

Effect of Aerial Exposure on Brain and Liver of *Heteropneustes fossilis*

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ABSTRACT: Mostly fish cannot survive more than few hours out of the water, but in the *Heteropneustes fossilis* survives up to 60-70hrs under water deprived condition. Such fishes contain high level of ammonia in gills that may induce ammonia-mediated toxicity. Therefore, it has been intended to analyze oxidative and high ammonia stress markers in brain and liver of *Heteropneustes fossilis* after aerial exposure. The experimental group of *Heteropneustes fossilis* was exposed to air and tissues were collected at Zero hour as control and after 20 hrs as experimental. Super oxide dismutase and catalase were selected as oxidative stress markers, lactate dehydrogenase was evaluated for bioenergetics and expression of arginase was analysed through reverse-transcriptase-polymerase chain reaction at transcript level and by enzyme assay at the protein level. The affect of aerial exposure on the protein profile of control and aerial exposed liver and brain of fish were also evaluated by SDS-PAGE followed by MALDI-MS/MS. After 20 hours of aerial exposure, the expression of mRNA of super oxide dismutase, and catalase was up-regulated while lactate dehydrogenase, arginase-I, and arginase-II were down-regulated than the control in the liver as well as the brain. The activities of Cu-Zn-super oxide dismutase and catalase were decreased while Mn- super oxide dismutase, lactate dehydrogenase, malate dehydrogenase, arginase-I, and arginase-II were increased after aerial exposure in liver but the level of all enzymes was upregulated in the brain as compared to control. The increased intensity of band of 40kDa and 25kDa in liver and 25kDa in the brain that was identified as proteins either associated with actin or myosin or apoptosis. The enzymatic and proteomic analysis confirms that aerial exposure has the prominent effect on the cellular organization and cytoskeleton formation and maintenance by induction ammonia level followed by ROS.

KEYWORDS: Aerial exposure, Arginase, Brain, Liver, Actin

I. INTRODUCTION

Fish generally get exposed to air during summer as well as during the commercial handling that leads a condition of stress for an aquatic organism. As they generally respire through gills and take dissolve oxygen from water [1], [2], during aerial exposure or water shortage they are unable to respire and simultaneously exchange the excretory product like ammonia, which leads to several complications as generation of the oxidative stress [3], increased lipid peroxidation and protein carbonylation [4] and ammonia loading [5]. They show disruption of gill [6] and damage in thin-walled blood capillaries of air-breathing organ [7]. Most of the fish cannot tolerate the aerial exposure and died within hours, but some air breathing catfish can tolerate aerial exposure up to few hours like *Galaxias 'nebula'* [8], *Periophthalmodon schlosseri* and *Boleophthalmus boddarti* can survive up to 36 hrs [9], swamp eel *Monopterus albus* can survive up to 144 hrs [2], however in context to Indian sub-continent *Heteropneustes fossilis* survives up to 60-70 hrs under water deprivation conditions [4], [10], [11]. *H. fossilis* mainly lives in ponds, ditches, swamps, marshes and sometimes occurs in muddy rivers and face aerial exposure in summer season naturally and due to its easy availability and affordability exposed to air commercially.

Since, the effect of aerial exposure is known to alter the activity of respiratory chain enzymes [4], [12] and ammonia accumulation [5]. It is also explained that liver is the prime site for ammonia detoxification but dysfunction of liver high level of ammonia induces neuronal degeneration and induction of oxidative and nitrosative stress [13]. In this context, there is no study about the aerial exposure induced neuronal degeneration or status of oxidative stress enzymes

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and hyperammonemia markers in liver and brain in aerial exposure induced stress? Therefore, it was aimed to study the status of oxidative stress enzymes and Arginase, under the aerial exposure condition in brain and liver along with the comparison of the protein profile of aeri ally exposed brain and liver. The data would improve understanding on basic mechanism of stress due to aerial exposure. It would also be useful to identify molecular marker during the aerial exposure.

