2.1 Abstract

Neonatal anoxia at the time of birth can lead to mitochondrial dysfunction and further neurodevelopmental abnormalities. The present study investigated changes in mitochondrial bioenergetics in the neonatal rat whole brain at 30 min, 6 hr. and 24 hr. post first and second anoxic exposure. Neonatal anoxia was induced in rat pups on the day of birth by subjecting them to two episodes (10 min each) of anoxia, 24 hr. by passing 100% N_2 into an enclosed chamber. Bioenergetics was evaluated using a Clark-type electrode and results were analyzed as a function of injury severity and time post-injury. Respiratory control ratio (RCR) was significantly affected by all injury severity levels compared to uninjured tissue after second anoxic exposure. Complex-I and complex-II driven respirations were altered at 30 min, 6 hr. and 24 hr. time points respectively depicting mitochondrial dysfunction. Moreover, we did not observe any significant sensorimotor changes and loss of mitochondrial bioenergetics or elevated nitric levels after first anoxic episode. However there was a marked decrease in sensorimotor activity and mitochondrial respiration along with significant increase in nitric oxide levels following second anoxic episode. Therefore, the two episodic model shows mitochondrial linked mechanism underlying neonatal anoxia apart from sensorimotor deficits and therefore could be used to evaluate novel therapeutic strategies targeting mitochondria.

Keywords: Neonatal anoxia, animal model, mitochondrial bioenergetics, nitric oxide, sensorimotor activity.

2.2 Introduction

Neonatal asphyxia is a condition arising due to the severe deprivation of oxygen at the time of birth which may contribute to permanent neurodevelopmental disability. It is considered as a global clinical problem and has been implicated in causing motor and cognitive alterations in neonates of variable severity like cerebral palsy, epilepsy, dystonia in the early age and attention deficit disorder. Further, mental retardation and other neuropsychiatric syndromes at school age [20, 56].

The brain as an active metabolizing tissue requires an adequate provision of glucose and oxygen to maintain its normal functions is less during anoxia, triggering a cascade of biochemical events [57, 58]. This phenomenon can result in excitotoxicity which leads to an influx of Ca^{2+} and overproduction of nitric oxide (•NO) and other reactive oxygen species (ROS) within mitochondria [56]. As a result of the opening of membrane permeability transition pore took place and increased the release of cytochrome-C which results in mitochondrial stress [59]. Thus, mitochondria may be an important target for the progression of primary to secondary insult. Brain injury as a result of anoxia progressively appears in two distinct phases, primarily as an initial phase which may result due to necrotic cell death and secondarily the latter phase due to apoptotic cell death. Overall, there is a state of energy deficiency that plays a key role in anoxia induced brain damage in newborns [60-62].

In the present study, we used a non-invasive two episodic model of anoxia in neonate rats [61]. This method is better optimized than other similar anoxic models as it uses two subsequent episodes of anoxia rather than a single prolonged episode. This increases the survival rate of neonates by decreasing the length of anoxic exposure. Several asphyxia models were evaluated for mitochondrial functions, but none of those have characterized

mitochondrial bioenergetics regarding oxygen consumption in different states post anoxic exposure [60, 62, 63]. We for the first time evaluate mitochondrial bioenergetics using the two episodic model of anoxia.

Thus, the present study characterizes the mitochondrial bioenergetics in the two distinct episodes of anoxia. This may lead to a precise understanding of substrates involved in mitochondrial dysfunction and therefore be helpful to evaluate a mitochondrial targeted pharmacological intervention for the treatment of anoxia.

2.3 Materials and methods

2.3.1 Animals

Charles Foster albino pregnant rats (180–220 g) were procured from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University (IMS-BHU). The animals were housed in polypropylene cages under controlled environmental conditions of a temperature of 25 ± 1 ℃ and 45 – 55 % relative humidity and a 12: 12 h light/dark cycle. Birth litter count ranged from 8 to 12 pups per rat of approximately 30 hr. age and weighing 6–8 g, was used. The experimental procedures were approved by the Institutional Animal Ethical Committee of BHU (Protocol No. Dean/11-12/CAEC/328). All experiments were performed as per the guidelines of laboratory animal care (National Research Council US Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011) guidelines.

