Original Research

ISSN (Print) : 0971-913X
ISSN (Online) : 2455–1244

Journal of Endocrinology and Reproduction, *Vol 22(1), 30-43, June 2018, DOI: 10.18311/jer/2018/24149*

Nitric Oxide Drives Mitochondrial Energetics in Heart and Liver Mitochondria of Hypoxia-Stressed Climbing Perch (*Anabas testudineus* Bloch)

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Abstract

Nitric oxide (NO), a gaseous free radical that functions as signal molecule, regulates several physiological functions. But in teleost fishes, the influence of NO on mitochondrial energetics is not yet understood. With a view to understanding the short-term *in vivo* action of NO on mitochondrial energetics in fish, we examined the effects of sodium nitroprusside, a NO donor (SNP) and N-omega-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of nitric oxide synthase (L-NAME), on major electron carriers and oxidative status in heart and liver mitochondria of an obligate air-breathing fish (*Anabas testudineus* Bloch) kept at either non-stressed or hypoxia-stressed condition. The total nitrate/nitrite (NO_3^-/NO_2^-) level that corresponds to NO content showed a rise after SNP (5 µg g^{-1}) and a decline in the heart and liver of non-stressed fish after L-NAME (100 ng g^{-1}) treatments for 30 min. Water immersion for 30 min that induced hypoxia lowered NO_3^-/NO_2^- level in heart and liver, but showed a rise in NO_3^-/NO_2^- level after SNP treatment of immersion-stressed fish. Reactive Oxygen Species (ROS) production increased after SNP treatment but decreased after L-NAME treatment in heart of hypoxia-stressed fish where as in liver both SNP and L-NAME treatments caused decrease of ROS in stressed fish. SNP treatment increased and L-NAME treatment lowered peroxynitrite ($ONOO^-$) level in heart and liver of non-stressed fish. SNP treatment lowered the activity of cytochrome c oxidase (COX) but L-NAME treatment increased its activity in mitochondria of heart of hypoxia-stressed fish. In liver mitochondria, however, COX activity showed a rise after these treatments. On the contrary, SNP and L-NAME treatments in stressed fish elevated succinate dehydrogenase (SDH) activity in both heart and liver mitochondria. In heart, LDH activity increased after SNP and L-NAME treatments in both non-stressed and stressed conditions, but not in liver of stressed fish. Put together, the data provide evidence that NO exerts an integrative action on mitochondrial energetics in heart and liver mitochondria of air-breathing fish during their exposure to hypoxia-stress.

Keywords: *Anabas testudineus,* Cytochrome oxidase, Fish Stress, Hypoxia ROS, Mitochondria Energetics, Nitric Oxide

1. Introduction

Mitochondria, as the site of release of cellular energy, are critical due to their ability to sense the environmental changes. A complex molecular network system exists in mitochondria for adjusting their bioenergetic and oxidative responses for the reestablishment of homeostasis[1-2]. Given their crucial role in cellular physiology, mitochondria are among the first responders to various physiological stressors. It is known that the primary

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mediators of stress response such as glucocorticoids and catacholamines exert numerous effects on mitochondrial biogenesis, metabolism, ROS generation and apoptosis[1, 3-6]. In addition to that, mitochondria have also been described as the site of synthesis of hormones such as glucocorticoids and mineralocorticoids[7].

Nitric oxide (NO), as a gaseous signaling molecule, plays many roles in the cardiovascular system and hepatic system[8-10]. Cardiac muscle has the highest density of mitochondria among all vertebrate organs due to its high-energy demand. NO has been shown to regulate mitochondrial respiration in vertebrate heart as these mitochondria can generate all the ATP required for muscle contraction in the heart[11]. It is also known that NO can play a key role in liver physiology where it acts as a protective agent against onset of disease[12].

In mitochondria NO is known to regulate O_2 consumption, energy metabolism and ROS formation at physiological levels[13-14]. Once NO is produced it may rapidly react with ferrous iron which is either free iron or iron within iron-sulphur centres or within haemoproteins[15]. In biological systems, the mode and rate of NO metabolism is dependent on its diffusibility, its concentration and the surrounding concentration of other bioreactants[16-17]. The main route by which NO is broken down in the body of vertebrates is thought to be the reaction with hemoglobin or myoglobin[17]. In addition, NO would also directly react with oxygen to produce NO_2 and N_2O_3 which is thought to be relatively slow at physiological levels of NO[18]. Furthermore, NO reacts with superoxide ($O2^-$) to produce peroxynitrite ($ONOO^-$), a strong oxidant that creates cytotoxicity[19]. NO can also react indirectly with thiols to produce S-nitrosothiols (SNO)[20-21]. As potential physiological regulator of mitochondrial respiration NO at physiological concentrations can cause rapid and reversible inhibition of COX depending on the intracellular O_2 concentration and redox state of COX[22]. When O_2 concentration is high, COX favors oxidized state and consumes NO and converts it into nitrite[23]. On the contrary, when the concentration of O_2 is low, the COX is in its reduced state, and NO accumulates in the microenvironment[24].