II. MATERIALS AND METHODS

A. ACCLIMATIZATION OF FISH, AERIAL EXPOSURE, AND PREPARATION OF SAMPLES

Heteropneustes fossilis were purchased from the local fish market and acclimatized in animal house facility of the Department. They were divided into two groups (n=10), control and 20hrs aerial exposed experimental group. The fishes were sacrificed by decapitation and tissues (brain and liver) were dissected out at 0hr (Control) and 20hrs (Experimental) interval.

For the enzymatic analysis, 20% homogenate of tissue was prepared in homogenizing medium 30mM Tris-HCl pH 7.2 containing 1mM EDTA, 250mM Sucrose, 50mM Mannitol, 150mM KCl and protease inhibitor cocktail (Sigma-Aldrich) using a Potter-Elvehjem type glass homogenizer with a motor-driven teflon pestle [13]. The protein concentration was estimated by the method of [14] using bovine serum albumin as the standard. The tissue lysates of 0hr and 20hrs were resolved on 12% denaturing polyacrylamide gel for comparison of the protein profile.

B. ENZYME ASSAY OF SUPEROXIDE DISMUTASE (SOD), CATALASE, LACTATE DEHYDROGENASE (LDH) AND MALATE DEHYDROGENASE (MDH) AND ARGINASE (ARG)

Enzyme specific staining of the SOD (EC 1.15.1.1), Catalase (EC 1.11.1.6) and LDH were performed by the described modified method of [14]. In brief, 10% native polyacrylamide gel electrophoresis was performed to analyze the active fraction of LDH, SOD, and 7% for Catalase. Electrophoresis was carried out at 4°C for three hours, applying a current of 100mV. The gels were stained for specific enzymes. The staining solution for LDH comprised 0.1M Tris-Cl buffer (pH 8.4), 1mg ml⁻¹ Nicotinamide adenine dinucleotide (NAD⁺), 0.5mg ml⁻¹ Nitro blue tetrazolium (NBT), 0.1mg ml⁻¹ Phenazine methosulphate (PMS) and 0.05M Lithium lactate. The staining solution for MDH comprised 0.1M Tris-Cl buffer (pH 8.4), 1mg ml⁻¹ Nicotinamide adenine dinucleotide reduced (NAD⁺), 0.5mg ml⁻¹ Nitro blue tetrazolium (NBT), 0.1mg ml⁻¹ Phenazine methosulphate (PMS) and 0.05M Oxaloacetate. The staining mixture for SOD consists of phosphate buffer 0.1M (pH 7.4), 2.34mM NBT, 28μM riboflavin and 28mM NNN'N' Tetramethylethylenediamine (TEMED). The gel for the Catalase specific staining was incubated in 0.03% H₂O₂ in dark for 15 min. and stained in 2% potassium ferricyanide and 2% ferric chloride until the achromatic bands appeared against the dark green background.

Arginase (EC 3.5.3.1) assay was performed according to the method of [16]. The reaction mixture consisting of 25 mM sodium glycinate buffer (pH 9.5), 2.5 mM MnCl₂, 25 mM L-arginine and suitably diluted enzyme extract in the total volume of 2 ml was incubated for 15 minutes at 30°C. The reaction was terminated by adding 10% Perchloric acid, and protein was removed by centrifugation. Urea was determined in the supernatant. A unit of ARG activity was defined as that amount of enzyme, which produced one μmole of urea per hour at 30°C.