2.3.2 Anoxia model

The anoxic procedure was performed as previously defined [20] with some slight modifications. Briefly, the pups were kept in an anoxic chamber (plexiglass with dimensions

of 21 cm ×18 cm ×11 cm) contained a gas inlet and outlet with an airtight lid equipped with a flowmeter and a nitrogen gas cylinder. The chamber was partially immersed in warm water (35 and 37°C) to avoid hypothermia [64]. To induce anoxia, six pups (neonates) of approximate 30 hr. age were placed inside the chamber with continuous 100% nitrogen gas flow at 3 L/min at a pressure of approximately 101.7 kPa into the chamber. Two anoxic episodes of 10 min each at an interval of 24 hr. was used. The lid was removed immediately after each episode and pups were exposed to the atmosphere and resuscitated by laying them on their back and spreading their limbs for recovery. After recovery, confirmed by skin color, respiration and locomotor behavior for 5 minutes after removal from the chamber, the animals were returned to the dams and killed at different time points (30 min, 6 hr. and 24 hr. respectively) following first and second anoxic exposure. The same experimental procedure was followed for the control group, but the chamber contained air instead of nitrogen. The brains were rapidly dissected out at different time points and stored immediately at -80 °C till further experimentation.

2.3.3 Reagents

Mannitol, sucrose, bovine serum albumin (BSA), EGTA, HEPES potassium salt (St. Louis, MO, USA), potassium phosphate monobasic anhydrous (KH2PO4), $MgCl_2$, malate, pyruvate, ADP, succinate, oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), Rotenone and and Griess reagent were procured from Sigma-Aldrich.

2.3.4 Behavioral parameter assessment

2.3.4.1 Righting reflex

The assessment was done by placing the pups on their back and the time taken to turn on to their belly. The time for a complete turn on to four limbs touching platform was measured [65].

2.3.5 Mitochondrial Isolation

Isolation of mitochondria from whole brain of neonatal rat was performed by the method previously defined [66] with some slight modifications. Briefly, brains dissected at different time points (30 min, 6 hr. and 24 hr. respectively) were homogenated in isolation buffer (consisting of 215 mM mannitol, 75 mM sucrose, 0.1% w/v bovine serum albumin, 20 mM HEPES buffer and 1 mM of EGTA in 100 ml of distilled water and pH adjusted to 7.2 with KOH) and first centrifuged at 1300 × g for 5 m at 4°C. Each supernatant was then topped off with isolation buffer with EGTA and centrifuged at 14,000 × g for 10 min at 4°C to get a tighter mitochondrial pellet. Further, a washing step was performed by suspending the pellets in isolation buffer without EGTA and again centrifuged at 14,000 × g for 10 min to remove EGTA from the pellets. Mitochondrial protein was estimated colorimetrically [67] with a microplate reader (Biotek, USA).

2.3.6 Measurement of mitochondrial function

Mitochondrial functionality was assessed using an Oxytherm Clark-type oxygen electrode (OXYT1/ED, Hansatech Instruments, Norfolk, UK). Mitochondria (180-200 µg) were placed in the sealed Oxytherm chamber containing respiration buffer (125 mMKCl, 0.1% BSA, 20 mM HEPES, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, pH 7.2) and continuously stirred at

37 ºC. The rate of oxygen consumption was defined as the slope of the response of isolated mitochondria to the consecutive administrations of oxidative substrates as previously described [68].

2.3.7 Assessment of brain mitochondrial nitrite level

Nitrite levels were determined by a colorimetric assay using Greiss reagent (0.1% at 540nm) as described previously [69]. Equal volumes of supernatant and Greiss reagent were mixed, and this mixture was incubated for 10 min at room temperature in the dark. Absorbance at 540 nm was measured with a microplate reader (Biotek, USA). The nitrite amount was calculated in comparison to the standard nitrite curve, and the results were expressed as nanomoles of nitrite formed per milligram of protein.