Induction of stress in teleosts evokes an array of modulations in hypothalamo-pituitary-inter-renal (HPI) axis that causes adaptive physiological disturbances[25-26], allowing the fish to regain homeostasis[27]. The release of stress mediators such as catacholamines and glucocorticoids serve to maintain effective blood supply to the brain and cardiac muscle, which increases energy production through the recruitment of substrates such as glucose, fatty acids and amino acids from liver and adipose tissue in vertebrates[28]. Likewise, organs with high metabolic demand, like liver, initially cope with the initial energy imbalance during stress by switching to fatty acid oxidation[29]. It is likely that the physiological response of these organs may serve as early markers of developing stress response in animals. In the present study, we examined the effect of NO on mitochondrial function in heart and liver mitochondria of climbing perch in their non-stressed and stressed states. We employed SNP, an agonist that generates exogenous NO or L-NAME, an inhibitor of NOS that depletes endogenous NO to delineate the action of NO on mitochondrial energetics of an obligate air-breathing fish kept immersed in water.

2. Materials and Methods

2.1 Fish Holding Conditions

Tropical freshwater air-breathing fish, commonly known as climbing perch (*Anabas testudineus* Bloch) belonging to order Perciformes and family Anabantidae, was used as the test species. This native teleost fish inhabiting the backwaters of Kerala is an obligate air-breathing fish equipped to live in demanding environmental conditions with their well defined physiological and biochemical mechanisms[30-31]. These fish in their post-spawning phase were collected from the wild and maintained in the laboratory for three weeks under natural photoperiod (12h L: 12h D) at water temperature of 28±1°C with a mean water pH of 6.2. Fish were fed with dry commercial fish feed at 1.5% of body mass and transferred to 50L glass tanks. Prior to experiment, the fish were kept in 100 L aquaria for two weeks, and food was withdrawn since 24h prior to sacrifice to ensure optimum experimental conditions. There was no mortality during the experiments and the stipulations of Animal Ethical Committee of the University were followed.

2.2 Experimental Design

Two independent *in vivo* experiments were conducted. In the first experiment the effect of NO agonist SNP was tested and in the second experiment the effect of NO antagonist L-NAME was tested in stressed and non-stressed fish. Forty-eight laboratory-acclimated fish were assigned to four groups of six each (each in replicate) in separate glass tanks (60 × 30 × 30 cm). The fish in first and second groups formed non-stressed fish whereas those in third and fourth groups formed hypoxia-stressed fish. The fish were immersion-stressed for 30 min by placing them in water and preventing them from gulping air as reported earlier[32]. This was accomplished by keeping iron nets just below the water surface. Fish in the first group of both experiments were given saline injections (0.65% saline in 100 µL) and they served as non-stressed control fish. A selected dose of SNP (5 µg g^{-1}) was used in experiment 1, and a selected dose of L-NAME (100 ng g^{-1}) was used in the experiment 2. The doses were selected based on the ability to induce physiological modifications including Na^+/K^+ ATP-ase activity and nitrate/nitrite (NO_3^-/NO_2^-) levels (unpublished). For both experiments 1 and 2, fish in the third group were given saline injection and served as hypoxia-stressed control fish, whereas fourth group of fish was given intraperitoneal injection of the selected dose of either SNP (5 µg g^{-1}) or L-NAME (100 ng g^{-1}). Care was taken to minimize stress due to injection and handling. All fish were sampled 30 min after injection.

2.3 Sampling and Analysis

After treatments, fish were caught in nets and anesthetized in a 0.1% 2-phenoxyethanol solution (SRL, Mumbai). Blood was drawn from the caudal artery and fish were then sacrificed by spinal transection. Liver and heart tissues were carefully excised. The tissues were then stored in ice-cold 0.25 M SEI buffer (0.3 mM sucrose, 20 mM EDTA, 0.1 M imidazole, pH 7.1) for analyzing total nitrate/nitrite and peroxynitrite levels and for the isolation of mitochondrial fraction. Likewise, tissue samples were also placed in 40 mM Tris-HCl buffer (pH 7.4) for quantifying ROS. A portion of the tissue samples was also placed in 0.05 M potassium phosphate buffer (pH 7.0) for the LDH assay.

2.3.1 Preparation of H_0 Fraction and Mitochondrial Suspension

Mitochondria were isolated from heart and liver tissues following the method of Peter *et al.*,[33]. Briefly, 10% tissue homogenates were prepared in 0.25 M sucrose EDTA Imdazole (SEI, pH 7.4) buffer in a teflon pestle glass homogenizer fitted with a motor (Remi, Mumbai). The homogenate was centrifuged (Eppendorf, 5430R) at 700 × g for 10 minutes at 4°C that separated cell debris and nuclei. A portion of the collected supernatant as homogenization-zero fraction (H_0) was used for analyzing the levels of total tissue nitrate/nitrite (NO_3^-/NO_2^-) and peroxynitrite ($ONOO^-$) in tissues. The remaining portion was then centrifuged at 10,000 × g for 10 min at 4°C (Eppendorf, 5430R) and the pellets were recovered, and washed thrice by repeating the centrifugation. The final pellet was then resuspended in fresh ice-cold 0.25 M SEI buffer which served as the mitochondrial suspension and used for analysis of the kinetics of activities of COX and SDH in mitochondria. The protein concentrations in H_0 fraction and mitochondrial suspension were determined by Biuret method using bovine serum albumin as the standard.

