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In Vivo Action of Ammonia on Ion Transport Function in Liver and Heart Mitochondria of Immersion-Stressed Air-Breathing Fish (*Anabas testudineus* Bloch)

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Abstract

Ammonia, as an endogenous respiratory gas, is produced during protein and amino acid metabolism. Accumulation of excess ammonia is toxic, and fishes have developed mechanisms to defend against ammonia toxicity. Here, we tested the *in vivo* action of ammonia in an air-breathing fish to find how it modulates mitochondrial ion transport in fish heart and liver. We thus analysed the activity pattern of mitochondrial ion transporters such as mitochondrial Ca^{2+}ATPase, mitochondrial H^{+}ATPase and mitochondrial F_1F_0 ATPase in heart and liver of air-breathing fish *Anabas testudineus* which were kept for immersion-induced hypoxia stress. In addition, plasma metabolites such as glucose and lactate were also quantified. Oral administration of ammonia solution [$(NH_4)_2SO_4$; 50ng g^{-1}] for 30 min increased mit.Ca^{2+}ATPase activity in heart but lowered its activity in liver mitochondria. A reduced mit.H^{+}ATPase activity was found in heart but in liver its activity showed increase after ammonia treatment. F_1F_0 ATPase increased significantly in heart but showed reduced activity in liver mitochondria. Administration of ammonia in immersion-stressed fish, however, nullified the excitatory response of heart and liver mitochondria in the immersion-stressed fish. Overall, the data indicate that ammonia can play a significant physiological role in the regulation of mitochondrial ion homeostasis in the liver and heart of air-breathing fish during their acclimation to hypoxia stress.

Keywords: Ammonia, *Anabas testudineus*, Fish, Hypoxia, Ion Transport, Mitochondria, Stress

1. Introduction

Ammonia, a highly diffusible gas, is endogenously generated in all cells and that diffuses through the lipophilic membrane across a concentration gradient and gets protonated to ammonium ion, NH_4^+. It can exist either as a weak base in gaseous form (NH_3) or a weak acid in salt (NH_4^+) form, and in teleost fish passive diffusion of ammonia occurs across the gills[1-2]. Ammonia can freely diffuse through the plasma membrane and can influence physiological processes[3]. However, no study has addressed this question, particularly in fishes.

Liver as the major site of ammonia production and detoxification centre, plays a central role in the regulation of plasma ammonia concentration of the body. Dietary proteins and nitrogen metabolism act as a major source of ammonia, and when released ammonia gets absorbed into portal veins and is transported to liver where inside the mitochondria glutamate dehydrogenase catalyzes its conversion to glutamate[4]. Glutamine and alanine in various tissues act as carrier of ammonia and get circulated to the liver. The metabolic pathways which result in ammonia production also provide the precursors essential for gluconeogenesis and lipogenesis[5]. In fishes, circulatory

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system plays an important role in ammonia transport and ammonia production occurs in the liver and gets transported to the gills and kidney for excretion. But in mammals, it gets converted to urea and transported to the kidney for elimination through urine[6].

As in vertebrates, teleost heart circulates the metabolites and by-products of various metabolic processes and assists in their transport and detoxification in various tissues[7]. In fishes, heart pumps venous blood to ventral aorta into gills and to the somatic vasculature[8]. The cardiac cycle is characterized by the sequential contraction of the atrium followed by the ventricle, where the maintenance of electrochemical gradients of Na^+, K^+ and Ca^{2+} ions is essential for normal physiological functioning[9]. The cardiac excitations and contractions are energy-dependent activites which utilize the energy released for proper transport of ions across the membrane, and the performance of ion-dependent ATPase acts as an index for cardiac excitation[10].