2.3.8 Experimental design

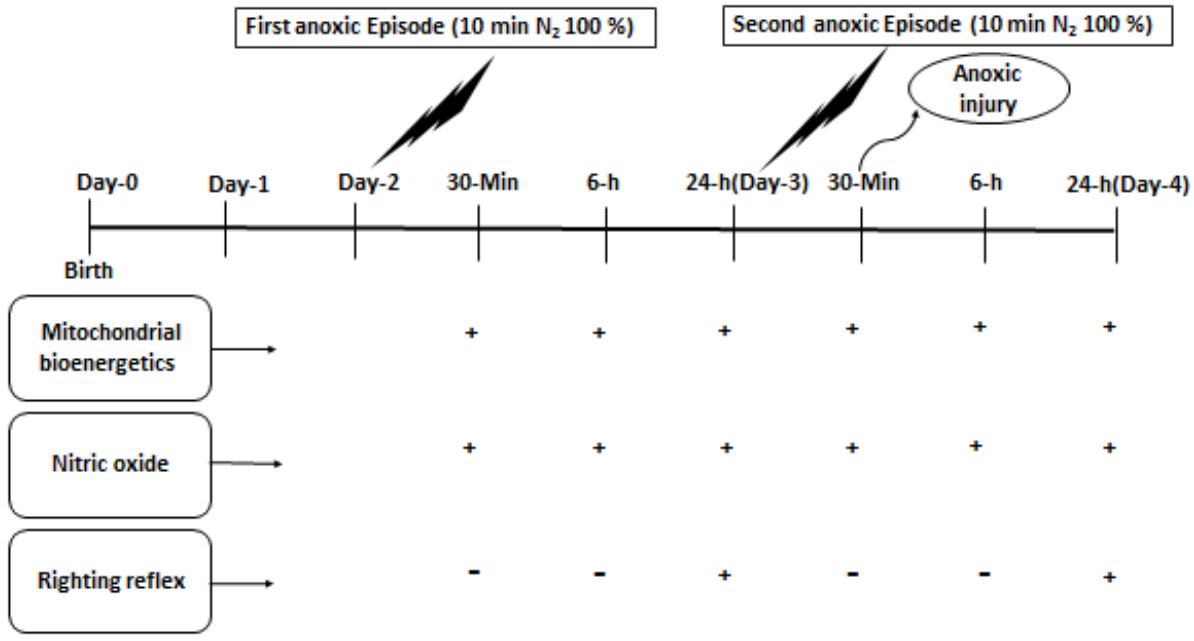

Figure 2.1 Schematic representation of the experimental design. '+' denotes performed and '-'denotes not performed

2.3.9 Statistical analysis

The data are expressed as means ± SD. For the statistical analysis of mitochondrial bioenergetics, mitochondrial RCR and brain nitrite level a one-way ANOVA followed by Newman-Keuls test was used. An unpaired t- test was used to compare the sensorimotor changes 24 hr. post first and second exposure to anoxia.

2.4 Results

2.4.1 Changes in mitochondrial respiration regarding oxygen consumption after first and second episodes of anoxia.

Fig. 2.2 shows a representative trace for the oxygen consumption of different groups and time points after (A) first and (B) second anoxic episode. Whereas, Fig. 2.3 shows different states of mitochondrial respiration and RCR in the (C, E) first episode and (E, F) and second episode. One-way ANOVA revealed no significant changes in different states of respiration post first anoxic exposure in s-2 [$F(3, 12) = 2.803$, $P>0.05$], s-3 [$F(3, 12) = 2.375$, $P>0.05$], s-4 [$F(3, 12) = 1.872$, $P>0.05$], s-5 complex-I [$F(3, 12) = 2.976$, $P>0.05$] and s-5 complex-II [$F(3, 12) = 2.293$, $P>0.05$] respiration at different time points. Similarly, there was no significant effect of first episode anoxic exposure on RCR [$F(3, 12) = 3.230$, $P>0.05$]. Whereas, a significant difference was observed in s-3 [$F(3, 12) = 18.97$, $P<0.05$] and s-4 respiration [$F(3, 12) = 5.021$, $P<0.05$] after second anoxic exposure. Post hoc analysis showed significant ($P<0.05$) decrease in s-3 respiration at all the time points. However, a marked increase in s-4 respiration was observed at 30 min but not in 6 hr. and 24 hr. compared to control group animals. Compared to 30 min groups. There was a marked decrease in s-5 complex-I [$F(3, 12) = 4.442$, $P<0.05$] and s-5 complex-II respiration [F (3,