2.3.2 Determination of Total Nitrate/Nitrite (NO_3^-/NO_2^-) Content in Tissue Samples

Total NO_3^-/NO_2^- content of liver and heart tissues were determined by the method of Sastry *et al.*,[34]. In brief, 100 µL of H_0 fraction of liver and heart tissues were mixed with 400 µL of 50 mM carbonate buffer (pH 9.0) and 0.15 g of activated copper-cadmium alloy filings and incubated for 1 h at room temperature. The reaction was stopped by addition of 100 µL of 0.35 M NaOH, followed by 120 mM $ZnSO_4$ solution under vortex and the solution was then allowed to stand for 10 min. The tubes were then centrifuged at 4000 × g for 10 min. Thereafter, 100 µL of clear supernatant in triplicate were added to the wells of 96-well microplate and Griess reagent (75 µL of 1% sulphanilamide prepared in 3 N HCl and 75 µL of 0.1% N-naphthyl ethylene diamine, prepared in distilled water) was added and incubated for 10 min. Then the absorbance was read at 545 nm in a microplate reader (Synergy HT, Biotek, USA). A standard graph was plotted against different concentrations of $NaNO_3$ and the NO_3^-/NO_2^- content was derived and expressed in nmol mg $protein^{-1}$.

2.3.3 Determination of Peroxynitrite ($ONOO^-$) in Tissue Samples

Peroxynitrite-mediated nitration of phenol was measured colorimetrically as described earlier[35] with some modifications. In brief, 5 µL of H_0 fraction of tissue homogenates was added to 5 mM phenol in 50 mM sodium phosphate buffer (pH 7.4). After 2 h incubation at 37°C in the dark, 2 µL of 0.1M NaOH was added and the absorbance was measured immediately at 412 nm (Synergy HT, Biotek, USA). The yield of nitrophenol was calculated utilizing $\varepsilon = 4400\ M^{-1}\ cm^{-1}$.

2.3.4 Determination of Reactive Oxygen Species (ROS) in Tissue Samples

A flourimetric assay was used to determine the levels of ROS such as superoxide radical, hydroxyl radical and hydrogen peroxide according to the method described earlier[36] with some modifications. Briefly, 10% tissue sonicate was prepared in ice-cold 40 mM Tris-HCl buffer, pH 7.4, using a sonicator (Δt = 15 sec; UP 50H, Hielscher Ultrasonics, Germany). These sonicates were then diluted to 0.25% with more ice-cold Tris-HCl buffer. A fraction of the sonicates was mixed with 40 µL of 1.25 mM 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes, USA) in methanol. Similarly, the other fraction was mixed with methanol-added blank controls, which were kept for detecting autoflourescence. All samples were incubated for 15 min at 37°C in water bath. After incubation, 240 µL of these samples were added to the flat bottomed transparent black 96-well microplates (Nunc, Thermo Fisher Scientific, USA) and the fluorescence at 485 nm excitation and 525 nm emission was measured in a fluorescence plate reader (Synergy HT, BioTek, USA). The assay measured the oxidative conversion of stable, non-fluorescent H_2DCFDA to the highly fluorescent 2', 7'-dichlorofluorescein (DCF) in the presence of ROS. Unknown fluorescence values were standardized using known concentrations of DCF (Sigma-Aldrich, USA) in methanol. Protein quantification was done using Bradford assay and the ROS production was expressed as nmol/mg protein.

2.3.5 COX activity

The activity of COX was measured using a spectrophotometric method[37] with some modifications. Briefly, mitochondrial suspension was incubated with 0.04% saponin for 5 min at 37°C in water bath. Samples in duplicates containing 1.0 µg protein were added to a 96-well microplate, followed by the addition of substrate medium containing 4mM 3, 3'-diamine benzidine tetrachloride (DAB), 100 µM reduced cytochrome C, and 2 µg/mL catalase in 0.1M $NaPO_4H_2$(pH 7.0). Reduced cytochrome C was prepared in a pre-equilibrated G25 M Sephadex column by adding 5mg ascorbic acid to 100 mg cytochrome C in 2.5 mL of 100 mM sodium phosphate, pH 7.0). Soon after the addition of substrate medium, the absorbance variation was measured at 450 nm for 15 min and the COX activity as the increase in OD was quantified. The change in absorbance (ΔOD), expressed as micromoles of cytochrome c oxidized mg $protein^{-1}\ min^{-1}$, was calculated after normalization with protein. The statistical significance among groups was calculated by considering the control as 100%.

2.3.6 SDH Activity

The activity of SDH was determined spectrophotometrically following the method of Hollywood *et al.*[38] with some modifications. 2, 6-dichlorophenol-indophenol (DCPIP) and sodium succinate acted as an artificial electron acceptor and the substrate were used in this assay. Briefly, 0.05 mg of mitochondrial suspension of tissue samples permeabilized with 1% sodium deoxycholate (DOC; Sigma-Aldrich, USA) were added to the microplate followed by the addition of reaction mixture containing 5 mM $C_4H_4O_4Na_2{\cdot}6H_2O$, 1 mM $K_4Fe(CN)_6{\cdot}3H_2O$ and 0.1 mM DCPIP in 50 mM potassium phosphate buffer, pH 7.5. After incubation for 2 min, the reduction of DCPIP was monitored for 15 min at 600 nm. Blanks were obtained in the absence of succinate. SDH activity was calculated using the molar absorption coefficient of reduced DCPIP, $\varepsilon = 21.0\ mM^{-1}\ cm^{-1}$. The specific activity was expressed in µmol DCPIP mg $protein^{-1}\ min^{-1}$.

