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ARTICLE

Ammonia ambiance induces SIRT5 regulated expression of EGF-AKT-mTOR axis in Asian stinging catfish *Heteropneustes fossilis* (Bloch) 1974

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ABSTRACT The present study was aimed to understand whether ammonia exposure induces oxidative stress in *Heteropneustes fossilis* and what is the fate of the excess ammonia in the skeletal muscle of the fish. The experiments were performed in two different sets as control and treated (repeated three times with fresh specimens) of aquaria with 25 mM of ammonium chloride treatment and the tissues were collected in different time intervals (24 h, 72 h and 7 days). The collected tissues were studied to understand the change of SIRT5 levels in liver and skeletal muscle tissues. The results that were obtained from investigation of MDA (malondialdehyde) and superoxide dismutase (SOD) revealed that the fish undergoes extensive oxidative stress when exposed to ammonia ambiance. Further, after 7 days of ammonia exposure increase in the levels of glutamate and glutamine revealed the fate of excess ammonia in the skeletal muscle of the fish. Moreover, the levels of cell proliferator proteins like EGF, AKT and mTOR were also analysed and found an increase in their expression with a time dependent manner. It indicates that the excess ammonia could be utilised in synthesising protein and triggering cell growth and proliferation even under such harsh condition of ambient ammonia.
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Introduction

An increase in ammonia (concentration) can generate reactive oxygen and nitrogen species in tissues. It is an already established fact that ammonia accumulation in the brain due to dysfunction in the liver can contribute to the pathogenesis of liver encephalopathy (Bobermin et al. 2015; Skowronska and Albrecht 2013). Ammonia, which is mainly produced from the deamination of amino acids is a toxic component in blood and tissues. It can significantly reduce cell viability and induce apoptosis in the cells (Wang et al. 2018). Ammonia is produced during protein catabolism and is an essential component for nucleic acid and protein biosynthesis. Inadequate ammonia detoxification can have adverse effects on muscle size and strength in mammals. On the other hand, ammonia can be utilized for muscle growth in avian species (Stern and Mozdziak 2019).

Among the teleosts, catfishes can tolerate stress to a great extent like thermal stress, salinity stress, heat stress, bacterial exposure, environmental and abiotic stressors like hypoxia (Burleson and Silva 2011; Meritha et al. 2018; Nepal and Fabrizio 2019; Saha et al. 2011; Sarma et al. 2012; Tan et al. 2019; Zhou et al. 2018). The walking catfish (*Clarias batrachus*) can survive in very high ambient ammonia concentration (25 mM) during certain seasons of the year in the natural habitat (Banerjee et al. 2019). They are predominantly ammoniotelic but can totally turn into ureotelism when exposed to higher ambient ammonia. This can be significantly evident from the increase of some of the key urea cycle enzymes from hepatic and non-hepatic tissues (Banerjee et al. 2020). That is why it is considered as a potential ureogenic teleost because of its significantly functional ornithine urea cycle (Saha et al. 2003; Saha and Das 1999). There are various other fish species that can tolerate ammonia stress. Detoxification of ammonia to urea has also been observed in elasmobranchs (Randall and Tsui 2002).

It is evident that a rise in the expression of inducible nitric oxide takes place in catfishes when exposed to ambient ammonia which is considered to be a potential strategy to enhance the adaptive capacity and survivability of catfish under various adverse environmental conditions in nature (Ajani 2008; Kumari et al. 2018; Zhang et al. 2018). Fish like *Clarias magur* and *Clarias batrachus* show a

differential expression of multiple glutamine synthetase genes and carbamoyl phosphate synthetase III gene during excess ambient ammonia condition (Banerjee et al. 2018). Studies have reported that ammonia toxicity can induce glutamine accumulation in catfishes (Li et al. 2016; Saha et al. 2007). Catfishes like *H. fossilis* and *C. batrachus* have immense capacity and physiological adaptive strategies related to amino acid metabolism along with the presence of functional and regulatory urea cycle when exposed to very high ambient ammonia in the air, water or in the mud in their habitat (Saha and Ratha 1998; Saha et al. 2000; Saha et al. 2002).