12) = 4.300, P<0.05] at all the time points. Post hoc analysis revealed a significant (P<0.05) change in complex-I driven s-5 respiration (24 hr.) and complex-II mediated s-5 respiration (30 min, 6 hr. and 24 hr.) as compared to control group animals. Similarly, a significant difference in RCR at different time points following the second episode of anoxic injury [F (3, 12) = 12.94, P<0.05] was observed. Post hoc analysis revealed that there was a significant decrease (P<0.05) in RCR at all-time points post second exposure compared to control group animals.

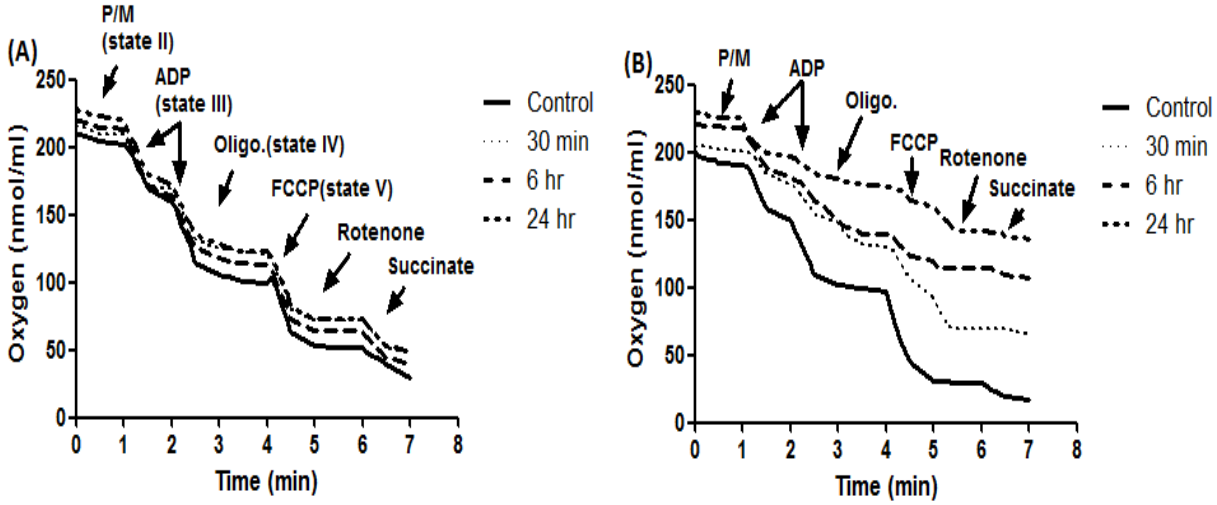

Figure 2.2 (A) A typical respiratory trace shows no significant changes in mitochondria bioenergetics at three different time points after first anoxic exposure. Mitochondrial oxygen consumption was measured using a Clark-type electrode in a continuously stirred sealed chamber (Oxygraph System; Hansatech Instruments Ltd.). Purified mitochondrial protein was suspended in respiration buffer in a final volume of 250 µL. State-2 (s-2) respiration was initiated by addition of pyruvate/malate (P/M), with a basal rate of respiration. State-3 (s-3) respiration was initiated by addition of ADP; the high level of oxygen utilization indicates that ADP is getting converted into ATP. State-4 (s-4) was measured by addition of oligomycin. The respiration returns to basal rate since the ATP synthase is shut down and no electrons are allowed to return to the matrix. The ETC continues only to maintain mitochondrial membrane potential due to loss of protons back into the matrix. State-5 (s-5) was measured by addition of FCCP. This represents the maximum rate of respiration, causing uncoupling of the ETC to ATP synthesis, and allows protons to rush back into the matrix. Rotenone was then added to shut down complex-I driven respiration. State-5 (succinate) was determined by addition of succinate. This is the maximum rate of respiration via complex-II since FCCP is present in the system. (B) Mitochondria isolated at all-time points after second anoxic exposure showed a compromised mitochondrial bioenergetics.