2.3.7 LDH Activity

The activity of LDH was assayed by measuring the rate of decrease of NADH absorbance at 340 nm (adopted from Sensabaugh and Kaplan[39]). Briefly, the stored tissues were homogenized in 0.05 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA sodium salt and 10% sucrose and then centrifuged at 27,000 × g for 15 min at 4°C. The supernatant was collected and

protein was measured using Bradford reagent. Samples in duplicate were added to 96-well microplates followed by the addition of reaction mixture buffer (0.1 M potassium phosphate buffer, pH 7.5, containing 0.14 mM NADH). The reaction was started with the addition of 1 mM $C_3H_3NaO_3$ in reaction mixture buffer, and changes in absorbance were measured using microplate reader (Synergy HT, Biotek, USA) for 5 min at 1 min intervals at 25°C. The LDH activity was expressed in nmoles NADH oxidized/min/mg protein.

2.4 Statistical Analysis

Before statistical analysis, data were checked for normal distribution and variance homogeneity. The values were represented as mean ± SEM and the statistical difference among groups was tested by means of one-way analysis of variance (ANOVA) followed by SNK comparison test. Significance between the groups was analyzed with the help of GraphPad software (Instat-3, San Diego) and the level of significance was accepted if $P < 0.05$.

3. Results

3.1 Effect of SNP and L-NAME on Total Tissue NO_3^-/NO_2^-, ROS and $ONOO^-$ in the Heart and Liver of Non-Stressed and Stressed Fish

Treatment of SNP for 30 min to non-stressed fish significantly increased (P<0.001) the total tissue NO_3^-/NO_2^-, ROS and $ONOO^-$ levels of heart (Tables 1, 2 & 3), whereas the L-NAME treatment significantly decreased (P<0.001) the levels of total NO_3^-/NO_2^- and $ONOO^-$ without altering the ROS level of heart (Tables 1, 2 & 3). Exposure of fish to immersion stress decreased the total NO_3^-/NO_2^- and $ONOO^-$

Table 1. Total tissue NO_3^-/NO_2^- (nmol. mg protein^{-1}) after SNP treatment (5 µg g^{-1}) and L-NAME (100 ng g^{-1}) for 30 min in heart and liver of non-stressed and immersion-stressed (IMR) climbing perch

Status	Total tissue NO_3^-/NO_2^- (nmol. mg protein^{-1})	
	Heart	Liver
SNP-treated fish		
Control	3.39 ± 0.87	3.56 ± 0.41
SNP	4.67 ± 0.56***	4.55 ± 0.16**
IMR	2.52 ± 0.14@	2.96 ± 0.26@
SNP + IMR	3.09 ± 0.69##	4.40 ± 0.14###
L-NAME-treated fish		
Control	3.36 ± 0.22	3.36 ± 0.18
L-NAME	1.03 ± 0.14***	2.20 ± 0.18***
IMR	2.80 ± 0.10@@@	2.03 ± 0.17@
L-NAME+IMR	2.46 ± 0.24	2.38 ± 0.21

Each value is mean ± SEM of 12 fish. **(P< 0.01) and ***(P<0.001) denote significant difference between control and SNP-treated/L-NAME treated fish. @ (P< 0.05) and @@@ (P < 0.001) denote significant difference between control and immersion-stressed (IMR) fish. ## (P < 0.01) and ### (P<0.001) denote significant difference between immersion-stressed (IMR) and SNP-treated/L-NAME treated fish.

levels in heart (Tables 1&3), whereas ROS level showed a significant rise in this fish (Table 2). SNP treatment further increased the ROS production after immersion-stress, while showing a rise in total NO_3^-/NO_2^- (Tables 1 & 2). On the contrary, L-NAME treatment decreased the ROS level in heart of immersion-stressed climbing perch without altering the total NO_3^-/NO_2^- and $ONOO^-$ levels (Tables 1, 2 & 3).

In the liver of non-stressed fish, the levels of total NO_3^-/NO_2^- ($P<0.01$) and $ONOO^-$($P<0.05$) showed significant rise after SNP treatment (Tables 1 & 3) but the levels declined after L-NAME treatment (Tables 1 & 3). Neither SNP nor L-NAME treatment affected the level of ROS in the liver of non-stressed fish (Table 2). However, immersion stress produced a rise in the level of ROS in liver ($P<0.001$) (Table2), whereas total NO_3^-/NO_2^- level decreased significantly ($P<0.05$) (Table 1). A decline in the production of ROS occurred after SNP ($P<0.05$) and L-NAME ($P<0.01$) treatments in the liver of stressed fish (Table 2). However, these treatments in stressed fish did not affect the levels of $ONOO^-$ and total NO_3^-/NO_2^- (Table 3).