However, a strong metabolic support is necessary for acquiring necessary energy pool to appropriately response such behaviours of catfish. The metabolic backup is further required to augment growth in catfish under stressed ammonia ambiance. In this context, deficiency of SIRT5 is reported to suppress mitochondrial NADH oxidation as well as inhibition of ATP synthase activity resulting in reduced formation of ATP and subsequently activates AMPK in cultured cells and mouse hearts (Zhang et al. 2019). Understanding SIRT 5 is important because it has a strong control over mitochondrial energy metabolism and its level regulated by PGC1 α and AMPK (Buler et al. 2014). This information are hardly available in fish, although energy supplement is shown as vital criteria in fish group. Understanding these kinds of adaptations to withstand adverse situation by catfish may further enlighten possibility of other similar strategy of fish growth in aquaculture practice.

With this background, we attempted to detect the oxidative stress in ambient ammonia in *Heteropneustes fossilis,* accordingly, and change in expression of mammalian SIRT5. To understand the growth under ammonia ambiance, expression of growth factors like epidermal growth factor (EGF) in skeletal muscle tissue and few of its downstream proteins AKT (Protein kinase B) and mTOR (Mechanistic target of rapamycin) of a specific pathway in *H. fossilis* exposed to ammonia ambiance were also analyzed. The amino acids like glutamate and glutamine are also measured in skeletal muscle tissue in ammonia exposed *H. fossilis*.

Materials and Methods

Animals

Live samples of *H. fossilis* (50 ± 10 g body mass) were purchased from commercial sources and acclimatized in the laboratory approximately for 15 days at 28 ± 2 °C with 12 h:12h light dark photoperiod before experiments. Minced pork liver and rice bran (5% of body wt) were given as food, and the water was changed on alternate days. Food was withdrawn 24 h prior to the experiment.

Experimental protocol

A set of fish (n = 15) of similar sizes (Length = 25 ± 5 cm, Weight = 50 ± 10 g) was weighed and placed in aquarium containing 1 L of 25 mM NH_4CL solution prepared in fresh water (pH 6.82 ± 0.11). Another set of fish (n = 15) were kept in aquarium containing 1 L of fresh water (pH 7.04 ± 0.10) that served as controls. Both NH_4CL solution and the fresh water from each aquarium (treated and control) were replaced with a fresh medium every day at a fixed time. On first, third and seventh day, 5-5 fish (n = 5) from each treatment were removed and sacrificed. No mortality of fish was observed during the period of experiment. Before tissue collection, fishes were narcotized using MS222. The overall experiment was repeated three times. Liver and skeletal muscle tissues were dissected out from all the treated as well as control fish and tissue samples were stored at -20 °C. All the assays were completed within 2 weeks of collecting the tissue.

Tissue collection and processing

Tissues (skeletal muscle, and liver) were collected from *H. fossilis* and processed for further analysis. The skeletal muscle tissues were collected from head region (HM), trunk region (TRM) and tail region (TLM) for estimating the total protein in these regions separately. Before every analysis, the pooled tissues (100 mg) kept in lysis buffer (0.1M phosphate buffer, pH 7.2) were homogenized (10% homogenate) using micro tissue homogenizer. The tissues homogenized were then centrifuged in 10000 g for 15 min. The supernatant of the 10% homogenate was collected and used for all biochemical assessments (MDA, SOD, glutamate and glutamine). For western blot analysis, tissue proteins of 100 μg from the lysate were used.

Biochemical assays

The biochemical assessment was performed for estimating total skeletal muscle protein, MDA (malondialdehyde), antioxidant enzyme SOD (superoxide dismutase), glutamate and glutamine. The total skeletal muscle protein was estimated using Lowry's (1951) method of protein estimation. MDA assay was performed according to the method of Aust (1985). MDA is a product of lipid peroxidation and reacts with TBA (thiobarbituric acid) to give a red species named TBARS (thiobarbituric acid reactive substance). The antioxidant enzyme SOD assay was performed following the method of Ewing and Janero (1995) while glutamate assay together with glutamine quantification were performed using assay kit (CCK037-100).