Air-breathing fishes dwell in an environment where the oxygen supply is challenged but they are assisted with accessory respiratory organs[11]. Therefore, fishes with bimodal breathing mechanisms utilize both gill and air-breathing organs to hold air in their buccal cavity during immersion which reduces the ventilation and ammonia excretion rates. Some of them have evolved the ability to emerge from water and make excursions to land or burry themselves in shallow mud during drought seasons. During such conditions fish face inability to properly excrete ammonia or when trapped in crevices or shallow water pools, the continuous excretion of endogenous ammonia into small volumes of external water leads to high external ammonia which in turn results in retention of endogenous ammonia and uptake of exogenous ammonia[11].

Fishes have developed various strategies to avoid ammonia-induced toxic manifestations which include conversion of ammonia to less toxic compounds for accumulation and excretion, facilitating ammonia excretion and reducing ammonia entry, reduction of ammonia production and tolerance of ammonia at cellular and tissue levels[12-14]. On the contrary, physiological levels of ammonia may be essential for major energy pathways in fish where internal ammonia homeostasis may maintain the tissue and plasma concentration of ammonia. It is likely that changes in environmental and physiological conditions along with water chemistry might influence this balance of endogenous ammonia and excretion mechanisms which is essential for a basal level that maintains internal ammonia homeostasis. Here, we tested how physiological doses of ammonia could affect the mitochondrial ion transport function in the heart and liver of air-breathing fish *Anabas testudineus* during immersion in water that resulted in hypoxia stress.

2. Materials and Methods

2.1 Fish Holding Conditions

Freshwater climbing perch *Anabas testudineus,* an obligate air-breathing fish, inhabits the backwaters of Kerala and is equipped to live in demanding environmental conditions with their well-defined physiological and biochemical mechanisms[15-16]. Fish of approximately 30 ± 5g body mass were obtained from a local supplier, brought to the laboratory and kept in 30L glass tanks for three weeks. These fish were acclimated to the tap water under natural photoperiod (12L/12D) and fed with fish feed daily at a ratio of 1% body mass. Food was withdrawn 24 hr prior to the experiment to ensure optimum experimental condition. No fish mortality was recorded during the experiment. Two experiments were carried out. Buccal ingestion was selected as the mode of treatment for the control and treated groups in all the experiments.

2.2 Experimental Protocol

2.2.1 Experiment 1: Dose-Responsive In Vivo Action of Ammonia

This experiment was meant to test the *in vivo* action of varied doses of ammonium sulphate $(NH_4)_2SO_4$ (5, 25, 50ng g^{-1}) on liver and heart mitochondria. Thirty-two fish were divided into four groups of eight each. First group of fish were treated with 0.65% saline and formed the control. Fish in groups 2, 3 and 4 were treated with 5, 25, 50ng g^{-1} $(NH_4)_2SO_4$ solutions respectively, for 30 min. Nominated doses of $(NH_4)_2SO_4$ were dissolved in 0.65% saline and delivered directly into the base of the oral cavity with the help of syringe fitted with P50 tubing.

2.2.2 Experiment 2: Action of Ammonia in Hypoxia-Stressed Fish

In the second experiment, the role of ammonia in mitochondrial ion transport in hypoxia-stressed fish was studied. Thirty-two fish were divided into four groups of eight each. Groups 1 and 2 were non-stressed fish and groups

3 and 4 formed stressed fish. The first fish the group in non-stressed batch served as control and received i.p. injection of 0.65% saline, and the second group of fish received i.p. injection of 50ng g^{-1} of $(NH_4)_2SO_4$ in solution and kept for 30 min. Fish of stressed batch (3 and 4 groups) were subjected to water immersion, that produced hypoxia stress, for 30 min. Fish in third group received first saline injection whereas fourth group fish were treated with 50ng g^{-1} $(NH_4)_2SO_4$ before giving water immersion stress for 30min.