3.2 Effect of SNP and L-NAME Treatments on COX Activity in Heart and Liver of Non-Stressed and Immersion-Stressed Fish

In heart, the activity of COX increased significantly ($P<0.05$) after SNP treatment in non-stressed fish (Figure 1A). Similar rise in its activity was found ($P<0.05$) after L-NAME treatment in non-stressed condition (Figure 1B). The immersion-stress significantly ($P<0.05$) increased the COX activity in heart mitochondria (Figure 1A & B). However, the SNP treatment resulted in decrease of COX activity ($P<0.05$) in stressed fish (Figure 1A), whereas the L-NAME treatment increased ($P<0.05$) its activity further in these fish (Figure 1B). In liver, the COX activity increased significantly ($P<0.001$) after

Table 2. Total tissue ROS (nmol. mg protein^{-1}) level after SNP treatment (5 µg g^{-1}) and L-NAME (100 ng g^{-1}) for 30 min in heart and liver of non-stressed and immersion-stressed (IMR) climbing perch

Status	Tissue ROS (nmol.mg protein^{-1})	
	Heart	Liver
SNP-treated fish		
Control	31.33 ± 3.26	19.43 ± 0.99
SNP	60.43 ± 2.28***	21.13 ± 3.42
IMR	42.02 ± 0.46@@	31.31 ± 1.22@@@
SNP + IMR	53.22 ± 1.35##	20.31 ± 2.73#
L-NAME-treated fish		
Control	29.81 ± 2.09	21.89 ± 1.51
L-NAME	30.51 ± 2.16	23.91 ± 1.37
IMR	39.16 ± 1.10@@	34.13 ± 0.66@@@
L-NAME + IMR	15.87 ± 1.4###	26.90 ± 1.81##

Each value is mean ± SEM of 12 fish. ***($P<0.001$) denotes significant difference between control and SNP-treated/ L-NAME treated fish. @@ ($P< 0.01$) and @@@ ($P< 0.001$)denote significant difference between control and immersion-stressed (IMR) fish. # ($P< 0.05$), ## ($P< 0.01$) and ### ($P<0.001$) denote significant difference between immersion-stressed (IMR) and SNP-treated/ L-NAME treated fish.

Table 3. Tissue $ONOO^-$ (nmol. mg $protein^{-1}$) level after SNP treatment (5 μg g^{-1}) and L-NAME (100 ng g^{-1}) for 30 min in heart and liver of non-stressed and immersion-stressed (IMR) climbing perch

Status	Tissue $ONOO^-$ (nmol.mg $protein^{-1}$)	
	Heart	Liver
SNP-treated fish		
Control	25.69 ± 2.59	30.89 ± 2.06
SNP	36.96 ± 0.71***	34.99 ± 1.3*
IMR	19.84 ± 0.8@@	32.73 ± 0.87
SNP + IMR	23.08 ± 1.74	30.13 ± 2.43
L-NAME-treated fish		
Control	24.56 ± 1.32	29.09 ± 1.56
L-NAME	12.47 ± 0.78***	26.24 ± 1.48*
IMR	20.05 ± 1.01@@	30.50 ± 0.56
L-NAME + IMR	18.31 ± 0.68	31.08 ± 1.06

Each value is mean ± SEM of 12 fish. *(P< 0.05) and ***(P<0.001) denote significant difference between control and SNP-treated/ L-NAME treated fish. @@ (P< 0.01) denotes significant difference between control and immersion-stressed (IMR) fish.

L-NAME treatment (Figure 1B), whereas SNP treatment did not affect its activity (Figure 1A). It was observed that the immersion stress increased the COX activity significantly (P<0.05) in liver, which increased further after SNP (P<0.05) (Figure 1A) and L-NAME (P<0.001) treatments in stressed fish (Figure 1B).

3.3 Effect of SNP and L-NAME Treatments on SDH Activity in the Heart and Liver of Non-Stressed and Immersion-Stressed Fish

In non-stressed fish, SNP treatment decreased the SDH activity significantly (P<0.05) in heart mitochondria (Figure 2A), whereas L-NAME treatment increased its activity (P<0.01) (Figure 2B). Immersion stress did not alter the SDH activity in heart mitochondria (Figure 2A & B). But the treatment of SNP in stressed fish produced a rise in SDH activity in heart mitochondria (P<0.001) (Figure 2A). Similar observation was made after L-NAME treatment in which the activity increased significantly (P<0.05) in heart mitochondria of stressed fish (Figure 2B). Neither SNP nor L-NAME treatment altered the SDH activity of liver mitochondria in non-stressed fish (Figure 2A & B). Likewise, immersion stress did not alter SDH activity in liver mitochondria. However, the SNP and L-NAME treatments increased the SDH activity significantly (P<0.001) in liver mitochondria of stressed fish (Figure 2A & B).