Electrophoresis and immunoblotting

The desired quantity of tissue protein (100 μg) was used in

Table 1. Antibodies used for immunoblotting of tissues from *H. fossilis*.

Primary antibody	Company	Art No.	Secondary antisera for immunoblotting	Company	Art No.
SIRT5	Elabscience	E-AB-15844	Anti-rabbit antibody	Sigma Aldrich	A3687
EGF	Abclonal	A2720			
AKT	Abclonal	A5523			
mTOR	Abclonal	A2445			
α-tubulin	Affinity Biosciences	AF7010			

10% SDS-PAGE and was transferred to PVDF membranes through transfer buffer (25 mM Tris, 193 mM glycine, 20% methanol, pH 8.5) for 1.5 h. Western blot analysis was performed for the proteins like SIRT5, EGF, AKT, mTOR using specific antibodies (Table 1). Membrane bound primary antibodies were visualized using corresponding secondary antibodies at 1:1000 dilutions, which was tagged with alkaline phosphatase and developed with corresponding substrates, 5-bromo-3-chloro-3-indolyl phosphate/ nitrobluetetrazolium (BCIP/NBT). Band intensities were quantified by utilizing Image J software (NIH, Bethesda, MD).

Statistical analysis

Homogeneity of variances of data sets was tested using Levene's statistics. The Kruskal-Wallis H test was computed where Levene's statistics did not comply to $p > 0.05$.

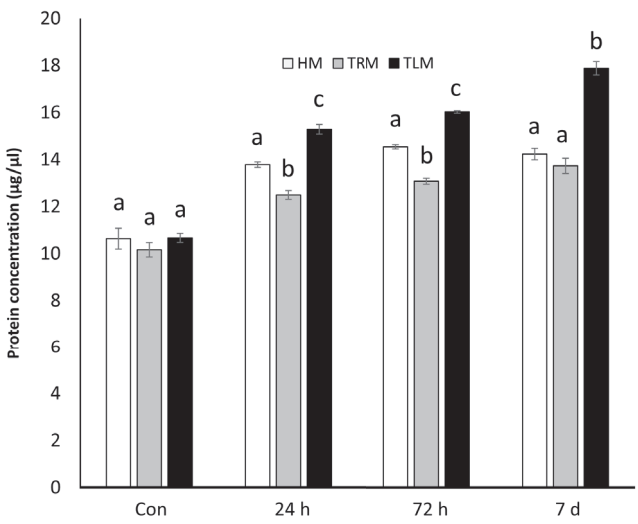

Figure 1. Determination of total protein concentration (µg/µl) in skeletal muscle of *H. fossilis* under three different time intervals of 25 mM ammonia exposed conditions compared with control: Control, 24 h, 72 h and 7 days. Data are shown as Mean ± SE. Comparison of mean are performed following Kruskal-Wallis non-parametric test. Bars with different lower-case letters (a, b, c) show statistically significant difference at $p < 0.05$. HM: Head Region Muscle; TRM: Trunk Region Muscle; TLM: Tail Region Muscle.

In case of all statistical analyses, α level was fixed at 0.05. SPSS 16.0 was used for all statistical analyses.