2.3 Tissue Sampling and Analysis

After treatments, fish in each group were quickly dip-netted and anesthetized using 0.1% 2-phenoxyethanol solution (SRL, Mumbai). Blood was drawn from the caudal artery using heparinised syringe fitted with #23 syringe. The heparinised blood was then centrifuged at 5,000xg for 5 min at 4ºC and the plasma was separated and stored at -80ºC until analyzed. Fish were then sacrificed by spinal transection and heart and liver tissues were excised immediately and kept in SEI buffer (pH 7.1) that contained 0.25 M sucrose, 10 mM Na2EDTA, and 0.1 M imidazole and stored at -80°C until analysis.

2.3.1 Estimation of Plasma Metabolites

Plasma glucose (GOD/POD test kit, Span Diagnostics Ltd., New Delhi) and lactate (Lactate liquid test kit, FAR Diagnostics, New Delhi) concentrations were measured in a microplate reader (Biotek, Germany) using commercial test kits and expressed as mmol L^{-1}.

2.3.2 Ion-Specific ATPases

The frozen tissue samples were weighed and homogenized (1:10 W/V) in SEI buffer (pH 7.1) using a homogenizer fitted with a Teflon pestle. The homogenate was subjected to centrifugation at 700xg for 10 min at 4ºC (Eppendorf 5430R) and the supernatant was further centrifuged at 10000xg for 10 min to isolate mitochondria as reported earlier[17]. The activity of mit.Ca^{2+} ATPase, mit.H^+ ATPase, and mit.F_1F_0 ATPase were quantified in the mitochondrial fraction as described earlier[17]. The mitochondrial protein concentration was estimated according to a modified Biuret assay[18] that used bovine serum albumin (BSA) as the standared.

2.3.2.1 Mit.Ca^{2+}ATPase Specific Activity

Vanadate-sensitive mitochondrial Ca^{2+}-ATPase activity was determined in the mitochondrial suspension as described earlier[17]. Samples in duplicate were added to a 96-well microplate containing either $CaCl_2$ as activator or vanadate as inhibitor. The reaction was initiated by the addition of ATP and terminated by adding 8.6% TCA. The inorganic phosphate content released was quantified and expressed in μM Pi h^{-1} mg $protein^{-1}$.

2.3.2.2 Mit.H^+-ATPase Specific Activity

Baffilomycin-sensitive mit.H^+-ATPase activity was quantified in mitochondrial fractions as described earlier where baffilomycin A was used as inhibitor[17]. The samples in duplicate were added to a 96-well microplate containing baffilomycin A. The inorganic phosphate content was determined as above and expressed in μM Pi h^{-1} mg $protein^{-1}$.

2.3.2.3 F_1F_0 ATPase Specific Activity

The specific activity of oligomycin-sensitive F_1F_0 ATPase in the mitochondrial fraction was determined as reported earlier where oligomycin was used as inhibitor[17]. Mitochondrial samples in duplicate were added to a 96-well microplate with or without oligomycin. The inorganic phosphate content released was measured. The change in absorbance between promoter and inhibitor activities was calculated, and regression analysis was employed to derive the rate of F_1F_0 ATPase activity and expressed in μM Pi h^{-1} mg $protein^{-1}$.

2.4 Statistical Analysis

Data were collected from all eight animals in each group. Statistical significance among fish groups was tested by means of one-way analysis of variance (ANOVA) followed by SNK comparison test. Significance between groups was analyed with the help of Graphpad Software (Graphpad Instat-3, San Diego) and the level of significance was accepted if $p<0.05$.

3. Results

3.1 *In Vivo* Action of Ammonia on Plasma Glucose and Lactate Status

The varied doses of *in vivo* ammonia produced significant modifications in the levels of plasma glucose and lactate. The low dose (5ng g^{-1}) of ammonia produced a significant rise ($p<0.001$) in glucose level, whereas the medium and

high doses (25 and 50ng g^{-1} respectively) showed significant decline ($p<0.001$) (Figure 1A) in its level. The lactate concentration showed significant rise ($p<0.001$) after medium and high doses of ammonia treatment (Figure 1A). A significant decline ($p<0.001$) in glucose content was observed after ammonia treatment in non-stressed fish which was not found in stressed fish (Figure 1B). Lactate level showed a rise after ammonia treatment in non-stressed fish, but showed a significant decline ($p<0.001$) in stressed fish (Figure 1B).