C. ANALYSIS OF EXPRESSION PATTERNS OF MARKER BY RT-PCR

Total RNA was extracted from brain and liver of aeri ally exposed fishes for 0hr (Control) and 20hrs (Experimental) using TRIzol (Invitrogen). The RNA was quantified by spectrophotometric analysis and the quality was checked on 1% agarose gel. The RNA was reverse transcribed using Oligo-dT primer according to suggested protocol for first strand cDNA synthesis kit (Applied Bio systems). The following primers LDHF:5'-CGGCTCAACCTGGT-3', LDHR:5'-TAGGCACTGTCCACCAC-3'; SODF:5'-TGGGGACAATACACAAGGCTGT-3', SODR:5'-TTTCCACCTTTGCCCAAGTCA-3'; CATALASE F:5'-CCTCCTCGTTCAGGATGTGGTT-3', CATALASE R:5'-CGAGGGTACGAAGTGTGTCAG-3'; βACTINF:5'-TGACGGGGTCAACCCACTGTGCCCATCTA-3', βACTINR:5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'; ARG IF: 5'-GTCCAGAAGAATGGAAGAGTCAG-3', ARG IR: 5'-GTTCCCAGGGTCTACGTCTCG-3'; ARG IIF: 5'-

GTCCAGAAGAATGGAAGAGTCAG-3', ARG IIR: 5'-GACTCCTTCAAACCTTACATG-3' were used for RT-PCR. The PCR products were analyzed on 2% agarose gel.

D. ANALYSIS OF PROTEIN PROFILE BY SDS-PAGE AND MALDI-MS/MS

The tissue lysates of 0hr and 20hrs were resolved on 12% denaturing Polyacrylamide gel for comparison of the protein profile. The gel is stained by Coomassie Brilliant Blue R-250 and selected bands were subjected to MALDI-MS/MS analysis. The in-gel trypsin digestion of selected protein bands was performed before the MALDI analysis as described earlier [17]. The protein database employed was NCBI nr (The National Center for Biotechnology Information non-redundant). The peptide sequences with highest scores of each spot were validated by BLAST P analysis. Data generated were analyzed in MASCOT database (www.matrixscience.com/) and Panther database (Pantherdb.org/).

E. STATISTICAL ANALYSIS

All experiments were carried out in triplicate and the enzyme activities and Integrated Density Value shown in mean \pm S.D. The significance of mean was analyzed by Student's t-test and $p < 0.05$ taken as the significance level.

III. RESULT

Levels of transcripts affected due to aerial exposure in liver

Aerial exposure in liver induces the activity of oxidative stress enzymes SOD (Fig 1B) and Catalase (Fig 1A). However, the mitochondrial SOD was upregulated, while cytosolic SOD was downregulated during aerial exposure significantly (Fig 1B). The upregulation of MDH (Fig 1C) activity was significantly higher than LDH (Fig 1D). Similarly, activity of mitochondrial arginase (ARG II) was significantly induced than cytosolic arginase (ARG I) (Fig 1E).

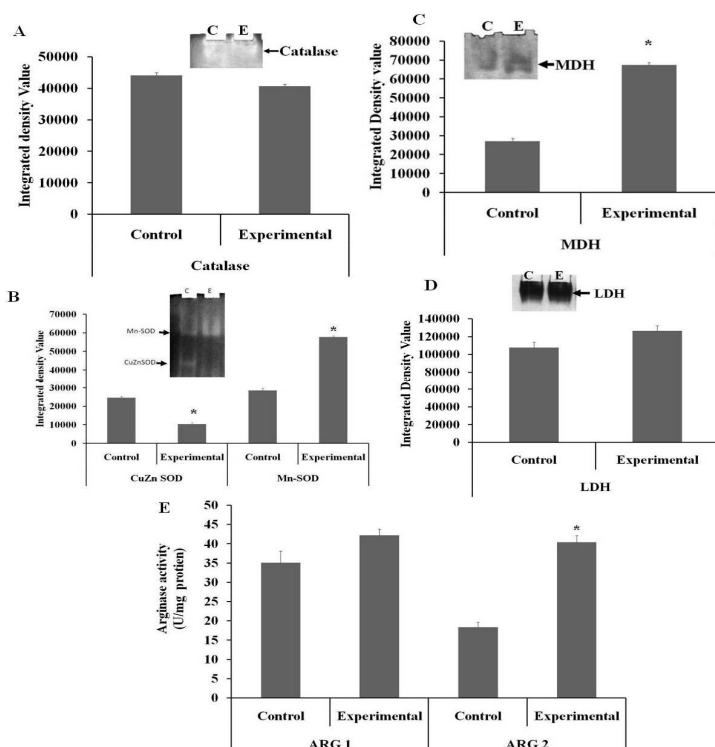


Figure 1 Shown the enzyme analysis of (A) Catalase, (B) Super oxide dismutase (SOD), (C) Malate Dehydrogenase (MDH), (D) Lactate Dehydrogenase (LDH) (E) Arginase I (ARG I), and Arginase II (ARG I) in *H. fossilis* liver. Three independent experiments were performed and the data presented in histogram is mean \pm SD. * represent the $p < 0.05$ by Student's t-test.