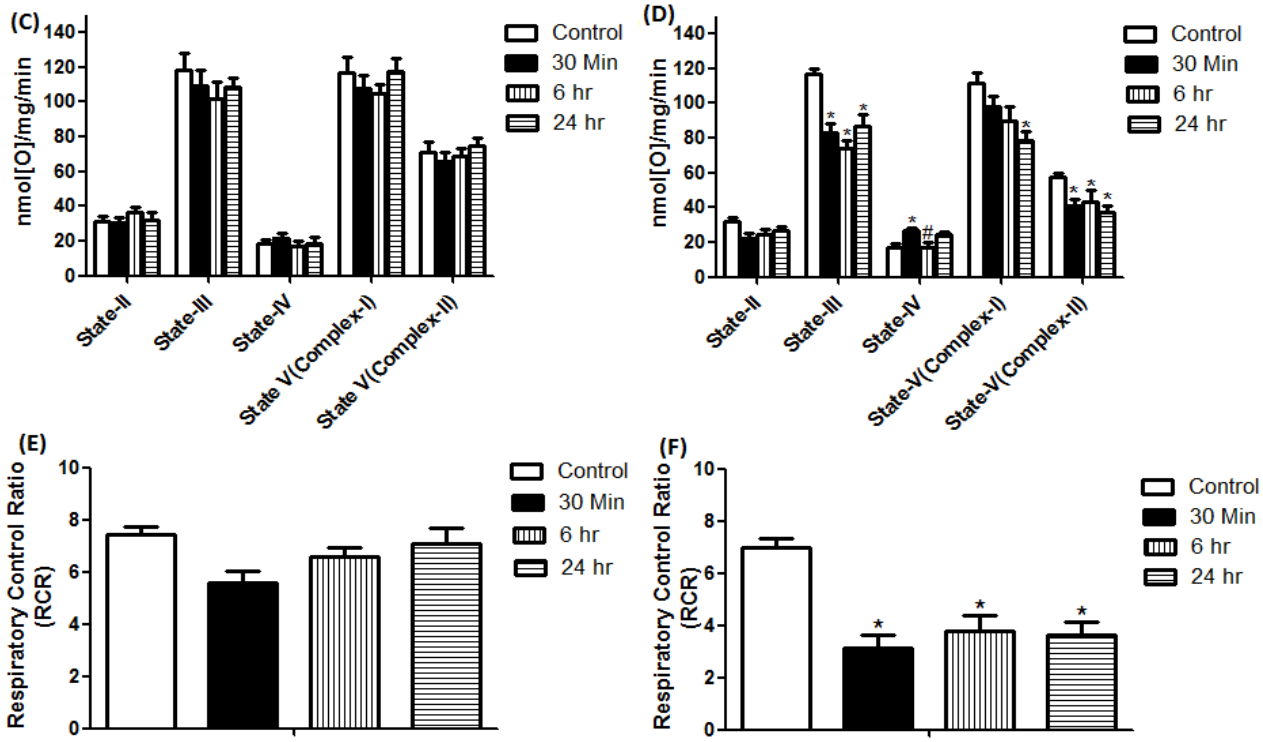

Figure 2.3 The effect of first and second episodes of anoxia on oxygen consumption in different states of mitochondrial respiration (C and D respectively) and respiratory control ratio (RCR; E and F respectively) in whole brain mitochondria after 30 min, 6 hr. and 24 hr. Bars represent group means ± SD (n=4 in each group). $^{*}P<0.05$ compared to control and $^{\#}P<0.05$ compared to anoxic 30 min post exposure groups respectively [One-way ANOVA followed by Student–Newman–Keuls test].

2.4.2 Changes in brain mitochondrial nitrite levels after first and second anoxic episode.

Fig. 2.4 shows the changes in brain mitochondrial nitrite levels after (A) first and (B) second anoxic episode. A one way ANOVA depicted no significant difference in nitrite levels at different time points [$F(3, 12) = 0.7716$, $P>0.05$] after first anoxic exposure. Whereas, a significant difference is observed in nitrite level at all-time points following the second episode anoxic exposure [$F(3, 12) = 16.75$, $P<0.05$]. Post hoc analysis showed a significant ($P<0.05$) elevation of nitrite levels at all time points compared to control group and 6 hr. compared to 24 hr. group animals after second episode post exposure.