3.2 *In Vivo* Action of Ammonia in Heart and Liver Mitochondria

The mit.Ca^{2+}ATPase activity showed a significant ($p<0.01$) decline in both heart and liver after low ($p<0.01$) and medium doses ($p<0.001$) of ammonia treatment, respectively. But its activity showed a significant rise ($p<0.05$) in heart after the high dose of ammonia, whereas a significant decrease ($p<0.001$) in its activity was found in liver (Figure 2A). Medium dose of ammonia produced a

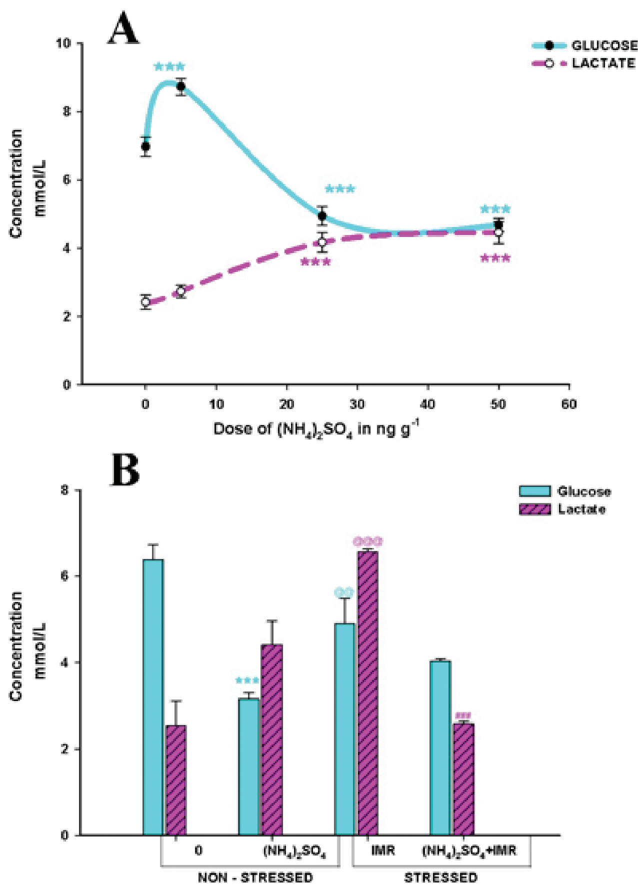

Figure 1. Dose-responsive *in vivo* action of ammonia ($(NH_4)_2SO_4$) treatment for 30 min on plasma glucose and lactate concentrations (**A**) in climbing perch. Action of a selected dose of ammonia (50ng g^{-1}) on plasma glucose and lactate in immersion-stressed (IMR) fish (**B**). Each point/bar is mean ± SE for six fish. * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$) denotes significance when compared between control fish (0) and test. @ ($p<0.05$), @@ ($p<0.01$) or @@@ ($p<0.001$) represents significance when compared between ammonia-treated and immersion-stressed fish.