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The transcript level of oxidative stress enzymes (*SOD* and *CATALASE*) were up regulated (Fig 2A-B), however, expression of *LDH* was down regulated due to aerial stress (Fig 2C). The expression of arginase isoforms (*ARG I* and *ARG II*) were down regulated significantly (Fig 2D-E) aerielly exposed liver tissue of *Heteropneustes fossilis*. Aerial exposure condition were upregulated activity of *SOD* (Fig 3B) and Catalase (Fig 3A), *MDH* (Fig 3C), and *LDH* (Fig 3E) in brain. Whereas, activity of mitochondrial arginase (*ARG II*) and cytosolic arginase was increases in aerielly exposed brain (*ARG I*) (Fig 3D).

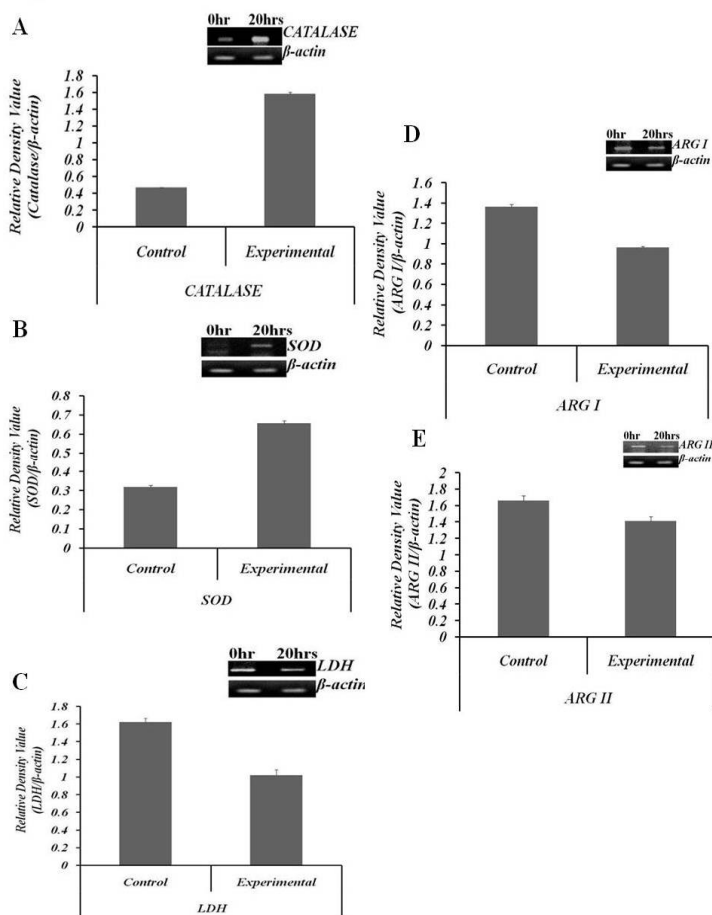


Figure 2 Shown the RT-PCR analysis of (A) CATALASE, (B) SOD (C) LDH, (D) ARG I, (E) ARG II in *H. fossilis* liver . Three independent experiments were performed and the data presented in histogram is mean \pm SD. * represent the $p < 0.05$ by Student's t-test.