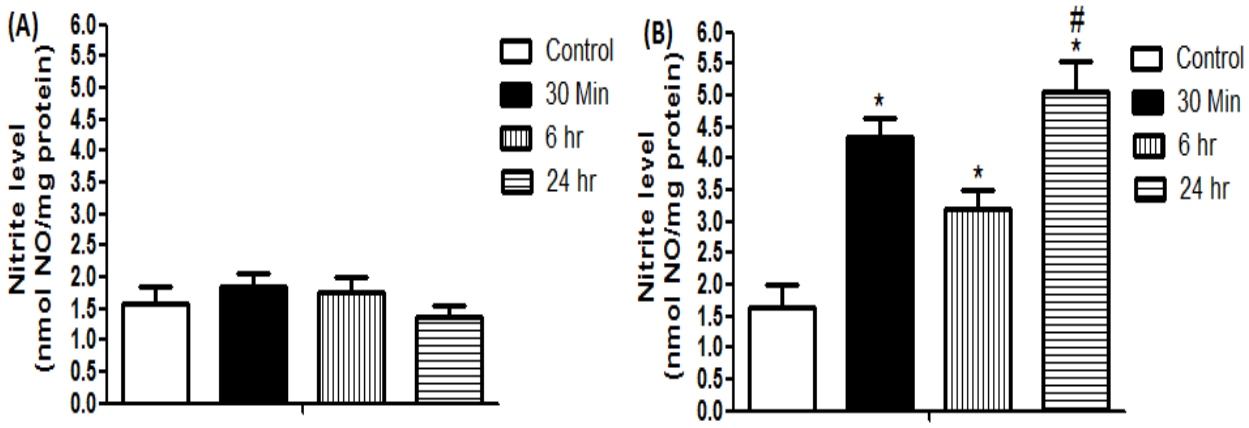

Figure 2.4 The effect of first and second episodes of anoxia on whole brain nitrite level (A and B respectively). Bars represent group means ± SD (n=4 in each group). $^{*}P<0.05$ compared to control and $^{\#}P<0.05$ compared to 6 hr. Post exposure groups respectively [One-way ANOVA followed by Student–Newman–Keuls test].

2.4.3 Sensorimotor changes 24 hr. after first and second anoxic exposure.

Fig. 2.5 illustrates changes in reflex latency after (A) first and (B) second episode of anoxic exposure. An unpaired t-test revealed no significant difference in the reflex latency of control and anoxic group [$t_6 = 0.7313$, $P>0.05$] 24 hr. after first episode of anoxia. Whereas, a significant difference was observed [$t_6 = 6.708$, $P<0.05$] 24 hr. after second episode.