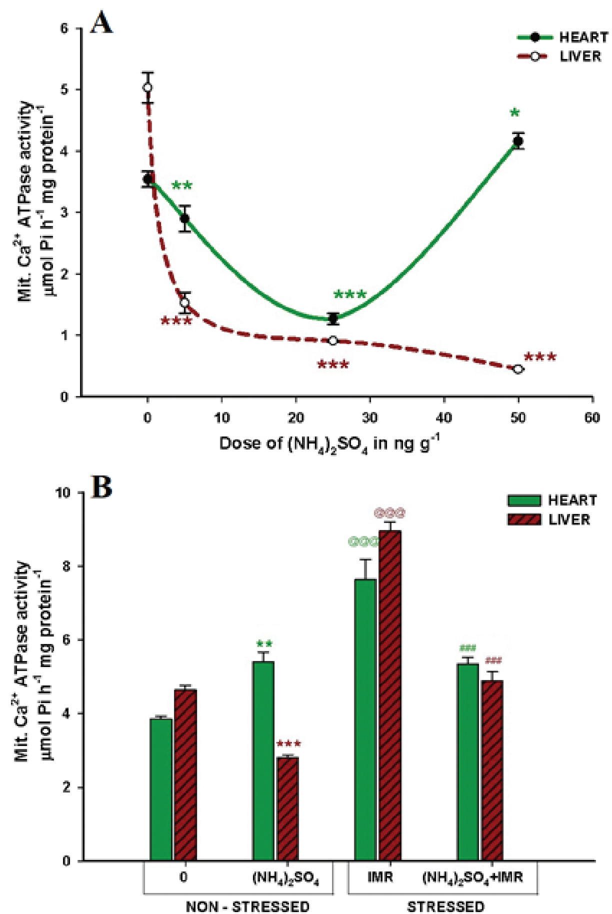

Figure 2. Dose-responsive *in vivo* action of ammonia ($(NH_4)_2SO_4$) treatment for 30 min on activity pattern of mitochondrial Ca^{2+}ATPase in heart and liver (**A**) in climbing perch. Action of a selected dose of ammonia (50ng g^{-1}) on activity pattern of mitochondrial Ca^{2+}ATPase in the heart and liver in immersion-stressed (IMR) fish (**B**). Each point/bar is mean ± SE for six fish. * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$) denotes significance when compared between control fish (0) and test. @ ($p<0.05$), @@ ($p<0.01$) or @@@ ($p<0.001$) represents significance when compared between ammonia-treated and immersion-stressed fish.

significant decrease (p<0.001) in the mit.H^+ATPase activity in heart, whereas its activity remained unaffected after low and high doses of ammonia treatment (Figure 3A). All the three doses of ammonia, however, caused significant fall (p<0.001) in its activity in liver (Figure 3A). The F_1F_0 ATPase activtiy showed a significant increase (p<0.001) in heart after low and medium doses of ammonia treatment, whereas the high doses failed to produce any variation in its activity (Figure 4A). In liver, all the three doses of ammonia produced significant (p<0.001) decrease in F_1F_0 ATPase activity (Figure 4A).