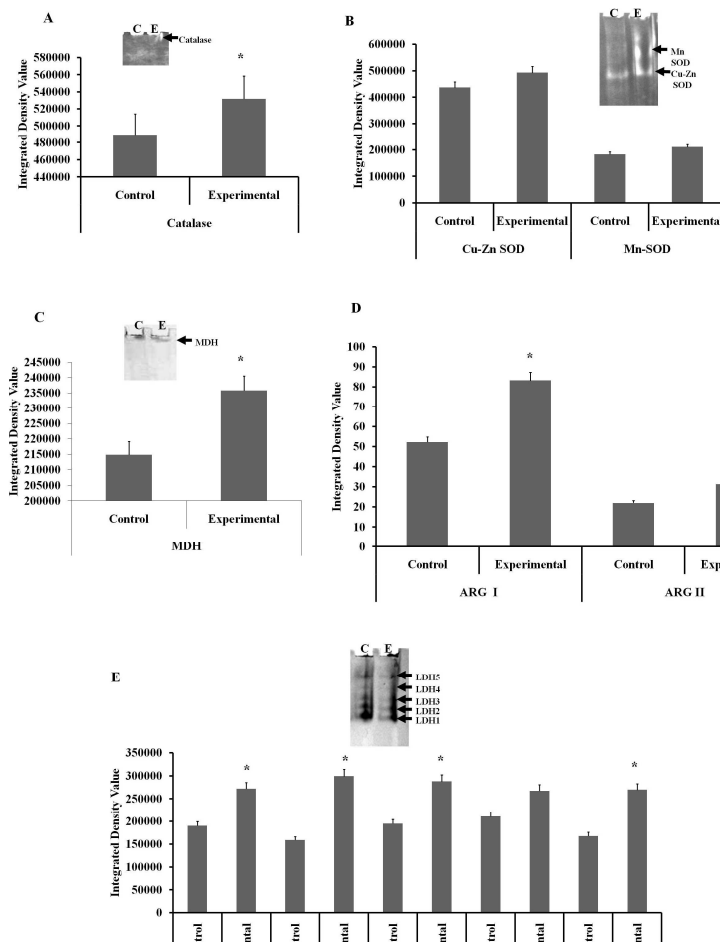
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Figure 3 Shows the enzyme analysis of (A) Catalase, (B) Super oxide dismutase (SOD), (C) Malate Dehydrogenase (MDH) (D) Arginase I (ARG I), and Arginase II (ARG II), (E) Lactate Dehydrogenase (LDH) in *H. fossilis* brain. Three independent experiments were performed and the data presented in histogram is mean \pm SD. * represent the $p < 0.05$ by Student's t-test.



The transcript level of oxidative stress enzymes (*SOD* and *CATALASE*) were up regulated (Fig 4A-B), however, expression of *LDH* was down regulated due to aerial stress (Fig 4C). The expression of arginase isoforms (*ARG I* and *ARG II*) were down regulated significantly (Fig 4D-E) aerially exposed liver tissue of *Heteropneustes fossilis*.