Results

The present study observed some effects in *H. fossilis* on exposure to ammonia (25 mM/L) for three different time intervals. On exposure for 7 days to ammonia, the total muscle protein level in the skeletal muscle showed an increase in concentration of protein. When analysed in head, trunk, and tail regions, from the skeletal muscles of *H. fossilis,* the treatments showed overall significant increase of protein concentration compared to the control (Kruskal-Wallis H Test, $\chi 2 = 113.96$, $p = 0.00$; Fig. 1). However, skeletal muscle from tail region, showed comparatively high level of protein concentration than the skeletal muscle of head and trunk regions. It was also observed that the fish was under oxidative stress during ammonia exposure, indicated, in terms of increase in MDA and antioxidant enzyme SOD in liver and skeletal muscle tissue (Fig. 2). In liver tissue, the MDA level was maximum and significantly increased at 24 h of ammonia exposure as compared to the control (One-way ANOVA, $F = 131.093$; 3, 36; $p < 0.05$; Fig. 2). Another significant increase of MDA was observed after 7 days of ammonia exposure giving a minor hint of chronic oxidative stress in the fish. The antioxidant enzyme SOD was found to increase prominently after 7 days of ammonia exposure in liver tissue (Kruskal-Wallis H Test, $\chi 2 = 36.585$, $p = 0.00$; Fig. 2). In skeletal muscle, significant increase of MDA was observed after 72 h of ammonia exposure as compared to the control which confirms that the fish was under oxidative stress, until it falls sharply after 7 days of ammonia exposure (One-way ANOVA, $F = 114.503$; 3, 36; $p < 0.05$; Fig. 2). In liver the results of antioxidant enzyme SOD showed a similar pattern in skeletal muscle signifying a constant stress at 7 days of ammonia exposure (Kruskal-Wallis H Test, $\chi 2 = 36.585$, $p = 0.00$; Fig 2). A more or less similar pattern of expression of SOD was also observed in skeletal muscle of the fish (Fig. 2).

In both liver and skeletal muscle tissues, increase in expression of SIRT 5 after 24 h of ammonia exposure