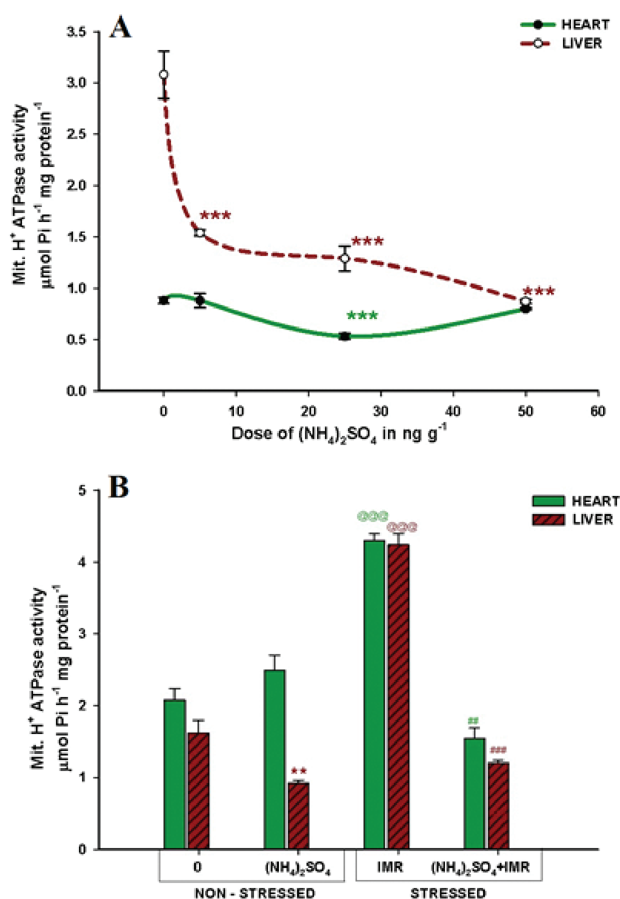

Figure 3. Dose-responsive *in vivo* action of ammonia ($(NH_4)_2SO_4$) treatment for 30 min on activity pattern of mitochondrial H^+ATPase in heart and liver (**A**) in climbing perch. Action of a selected dose of ammonia (50ng g^{-1}) on activity pattern of mitochondrial H^+ATPase in the heart and liver in immersion-stressed (IMR) fish (**B**). Each point/bar is mean ± SE for six fish. * (p<0.05), ** (p<0.01) or *** (p<0.001) denotes significance when compared between control fish (0) and test. @ (p<0.05), @@ (p<0.01) or @@@ (p<0.001) represents significance when compared between ammonia-treated and immersion-stressed fish.

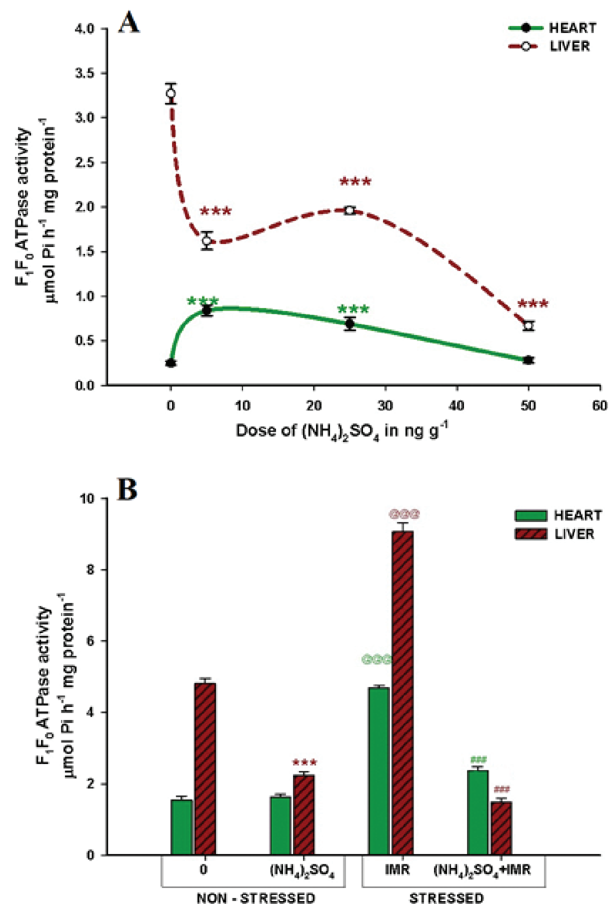

Figure 4. Dose-responsive *in vivo* action of ammonia ($(NH_4)_2SO_4$) treatment for 30 min on activity pattern of mitochondrial F_1F_0 ATPase in heart and liver (**A**) in climbing perch. Action of a selected dose of ammonia (50ng g^{-1}) on activity pattern of mitochondrial F_1F_0 ATPase in the heart and liver in immersion-stressed (IMR) fish (**B**). Each point/bar is mean ± SE for six fish. * (p<0.05), ** (p<0.01) or *** (p<0.001) denotes significance when compared between control fish (0) and test. @ (p<0.05), @@ (p<0.01) or @@@ (p<0.001) represents significance when compared between ammonia-treated and immersion-stressed fish.