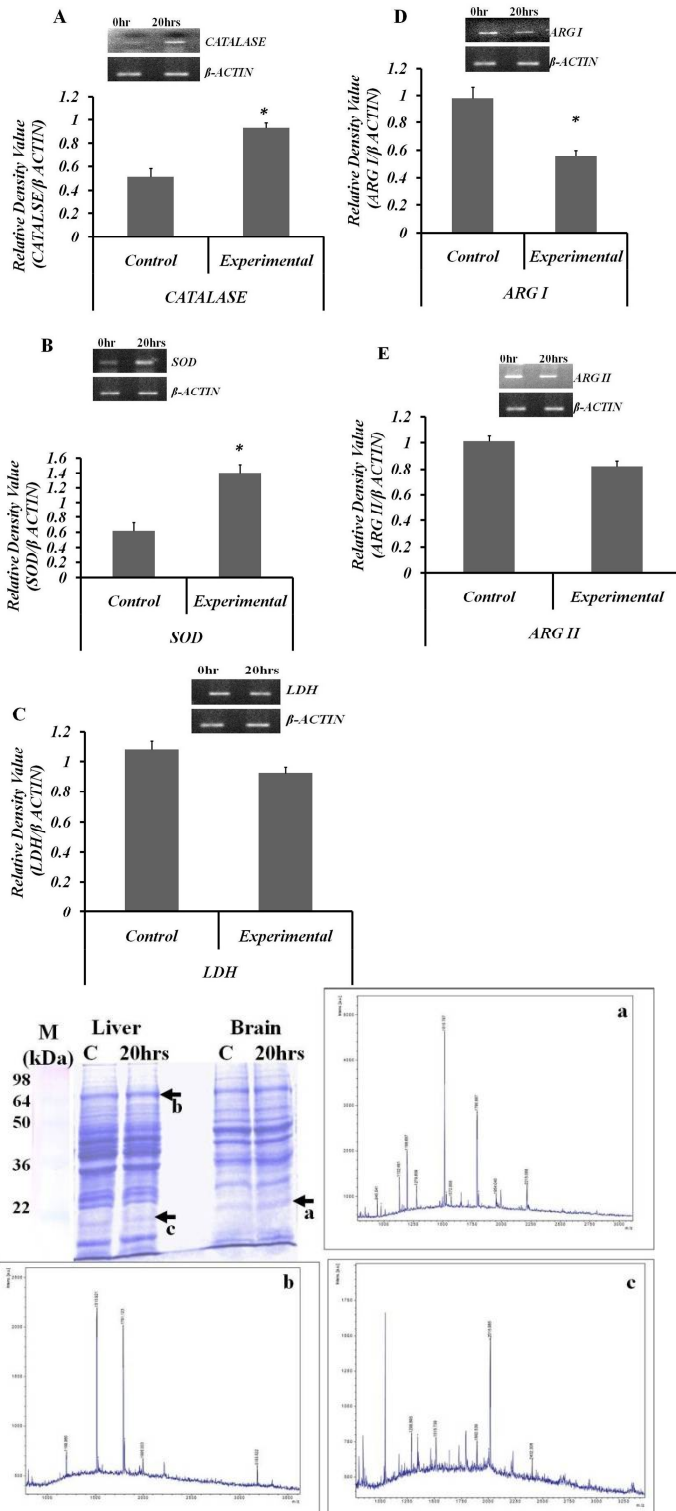


Figure 4 Shown the RT-PCR analysis of (A) *CATALASE*, (B) *SOD* (C) *LDH*, (D) *ARG I* and (E) *ARG II* in *H. fossilis* brain. Three independent experiments were performed and the data presented in histogram is mean \pm SD. * represent the $p < 0.05$ by Student's t-test.

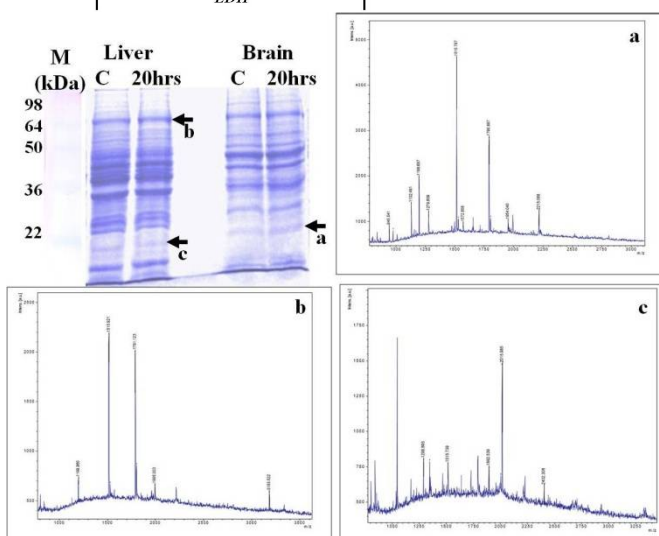


Figure 5 Protein profile analysis of control and 20hours aerial exposed liver (A) SDS-PAGE of control and 20hours aerial exposed brain and liver, (B) MS spectra of selected bands (a) Spot 1 40kDa, (b) Spot 2 25 kDa, and (c) spot 3in brain 25kDa,