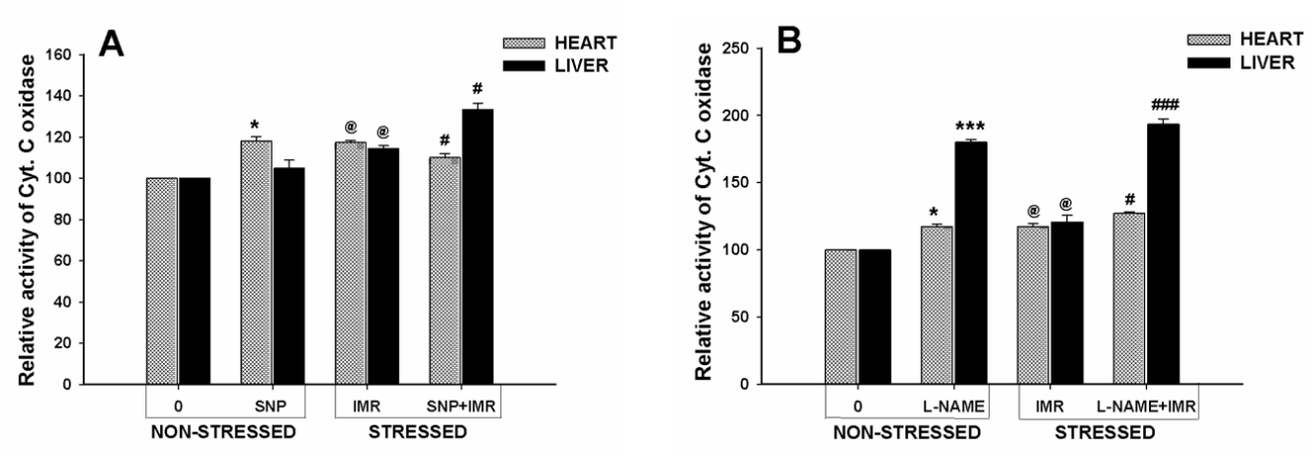

Figure 1. Relative COX activity following SNP **(A)** and L-NAME **(B)** treatments in heart and liver tissues of non-stressed and immersion-stressed climbing perch.

Each bar is mean ± SE (n = 12). Statistical significance between non-stressed control (0) and non-stressed SNP-treated fish were analyzed. Here '*' denotes (P<0.05) and '***' denotes (P<0.001) when compared to control. Similarly, significance between stressed control (IMR) and stress plus SNP/L-NAME were also analyzed. Here, '#' denotes (P<0.05) and '###' denotes (P<0.001) when compared to stressed control. The statistical significance between non-stressed control (0) and stressed control (IMR) were also analyzed. '@' denotes (P<0.05) when compared to non-stressed control.

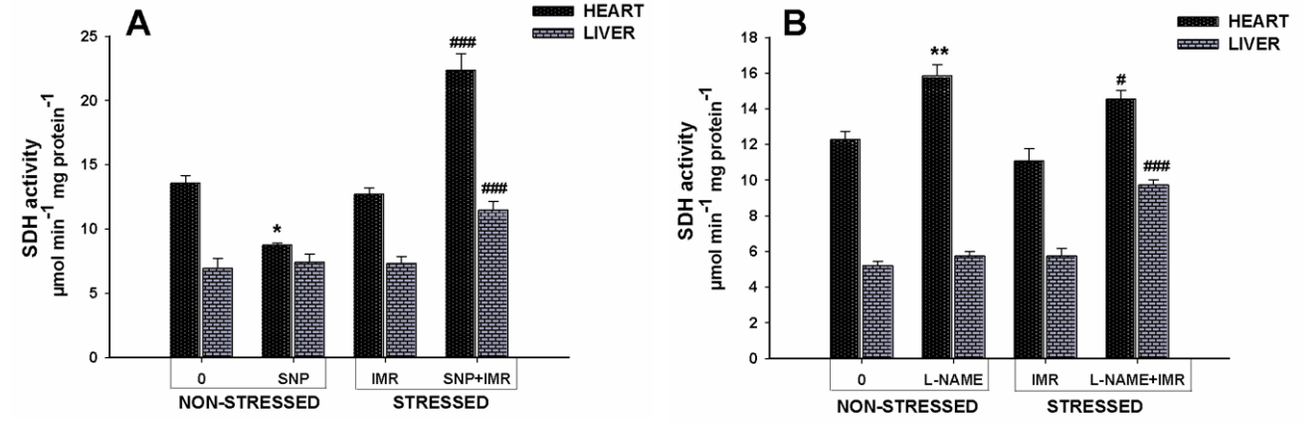

Figure 2. SDH activity following SNP **(A)** and L-NAME **(B)** treatment in liver and heart tissues of non-stressed and immersion-stressed climbing perch.

Each bar is mean ± SE (n = 12). Statistical significance between non-stressed control (0) and non-stressed SNP/L-NAME-treated fish were analyzed. Here '*' denotes (P<0.05) and '**' denotes (P<0.01) when compared to control. Similarly significance between stressed control (IMR) and stress plus SNP/ L-NAME were also analyzed. Here, '#' denotes (P<0.05) and '###' denotes (P<0.001) when compared to stressed control.

3.4 Effect of SNP and L-NAME Treatment on LDH Activity in the Heart and Liver of Non-Stressed and Immersion-Stressed Fish

The LDH activity of liver remained unaltered after SNP and L-NAME treatments in non-stressed fish (Figure 3A & B), whereas a significant rise in the activity of LDH was found after immersion-stress (Figure 3A & B). Treatments of SNP and L-NAME in immersion-stressed fish further decreased the activity of LDH (Figure 3A & B). The LDH activity in heart, however, increased significantly ($P<0.001$) after SNP and L-NAME treatments in both non-stressed and stressed fish (Figure 3A & B). On the contrary, immersion stress did not produce any alteration in the activity of LDH in heart tissue of climbing perch (Figure 3A & B).