3.3 *In vivo* Action of Ammonia in Hypoxia-Stressed Fish

In non-stressed fish, mit.Ca^{2+}ATPase activity in the heart showed a significant increase (p<0.01) following administration of a selected dose (50ng g^{-1}) of ammonia (Figure 2B), whereas in the stressed fish, ammonia administration significantly lowered (p<0.001) its activity (Figure 2B). In contrast, mit.Ca^{2+}ATPase activity in liver decreased significantly (p<0.001) after ammonia treatment in both non-stressed and stressed fish (Figure 2B). A significant decline (p<0.01) in mit.H^+ATPase occurred

after ammonia treatment in the liver of stressed fish but not in heart (Figure 3B). Mit.H^+ATPase activity in liver showed significant decrease after ammonia treatment in both stressed ($p<0.001$) and non-stressed ($p<0.01$) fish (Figure 3B). In heart, F_1F_0 ATPase activity showed a significant ($p<0.001$) decrease in stressed fish but not in ammonia-treated fish (Figure 4B). Liver showed a significant decrease ($p<0.001$) in F_1F_0 ATPase activity after the administration of ammonia in both non-stressed and stressed fish (Figure 4B).

4. Discussion

Plasma metabolites such as glucose and lactate are indicators of health. In our study, the levels of plasma metabolites responded to *in vivo* ammonia treatment in both stressed and non-stressed fish, indicating the metabolite-mobilizing action of ammonia in the test species. Physiological doses of (5, 25, 50ng g^{-1}) ammonia lowered glucose status in plasma and that implies a demand for higer utilization of glucose by oxidation which would appear as a protective mechanism. This further indicates that the doses of ammonia employed in our study could be in physiological range as previous studies have reported an increase in glucose production after high ammonia exposure[21]. Likewise, the lactate content that showed a rise in non-stressed fish indicates that ammonia might activate anaerobic energetics. It is likely that the rise in lactate production may also account for adrenergic stimulation which would favor the anaerobic pathway. Further, ammonia would also lower lactate production in immersion-stressed fish, an indication of protective energetics during hypoxia stress. Hypoxia has been shown to affect ammonia tolerance in fish[22]. Studies on goldfish have shown that hypoxia increases their capacity for nitrite uptake[23]. Climbing perch as an obligatory air-breathing fish have the capacity to operate urea cycle at the time of ammonia loading and actively converts ammonia to less toxic urea. Studies on the marine fish *Allenbatrachus grunniens* has shown a low urea excretion capacity despite significant elevation of plasma and tissue ammonia content induced by high pH and high ammonia treatments[24]. It has been proposed that low doses of ammonia promote growth and nitrogen retention in certain fishes[12] but its exact role in the physiological homeostasis has not yet been investigated.

In fish, liver is the major organ involved in ammonia synthesis and detoxification of major metabolites[19]. In hepatocytes, ammonia is produced mainly by the catabolism of dietary amino acids and by the combined action of cytosolic amino transferases and mitochondrial glutamate dehydrogenase[20]. Here, NH_4^+ is released through the deamination of glutamate in the presence of glutamate dehydrogenase, whereas NH_3 is released from the amide N of glutamine in the presence of glutaminase. Ammonia produced in the mitochondrial matrix of the liver gets transported through mitochondria and plasma membrane to reach the blood. Permeation of NH_3 across the inner mitochondrial membrane uncouples the oxidative phosphorylation and combines with H^+ outside the mitochondria and disrupts the proton gradient[13]. However, in the liver of ammonotelic fish, ammonia exits liver mitochondria without uncoupling oxidative phosphorylation, and which remains an enigma[13]. Biological membranes are permeable to neutral NH_3 molecule, and it readily transits through the membrane, but in its ionic form NH_4^+ requires a transport protein for this transit. In liver, there operates an active detoxification mechanism that relies on urea cycle, GDH and amino acid synthesis and protein metabolism[5]. Considering the central role of liver in ammonia synthesis and detoxification, it is likely that exogenous ammonia can influence ion transport across liver mitochondria as evident in the lowered mitochondrial ion-specific ATPase activities. The lowered mit.Ca^{2+}ATPase activity in liver further reveals a modulatory action of exogenous ammonia on calcium ion homeostasis in the target cell. A tight regulation of extracellular and intracellular calcium transport has been acheieved by the modulation of Ca^{2+}ATPase and calcium channel functions. The cell membrane permeability and mitochondrial integrity are associated with the ability of the cell to maintain Ca^{2+} homeostasis where high calcium level generally decreases and low calcium increases permeability[25]. Further it is known that Ca^{2+} entry drives in a huge transmembrane electrochemical gradient and thus cells ensure low Ca^{2+} concentrations for proper cell signalling[26]. Dose-responsive action of exogenous ammonia on mitochondrial Ca^{2+} transport in the liver thus signifies a regulatory role of ammonia in liver calcium homeostasis.