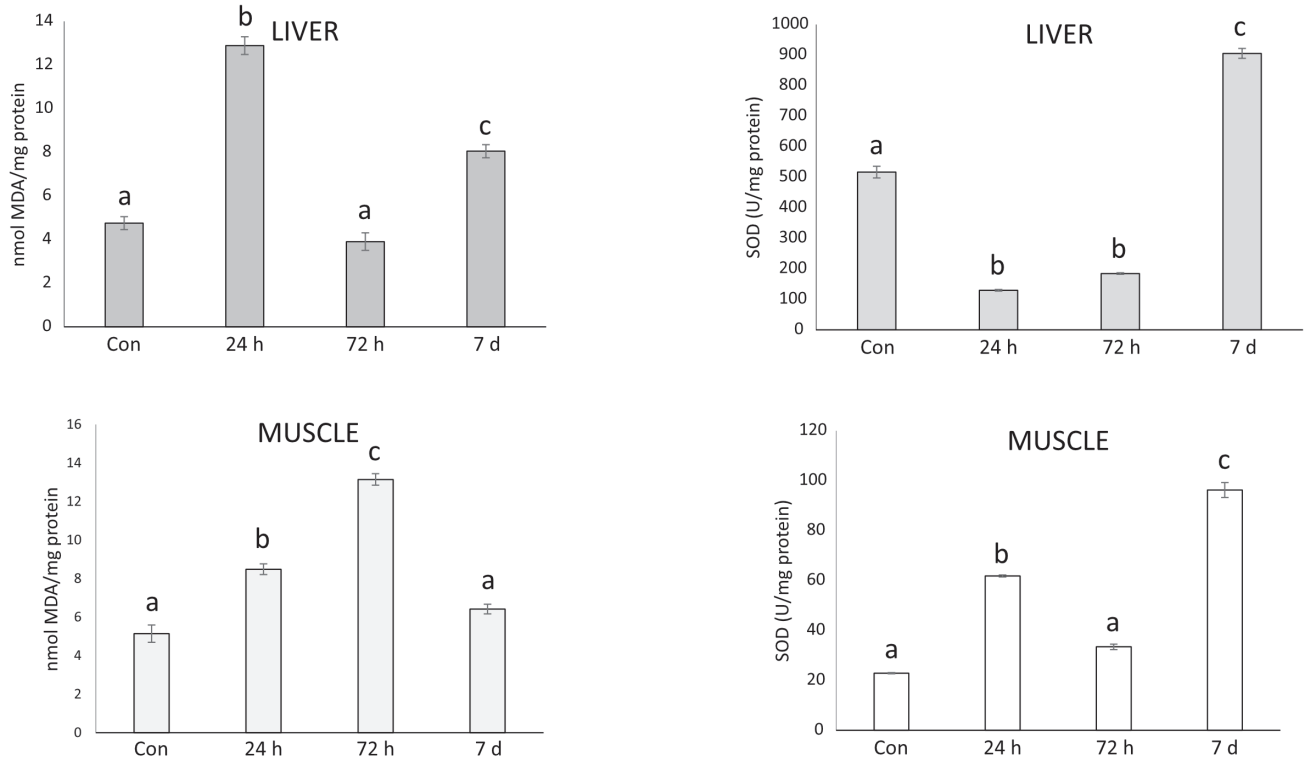

Figure 2. Determination of SOD (U/mg protein) and MDA (nmol/mg protein) in liver and skeletal muscle of *H. fossilis* under three different time intervals of 25 mM ammonia exposed conditions compared with control: Control, 24 h, 72 h and 7 days. Data are shown as Mean ± SE. Comparison of mean are performed following Kruskal-Wallis non-parametric test for SOD and Comparison of mean are performed following One-way ANOVA parametric test for MDA. Bars with different lower-case letters (a, b, c) show statistically significant difference at $p < 0.05$.

was prominent. Similar to MDA, the expression of SIRT 5 was peaked again after 7 days of ammonia exposure (One-way ANOVA, $F = 36.191$; 3, 20; $p < 0.05$ for SIRT5 in liver tissue; One-way ANOVA, $F= 195.803$; 3, 20; $p < 0.05$ for SIRT5 in skeletal muscle tissue; Fig. 3). On the other hand, proteins which are involved in the pathway of cell proliferation and growth (EGF, AKT, mTOR) were examined after exposure to ammonia at 24 h, 72 h and 7 days and found to exhibit time dependent significant upregulation in their expression (Kruskal-Wallis H Test, $\chi2 = 19.547$, $p = 0.00$ for EGF in skeletal muscle; One-way ANOVA, $F = 28.035$; 3, 20; $p < 0.05$ for AKT in skeletal muscle tissue and One-way ANOVA, $F = 76.588$; 3, 20; $p < 0.05$ for mTOR in skeletal muscle tissue; Fig. 4). Simultaneously, the levels of glutamate and glutamine also showed gradual increase from 24 h to 7 days of ammonia exposure (Kruskal-Wallis H Test, $\chi2 = 9.974$, $p = 0.019$ for glutamate in skeletal muscle tissue; Kruskal-Wallis H Test, $\chi2 = 10.385$, $p = 0.016$ for glutamine in skeletal muscle tissue; Fig. 5).

Discussion

It is obvious from the results that *H. fossilis* experiences oxidative stress when exposed to 25 mM of ambient ammonia. Oxidative indicators like MDA and SOD showed clear indication, both in skeletal muscle and liver that on 7th day of ammonia exposure, the fish underwent oxidative stress. Both MDA and SOD are already considered as efficient stress marker for animals. However, the results also depict that under ammonia exposure the total protein of the skeletal muscle increase significantly, which markedly visible from the tail region. How the augmentation of such increase in total protein under ammonia ambiance in catfish happens needs a clarification.