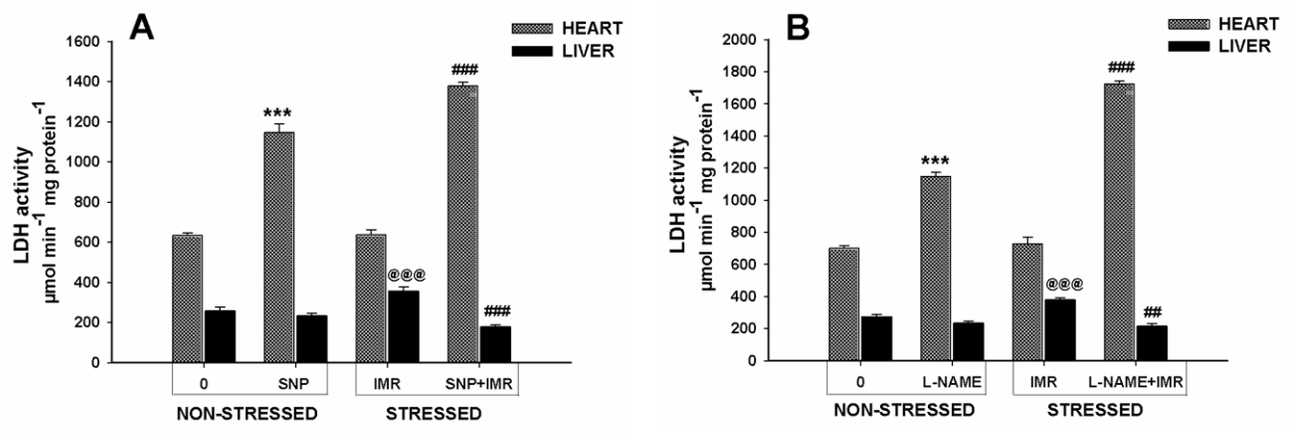

Figure 3. LDH activity following SNP **(A)** and L-NAME (B) treatment in liver and heart tissues of non-stressed and immersion-stressed climbing perch.

Each bar is mean ± SE (n = 12). Statistical significance between non-stressed control (0) and non-stressed SNP-treated fish were analyzed. Here '*' denotes ($P<0.05$) and '***' denotes ($P<0.001$) when compared to control. Similarly, significance between stressed control (IMR) and stress plus SNP/ L-NAME were also analyzed. Here, '#' denotes ($P<0.05$) and '###' denotes ($P<0.001$) when compared to stressed control. The statistical significance between non-stressed control (0) and stressed control (IMR) were also analyzed. '@@@' denotes ($P<0.001$) when compared to non-stressed control.

4. Discussion

Mitochondria are the sensitive target of NO action as they regulate mitochondrial complexes[40-41]. The rise in total NO_3^-/NO_2^- level that corresponds to NO availability in heart and liver of SNP-treated fish provides convincing evidence for the presence of physiological concentrations of NO in these tissues after SNP treatment. Similar rise in NO concentration has been found after *in vivo* administration of SNP in the cells of higher animals, where the pattern of NO action mainly depends on its concentration in tissues irrespective of its origin[42]. Administration of L-NAME, on the contrary, lowered NO_3^-/NO_2^-content in the tested tissues, reflecting a lowered availability of endogenous NO due to the non-specific inhibition of NOS. It is interesting to note that induction of hypoxia-stress by water-immersion of perch resulted in lowered NO_3^-/NO_2^- level and it indicates a higher utilization of NO during hypoxia stress. This view is further confirmed in the lowest level of NO in the heart tissue of SNP-treated hypoxia-stressed fish.

Mitochondria play a critical role in O_2 sensing in many vital organs including heart. The substantial rise in ROS production in mitochondria of heart tissue by SNP and its retention in the immersion-stressed fish indicates a role for NO in ROS-mediated cellular activities. It is well known that ROS at physiological levels activates many signaling pathways that are involved in cellular activities[43].

However, the response of liver ROS to both SNP and L-NAME treatments seems negligible, indicating that NO may not depend on ROS in liver for effecting its actions. It is, however, interesting to find that an elevated ROS production is required to meet the mitochondrial energetics in the heart of stressed fish, though NO depletion by L-NAME treatment decreased the ROS production substantially. It has been shown that hypoxia stress generates more ROS from mitochondrial as well as extra-mitochondrial sources, providing clue to the effect that it can regulate cellular activity through redox modifications without affecting respiration[44-46]. Water immersion of air-breathing fish has been shown to induce systemic hypoxia due to the fall of blood PO_2 and total saturated O_2 level and a rise in PCO_2 (unpublished data). The rise in ROS in the heart and liver of climbing perch in immersion-induced hypoxic condition thus clearly indicates that the generation of more ROS is essential probably to meet the demand for cell signaling that favors energy reallocation during hypoxia. The rise in ROS production in mitochondria of heart due to SNP treatment and its decline by L-NAME treatment in hypoxia-stressed fish further indicates a contributing action of NO on ROS-induced mitochondrial energetics in heart.

Donors of NO, that generate exogenous NO in target tissues, have been shown to alter mitochondrial function[47] due to their entry into mitochondria as low molecular weight S-nitrosothiol (SNO) compounds[48-49]. These SNO proteins have been demonstrated to inhibit complex I of ETC and elevate ROS production in mammalian mitochondria[50]. In the current study, the higher turnover of ROS level together with a rise in total NO level in heart after SNP treatment in non-stressed condition appears to promote the production of $ONOO^-$. The lowered $ONOO^-$ level in L-NAME treated fish provides convincing evidence for its relationship with ROS and total tissue NO levels. Interestingly, immersion-stress lowered the $ONOO^-$ level and that may account for a lesser availability of NO. The reduced NO and $ONOO^-$ levels in immersion-stressed fish provide further evidence for a tight regulation of NO and nitrosative compounds during hypoxic stress, which is not directly related to ROS production.