Cardiac function requires proper maintenance of Ca^{2+} homeostasis and that has been established through the transport of Ca^{2+} across the cardiocyte membrane during excitation and contraction cycles. The myocyte contraction in fish is dependent on the intracellular free Ca^{2+} concentrations but in most cases extracellular Ca^{2+} acts as the primary source for cardiac muscle activation[27].

Na^+/Ca^{2+} exchangers also help in the delivery of Ca^{2+} ions during cardiac contractions in fish[28]. Ca^{2+} as the major cation is essential for the proper functioning of cardiac muscles. In the present study, the activity pattern of mitochondrial Ca^{2+} ATPase in the heart of both stressed and non-stressed fish after the treatment of the selected dose of ammonia (50ng g^{-1}) showed a reverse shift, pointing to the modulatory action of ammonia in Ca^{2+} transport during hypoxia-stressed fish. On the contrary, the lowered activity pattern of Ca^{2+} transport in liver of both non-stressed and stressed fish indicate a lesser degree of sensitivity of liver to exogenous ammonia. High ammonia exposure has been shown to cause excessive activation of NMDA receptors due to the accumulation of Ca^{2+} ions. In contrast, physiological dose of ammonia used in our study reveals a tight regulation of calcium transport as evident in mitochondrial Ca^{2+} ATPase that showed a nullifying effect during immersion stress.

F_1F_0 ATPase that assists in the transepithelial regulation of Mg^{2+} ions is essential for the cell membrane integrity and stabilization of cell permeability[29]. It imparts a specific role in energy synthesis through oxidative phosphorylation[30] where synthesis of ATP occurs utilizing energy released from proton movement[31]. This transporter at specific environment exports protons and creates a proton gradient across the membrane[32]. We found a lowered F_1F_0 ATPase activity in liver mitochondria but in heart mitochondria it produced a rise after ammonia treatment. This implies a preferential action of ammonia in modulating oxidative phosphorylation in these tissues and this futher suggests a protective acion of ammonia on energy synthesis in hypoxia-stressed fish. The mitochondrial H^+ATPase activity in heart and liver showed lowered activity pattern after ammonia treatment in both stressed and non-stressed fish. This indicates a lowered proton pump upon ammonia treatment which also corresponds to declined F_1F_0 ATPase activity observed in both heart and liver mitochondria of hypoxia-stressed fish. The interactive response of these mitochondrial transporters reveals that ammonia would lower the mitochondrial energetics of these tissues, which appears to be an important strategy to meet hypoxia stress.

Our study thus indicates a modulatory role of exogenous ammonia in ion transporter function in the mitochondria of liver and heart. Further, we found that exogenous ammonia would nullify the excitatory response of mitochondrial Ca^{2+}ATPase, H^+ATPase and F_1F_0 ATPase function in immersion-stressed fish. Likewise, the response pattern of plasma metabolites also points to a physiological role of exogenous ammonia in fish in their acclimation to hypoxia stress. Overall, our data support the hypothesis that ammonia can fine-tune and drive mitochondrial ion homeostasis in the liver and heart of air-breathing fish during their acclimation to immersion stress.

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