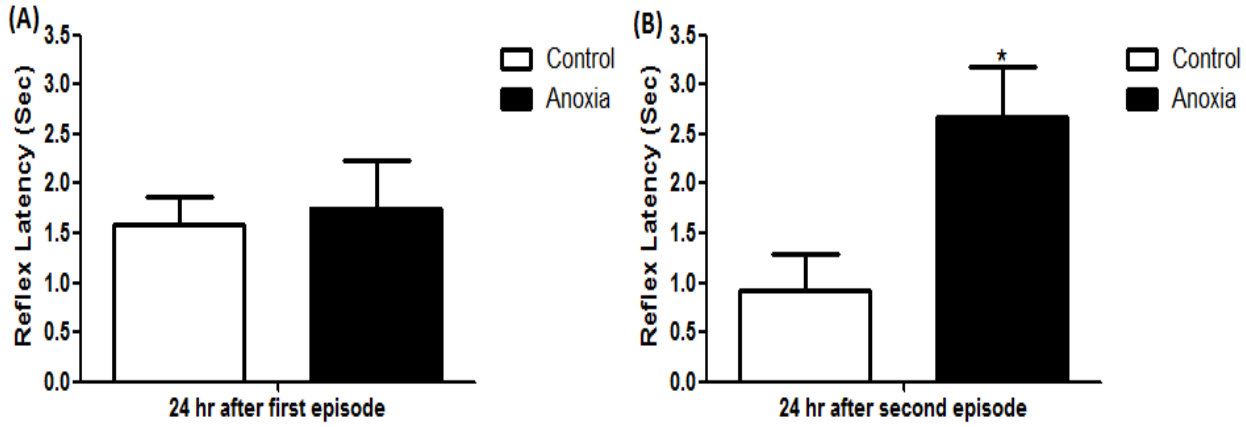

Figure 2.5 Changes in reflex latency after (A) first and (B) second episode of anoxic exposure. Bars represents group means ± SD (n=6 in each group). $^{*}P<0.05$ compared to control [Two tailed unpaired Student-t test].

2.5 Discussion

In this present study, we characterized for the first time the mitochondrial bioenergetics and associated sensorimotor changes using two subsequent episode model of neonatal anoxia in rats. The changes in mitochondrial respiration and sensorimotor deficit were observed after the second episode of anoxic injury which started as early as 30 min and continued up to 24 hr. post anoxia exposure.

The s-2 respiration depicts the consumption of substrate pyruvate/malate to fuel the mitochondrial ETC. No difference in s-2 respiration was observed following first and second episode of anoxia between any experimental groups. Further s-3 respiration was initiated by addition of ADP. In this study, s-3 respiration significantly declined at different time points. The reason may be a deficiency in the supply of respiratory substrates due to compromised ETC components and oxygen to produce ATP [28]. Previous studies targeting mitochondrial dysfunction have shown a decrease in s-3 respiration following anoxia which is in agreement with our finding [62]. We found an increase in s-4 respiration by using oligomycin (ATP-synthase inhibitor) after second anoxic episode. Similar finding has been

reported in focal traumatic brain injury model [70]. As s-4 respiration denotes the extent of coupling of proton motive force with ATP production versus maintaining the basal metabolic rate, increase in oxygen utilization denotes uncoupling of the ETC to ATP.

Subsequent addition of a mild uncoupler FCCP (s-5) detaches the ETC from oxidative phosphorylation. This determines the maximum respiration capabilities of the ETC in its attempts to restore the dissipated proton gradient [71]. As, mitochondria were not able to overcome this effect and maintain respiration due to a loss in function of complex I at 24 hr. time point, the oxygen consumption at s-5 (complex I) was significantly reduced [68]. Following the addition of rotenone, which acts as a competitive inhibitor to block complex I driven respiration, meanwhile succinate was added to determine if complex-II driven respiration is affected by second anoxic exposure. The significant loss of complex-II s-5 respiration was observed at all-time points post second anoxic exposure indicates that susceptibility of complex-II to anoxia. Previous experimental studies have shown compromised complex-II after anoxic exposure [60, 61]. RCR is the ratio of s-3 to s-4 respiration and is a measure of mitochondrial integrity [68]. There was a significant reduction in RCR as early as 30 min and up to 24 hr. post second anoxic exposure. Previous findings have shown that mitochondrial RCR was decreased immediately after hypoxia/anoxia [62]. This shows loss of mitochondrial integrity after the second episode but not after the first episode. There was a significant increment in nitric oxide regarding nitrite as early as 30 min, 6 hr. which was continued later up to 24 hr. post anoxic exposure. Nitric oxide forms peroxinitrite ($ONOO^-$) with superoxide ($O^{\cdot -}_2$), which causes mitochondrial oxidative stress and impair mitochondrial respiration [41].

Anoxic insult in the neonatal rats delayed the appearance of righting reflex at 24 hr. time point post second anoxic exposure. This reflects the sensorimotor deficiencies in early postnatal days in rats. It was reported that such deficits are likely caused by subcortical damage in the neonatal brain [65, 72]. The loss of sensorimotor function was observed along with a mitochondrial function post second episode of anoxia, but not after the first episode. Therefore, our results indicate that in anoxic condition mitochondria bioenergetics plays an important role in neurodevelopment [72].

Hence, the present study indicates that anoxic exposure using the two episode model causes mitochondrial dysfunction and sensorimotor changes post second anoxic exposure. The model will be helpful to evaluate mitochondrial targeted pharmacological interventions to treat neonatal anoxic conditions.