It led us to investigate the expression of a mammalian protein called SIRT5, one of the seven members of the nicotinamide adenine dinucleotide (NAD^+)-dependent sirtuin family of lysine deacetylases in mammals. SIRT5 is localized in the mitochondrial matrix and removes succinyl, malonyl, and glutaryl groups from protein targets in the mitochondrial matrix and other subcellular compartments (Bringman-Rodenbarger et al. 2018; Nakagawa et al. 2009). SIRT5 interacts with carbamoyl phosphate syn-

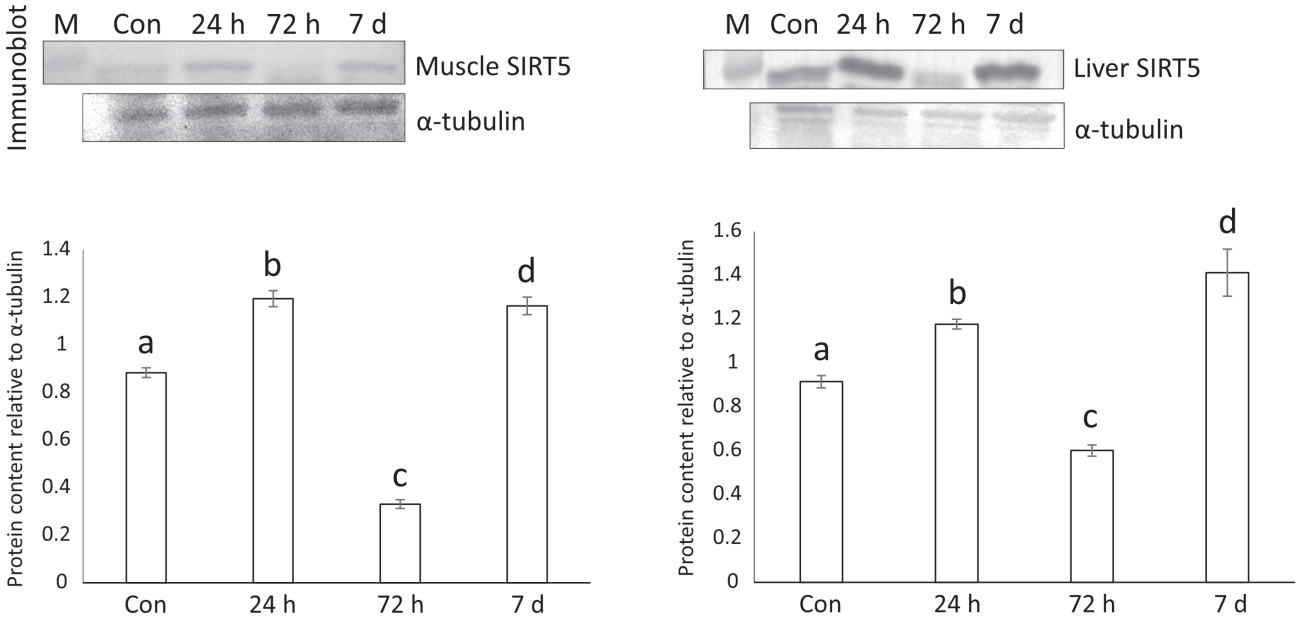

Figure 3. Immunoblot of SIRT5 in liver and skeletal muscle of *H. fossilis* under three different time intervals of 25 mM ammonia exposed conditions compared with control and its densitometry analysis: control, 24 h, 72 h and 7 days. Data are shown as Mean ± SE. Comparison of mean are performed following One-way ANOVA parametric test. Bars with different lower-case letters (a, b, c, d) show statistically significant difference at $p < 0.05$. M: molecular weight marker; Con: Control.