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Table 1 MALDI-MS/MS analysis of identified peptides

| Name of protein | Function | Family |
|---|---|---|
| Polypeptide 'a' (Brain) | | |
| actin, beta [Poecilia reticulata] | Cytoskeleton formation | Actin family |
| Polypeptide 'b' (Liver) | | |
| Cofilin-2-like [Clupea harengus] | Actin binding protein, activated during apoptosis | Cofilin family |
| olfactomedin-like protein 2A-like [Scleropages formosus] | Protein homodimerization | Olfactomedin family |
| Melanoma-associated antigen G1 [Osmerus mordax] | Activation of Ubiquitin ligases | Immunoglobulin |
| Ras-related protein Rab-33A [Poecilia formosa] | Vesicle transport | GTPase family |
| Rho guanine nucleotide exchange factor 18-like isoform X2 [Oryzias latipes] | Induces formation of Actin stress fibres, causes oxidative stress | GTPase family |
| N-acetyltransferase 14 [Clupea harengus] | Transfer acetyl group | Camillo family |
| Interferon-inducible GTPase 5-like [Danio rerio] | Hydrolysis of GTP | GTPase family |
| Glyceraldehyde-3-phosphate dehydrogenase, partial [Epinephelus coioides] | Glycolysis, apoptosis | Dehydrogenases |
| CDK5 regulatory subunit-associated protein 1-like [Lepisosteus oculatus] | Apoptosis | Methylthiotransferase family |
| Mitochondrial 2-oxoglutarate/malate carrier protein [Fundulus heteroclitus] | Aminoacid transport | mitochondrial carrier family |
| HCLS1-binding protein 3 isoform X3 [Esox lucius] | Lymphocyte activation, protein kinases | Receptor-interacting protein (RIP) family |
| N-chimaerin-like [Scleropages formosus] | GTPase activating protein | GTPase family |
| Phosphatidylcholine-sterol acyltransferase-like, partial [Lepisosteus oculatus] | Acetyl transferase | Transferases |
| Choline kinase alpha-like [Stegastes partitus] | Phosphatidylcholine biosynthesis | Choline Kinase family |

Alteration in proteomic profile of aerially exposed Liver and Brain

The protein profile of aerially exposed liver was shown the involvement of different mitochondrial peptides (Fig 5A). The several polypeptides of experimental liver were not showing any significant difference in the intensity of bands except few, one near the molecular weight 40kDa, and another near to 25kDa. These two bands were analyzed by MALDI-MS/MS analysis.

The peptides were identified by MALDI-MS/MS (Fig 5B) and further categorized by Panther (TABLE 1). The data mining suggested the impairment of mitochondrial 2-oxoglutarate/malate carrier protein, L-2-hydroxyglutarate

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dehydrogenase, mitochondrial metabolic pool; Ras-related protein Rab-33A, Interferon-inducible GTPase, N-chimaerin-like were related to GTPase super family; Calcium-binding protein 39-like, Receptor-interacting serine/threonine-protein kinase 3 and HCLS1-binding protein 3 isoform falls in Receptor-interacting protein (RIP) family; Phosphatidylcholine-sterol acyltransferase-like protein and Choline kinase alpha were involved in Phosphatidylcholine synthesis; Cofilin, Olfactomedin, Rho guanine nucleotide exchange factor, Myosin-7-were involved in cytoskeleton formation, CDK5 and MAGE were related to apoptosis.