The regulation of mitochondrial respiration is vital due to its role in ATP synthesis, and in heart it is critical for muscle contraction. Previous studies have shown that NO can inhibit COX activity, resulting in the inhibition of mitochondrial respiration in the heart of many vertebrates[51-54]. However, in the present *in vivo* study the rise in COX activity by NO donor in heart mitochondria of non-stressed fish points to an augmented mitochondrial respiration due to NO availability which was not found in hypoxia-stressed fish. It is likely that conversion of redox state of COX might happen during hypoxia stress. In respiring cells, hypoxia stress has been shown to modulate the redox state of COX from oxidized state to reduced state, due to alterred NO bioavailability[24]. The reduction of COX activity in the heart mitochondria of SNP-treated stressed fish may be thus attributed to a lowered mitochondrial respiration due to a modified NO availability. Alternatively, the elevated COX activity in these mitochondria of immersion-stressed fish could also be due to an activation of epinephrine release as increased ATP production by epinephrine has been shown in many tissues of vertebrates[55]. Similarly, the elevated COX activity may correspond to an elevated ATP production in heart mitochondria. The inverse correlation between the decrease of COX activity and the increase of SDH activity in the heart mitochondria of SNP-treated stressed fish implies that an uncoupling action of NO occurs in heart mitochondria, which is not found in NO-depleted mitochondria. L-NAME treatment that increased the COX and SDH activities in heart mitochondria of both non-stressed and stressed fish clearly indicates that similar to action of exogenous NO, absence of endogenous NO would also modulate mitochondrial energetics in heart. In addition, the rise in peroxynitrite that could inhibit SDH activity in heart mitochondria may account for an incomplete oxidative phosphorylation as reported earlier in many mammals[56]. This would also point to a direct action of NO on reallocation of mitochondrial energy partition particularly during hypoxia stress.

Unlike heart mitochondria, liver mitochondria have more diversified roles to play in energy metabolism. We found a rise in NO concentration caused by SNP in liver of non-stressed and stressed fish. However, such an increase in NO content does not reflect on the activities of COX and SDH, providing evidence to the effect that exogenous NO may have fewer roles in mitochondrial respiration in liver. On the contrary, depletion of NO by L-NAME treatment increased the COX activity in liver, pointing to a control of endogenous NO in hepatocyte mitochon-

drial respiration. The regulatory role of NO on complex IV / COX of the ETC is well established in mouse liver mitochondria[57]. The present study thus demonstrates a regulatory role of endogenous NO in the bioenergetics of fish liver. However, unaltered SDH activity in the NO-depleted state reveals a tight bioenergetic control of endogenous NO on complex III of the ETC. The immersion-induced hypoxia stress elevated the production of ROS in liver. It is likely that the increased production of ROS during stress would modulate the mitochondrial complexes as immersion-induced hypoxia-stress can increase the activity of COX without altering the SDH activity. Hypoxia has been shown to decrease the activities of complexes II and III of the respiratory chain in isolated rat liver mitochondria[58-59].

The interactive action of NO on heart mitochondria would demand participation of anaerobic respiratory markers as evident in the elevated LDH activity by SNP treatment. Simultaneous activation of LDH and COX activities would be probably due to the transfer of lactate to pyruvate in heart mitochondria. On the contrary, the rise in LDH activity together with elevated COX and SDH in the heart tissue of L-NAME-treated fish indicates an activation of energy metabolism in NO-depleted state. Similar to heart mitochondria, where activation of mitochondrial energetics occurs, liver mitochondria also show an active energetics during hypoxia as evident in the elevated COX and SDH activities, and not with LDH as it showed decreased activity. These results are in agreement with previous reports showing that NO can function as an endogenous protector against ischemia[60-61].

In summary, the *in vivo* treatment of SNP that releases higher NO_3^-/NO_2^-, ROS and $ONOO^-$ levels, modifies the mitochondrial complexes such as COX and SDH in heart mitochondria of non-stressed fish. On the contrary, L-NAME treatment that lowers NO_3^-/NO_2^- and $ONOO^-$ levels would also modulate the activities of COX and SDH but without affecting ROS production in non-stressed fish. It appears that NO would exert its action through ROS production which in turn might demand the involvement of other regulatory factors in the modulation of mitochondrial energetics. Our data also provide clues to the differential tissue response of NO to mitochondrial energetics. Furthermore, we found that NO demands energy reallocation in heart and liver mitochondria of stressed fish, pointing to a regulatory role of NO in the fish mitochondria. Overall, the present study provides evidence for an integrative action of NO on mitochondrial energetics in the heart and liver mitochondria of the air-breathing fish during their acclimation to water-immersion stress.

5. Acknowledgments

We gratefully acknowledge the financial support from iCEIB project (Higher Education Department, Govt. of Kerala). The authors are grateful to Dr. Jag Mohan, Central Avian Research Institute, Izatnagar, India, for providing the copper-cadmium alloy and Dr. Pankaj Kumar, Rajiv Gandhi University, Itanagar, India, for support. Thanks are due to UGC-SAP-DRS II facility in the Department of Zoology of the University of Kerala. VSP acknowledges the emeritus scientistship of Kerala State Council of Science, Technology and Environment (KSCSTE).

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