thetase 1 (CPS1), an enzyme that catalyzes the initial step of the urea cycle for ammonia detoxification and disposal (Saha and Ratha 1998). SIRT5 mainly deacetylates CPS1 (carbamoyl phosphate synthetase I) and upregulates its activity and plays a major role in ammonia detoxification and disposal by activating CPS1 (Nakagawa et al. 2009). There are studies which has reported the fact that catfishes under ammonia ambience upregulates SIRT5 and triggers the urea cycle for ammonia detoxification (Li et al. 2016). Increase of SIRT5 also is reported to be linked to ROS detoxification (Kumar and Lombard 2018). In the present study, it was found that SIRT5 expression was significantly upregulated in the liver and skeletal muscle of fish exposed to ammonia ambiance. The expression of SIRT5 was hiked twice with an intermittent gap at 72 h. The gap, at 72 h between these two hikes. i.e., at 24 h and 7 days of SIRT5 upregulation is significant, as the MDA during this period also remained high in both skeletal muscle and liver tissues. In a study, Liu et al. (2013) has showed that SIRT5 regulates oxidative stress induced apoptosis in cardiomyocytes. A similar function of SIRT5 may also be advocated here. As already mentioned in the introduction, the SIRT5 is mainly involved in activating the detoxification process through CPS1. It was necessary to examine the fate of glutamate and glutamine, being the substrate of the enzyme CPS1 through which it initiates detoxification cycle. It is possible that, as soon as the exposure of fish to ammonia toxicity, the SIRT5 gets upregulated in defence of the skeletal muscle cells, resulting in an increase in almost all the measured parameters. However, a chronic exposure may always induce a secondary defensive mechanism, which is visible from the occurrence of second spike of SIRT5. For first instance, safeguarding against the oxidative stress would be the best appropriate and possible explanation in the skeletal muscle cell. Consequently, the second instance may direct towards an alternative pathway of detoxification through protein metabolism.

This led us to measure the levels of glutamate and glutamine in the skeletal muscle of the fish. Both glutamate and glutamine are known to play important roles in metabolism. From the result, it is clear that, both glutamate and glutamine levels were increased from the first day with a significantly high level on 7th day in fish exposure to ammonia. It indicates that, these two by-products, advocates an alternative defence mechanism might be contributing to protein metabolism. Thus, under chronic ammonia exposure, the levels of glutamate and glutamine increased indicating that the excess ammonia not directed towards detoxification might be routed towards synthesis of glutamate and glutamine. This helps to maintain proteostasis under stress condition. If this is the case, there is possibility, that major growth proteins would be upregulated during this period.

Of the numerous protein synthesis and cell proliferation pathways, a significant pathway is the EGF-AKT-

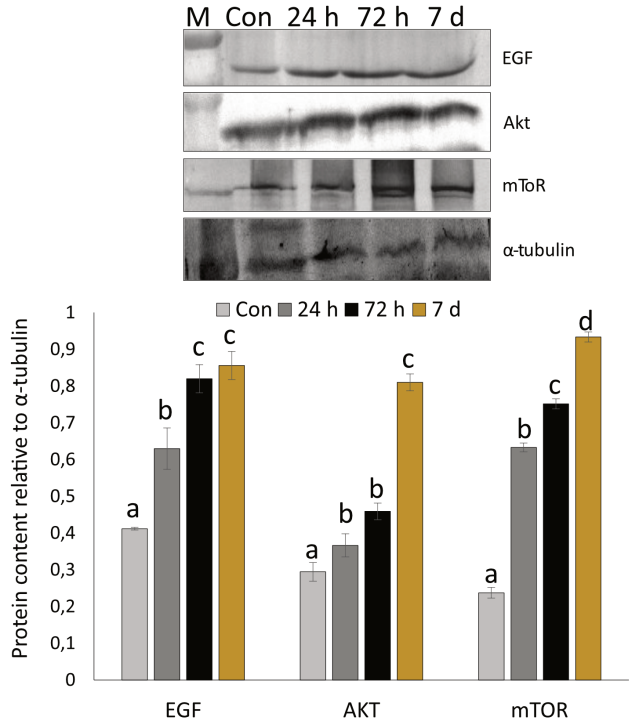

Figure 4. Immunoblot of EGF, AKT and mTOR in skeletal muscle of *H. fossilis* under three different time intervals of 25 mM ammonia exposed conditions compared with control and its densitometry analyses: control, 24 h, 72 h and 7 days. Data are shown as Mean ± SE. Comparison of mean are performed following Kruskal-Wallis non-parametric test for EGF. Comparison of mean are performed following One-way ANOVA parametric test for AKT and mTOR. Bars with different lower-case letters (a, b, c, d) show statistically significant difference at $p < 0.05$. M: molecular weight marker; Con: control.