IV. DISCUSSION

In India, most of the above habitats of *H. fossilis* become dried in summer season due to prolonged drought. However, this fish can burrow and live at least for 30 days inside mudflats to avoid total dehydration in summer [18], [19]. Besides the nutritional value of its flesh, the fish also gains attention for its medicinal value [11], [20]. It can also be found in slightly brackish water bodies [11], [21]. Fishes were adapt multiple strategies to overcome the aerial exposure induced stress such as aerial exposure induced glutamine and alanine synthesis were found in fish as an adaptive mechanism in *Protopterus dolloi* [1]. Aerial exposure in swamp eel leads to ammonia loading up to 144 hrs of aerial exposure but they do not adopt ureagenesis rather glutamine accumulation is the adaptive strategy [2]. Lim et al. [9] also suggest the reduction in protein and amino acid catabolism is an adaptive strategy in *P. schlosseri* and *B. boddaerti* during aerial exposure. In *H. fossilis* tissue-specific analysis suggest that urea-genesis could be an adaptive strategy to aerial stress except brain. However, it turns to ammonotelism when re-immersed in the water [5]. In contrast to this report, no change in concentration of urea in the plasma, brain and liver of Chinese loach *Paramisgurunus dabryanus* [22] was observed where volatilization of NH_3 and synthesis of alanine might be an adaptive strategy to aerial exposure.

Aerial exposure caused the significant decrease in SOD but no change in the level of Catalase up to 18 hrs of aerial exposure in brain of *H. fossilis* [4] whereas increased activity of Catalase was observed by Lushchak et al. [23]. Results of the present study indicate that after 20h aerial exposure, liver and brain of *H. fossilis* failed to maintain the balance between the generation and removal of ROS and acceleration of anti-oxidative capacity. The aerial stress impaired the mitochondrial energetic but the enhanced activity of MDH in brain and liver of this fish were contradictory to earlier reports [4] or induction in MDH also involved in amino acid synthesis adaption to restore the high ammonia level as observed in *P. dabryanus* [22]. The enhanced activity of arginase at the protein level might be the first level of defence against the aerial exposure stress. However, the down regulation of arginase expression is due to the differential effect of aerial exposure on the gene and protein of arginase isoforms in fish liver and brain. During the study of the effect of aerial exposure, the significant effect was observed on arginase-II than arginase-I. The enhanced arginase-II was might be due to decreased protein content and enhanced ROS generation. Decrease body weight was also reported earlier due to aerial exposure [4]. Arginase –II is involved in the synthesis of proline and glutamate which are the building blocks of collagen and proteins. However, we observed inverse relation in the pattern of oxidative stress enzymes, LDH, MDH, and arginase in brain and liver tissue at protein and transcript level. The similar pattern of alterations of the oxidative stress markers, bioenergetic markers and ammonia metabolizing markers in the brain and liver tissue suggest despite having different physiology both tissue adapt same mechanism (enhanced accumulation of urea) as an adaptive strategy during aerial stress.

The alteration in Cofilin, Olfactomedin, Rho guanine nucleotide exchange factor, Myosin-7-indicate impact on polymerization and de-polymerization of actin. Rho-guanine nucleotide exchange factor 18-like isoform X2 was also known for the generation of actin stress fiber that induces oxidative stress and leads to depolymerisation of actin fiber and finally resulted in muscle degradation. It is also in agreement to report of muscles degradation induced by aerial exposure [12]. The mitochondrial 2-oxoglutarate/malate carrier protein and L-2-hydroxyglutarate dehydrogenase were also identified that are involved in malate-aspartate shuttle indicating the imbalance of mitochondrial homeostasis, which we also observed in the biochemical analysis of MDH (Fig 1, 3) in brain and liver. However, CDK5 regulatory subunit-associated protein and cofilin induction suggested the induced apoptosis in the aerially exposed liver of *Heteropneustes fossilis*. In the brain, the actin is identified universally which also confirmed that aerial exposure leads to the deterioration of cytoskeleton in the brain similar to liver.

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IV. CONCLUSION

In conclusion, the aerial exposure severely damaged the actin filaments and may induce apoptosis by enhancing the ROS generation and ammonia toxicity in liver and brain of *Heteropneustes fossilis*. Despite urea genesis, synthesis of other amino acid may be a preferred adaptive mechanism in *Heteropneustes fossilis* during aerial stress.

V. ACKNOWLEDGEMENT

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VI. CONFLICT OF INTEREST

Authors have no conflict of interest.

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