression. It is depicted by results achieved in control subjected to hypoxia which is close to

pollutants induced hypoxia. Develi-Is and his collegues [76] indicated that HO-1 induction alleviated increased oxidative stress and inflammatory reactions together with deterioration in nitric oxide (NO) production by decreased asymmetric dimethylarginine (ADMA) levels in thioacetamide (TAA)-induced liver damage in rats. Our study results indicate that upregulation of heat shock proteins (HSP70 and HO-1) could suppress the decrease in cell viability, apoptotic kinase expression and retain cell survival due to pollutants induced stress condition. During *in-vitro* hypoxic condition, the level of HSPs (HSP70 and HO-1) was decreased in fish hepatocytes leading to cell death. In conlusion, our study proclaims that expression of HSPs (HSP70 and HO-1) is not precisely regulated beyond the threshold level as observed in test hepatocytes during *in-vitro* hypoxia. From the present study, results from the *in-vitro* hypoxic condition clearly demonstrate that HSPs may play a key role during hypoxic homeostasis in fish hepatocytes.

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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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