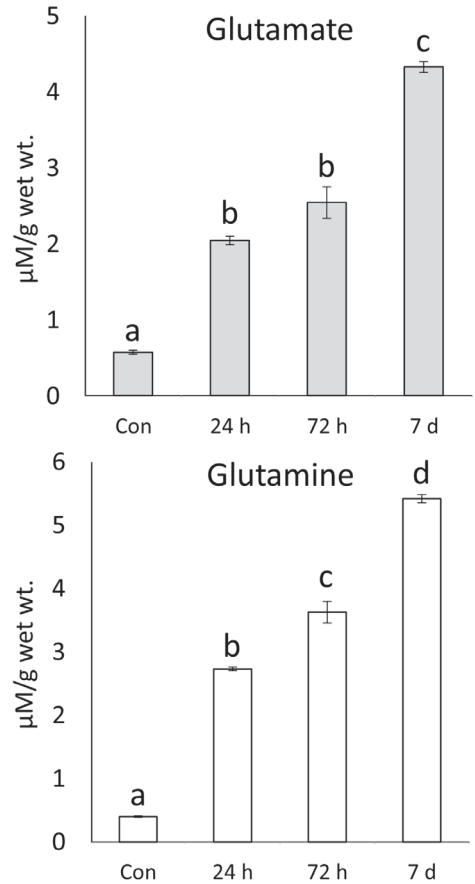

Figure 5. Determination of glutamate and glutamine (μM/g wet weight) in skeletal muscle of *H. fossilis* under three different time intervals of 25 mM ammonia exposed conditions compared with control: control, 24 h, 72 h and 7 days. Data are shown as Mean ± SE. Comparison of mean are performed following Kruskal-Wallis non-parametric test. Bars with different lower-case letters (a, b, c, d) show statistically significant difference at $p < 0.05$. Con: control.

mTOR pathway. In mammals, this pathway has been shown to play crucial role in cell differentiation, muscle development, environmental adaptation, cell growth, proliferation, apoptosis and promotes insulin-stimulated glucose uptake and storage which establishes a link between growth and development with metabolism (Xu et al. 2020). This study of the pathway also elucidates the pathogenesis of cerebrovascular diseases, neurodegenerative diseases, diabetes mellitus and other malignant tumours. It is a vital signalling pathway for growth that provides new molecular targets for diagnosis and treatments of various human diseases like ovarian cancer, haematologic malignancy, colorectal cancer, endometrial cancer, and lung tumorigenesis (Dobbin and Landen 2013; Kawauchi et al. 2009; Memmott and Dennis 2009; Slomovitz and Coleman 2012; Xu et al. 2020). In our study, it was observed that proteins in this pathway were upregulated on 7th day compared to control, reflecting a recovery of skeletal muscle growth compensating the loss during the exposure at 72 h. We could say that there is a time dependent increase in expression of these proteins. Thus, through the contributors like glutamate and glutamine, protein syntheses might get accelerated in *H. fossilis* under ammonia ambiance. Glutamine can even directly upregulate mTOR for cell growth and proliferation (Csibi et al. 2013; Zhu et al. 2020). So, from these outcomes, a relation of the increased levels of glutamate and glutamine under ammonia ambience indirectly initiated by SIRT5 may be established. With the increase in the levels of these amino acids, help us to explain the possible mechanism of growth and survival of stinging catfish in ammonia rich environment.

Conclusion

From the results it may be said that under chronic exposure to ammonia for 7 days the fish experiences oxidative stress, but the excess ammonia in the body of the fish is not directed towards detoxification, rather is used in synthesis of glutamate and glutamine which indirectly regulated by expression of SIRT5 and in turn, promotes protein synthesis and thereby cell growth and proliferation. From this, it may be said that even under chronic exposure to ammonia for 7 days, the catfish, *H. fossilis* has the potential to bypass the excess ammonia from the detoxification pathway by utilizing it in inducing protein synthesis and upregulating the pathway of cell growth and proliferation.

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