3.1 Abstract

Neonatal anoxia arises due to oxygen deprivation at the time of birth and results in lifelong neurodevelopmental deficits and sometimes may lead to death. The present study was investigated in the time-dependent cortical mitochondrial dysfunction linked apoptosis, related sensorimotor deficits and changes in cerebral blood flow (CBF) in neonates post-anoxia. Neonates (rat pups) of postnatal day 2 (P2) were subjected to anoxia of two episodes (10 min in each) at a time interval of 24 hr. by passing 100 % N_2 into an enclosed chamber as confirmed by pulse oximetry. Sensorimotor activities like reflex latency and hanging latency were carried out 24 hours after last anoxic episode i.e. from P4 (day-1; d-1) and continued up to P10 (day-7; d-7). Decreased regional CBF is assumed to be the prime factor that determines the level of tissue injury in the immature brain. The mean CBF was decreased from d-1 to d-7 resembling progression of the insult. Mitochondrial dysfunction after anoxia was evident by the decrease in respiration states, respiratory control ratio (RCR), antioxidant enzyme activity further increase in oxidative stress, lipid peroxidation and alteration in mitochondrial membrane potential (MMP) at different time points (30 min, 24 hr. (d-1) and d-7) were observed. A change in expression of Bcl-2 family proteins and the opening of mitochondrial transition pore (MPT) related to mitochondrial swelling was observed resulting in the release of cytochrome-C. This further activated intrinsic (mitochondrial) pathway of apoptosis through increased expression of caspase-9/3 as confirmed by flow cytometry. Therefore, strategies in limiting mitochondrial-linked apoptosis during the secondary insult input process may be useful in the treatment of long-term sensorimotor deficits following anoxia.

Keywords: Anoxia; mitochondrial respiration; neurobehavioral changes; respiratory control ratio; apoptosis; Flow cytometry.

3.2 Introduction

The mechanisms leading to brain damage after anoxia are complex and related to the developmental stage of the brain and the severity of the insult. We in the previous study have shown that anoxic exposure using the two episode model caused mitochondrial dysfunction and sensorimotor changes depicting that primary insult progression starts after second anoxic exposure. Since, it is evident that primary insult occurs more in the form of necrosis than apoptosis immediately after the hypoxic/anoxic exposure, resulting from the biomechanical forces directly damaging neuronal tissue. There are several reports to suggest that most of the cell death during secondary insult occurs through apoptosis [73-75]. Therefore, inhibition of this secondary insult processes may be one of the most important means for therapeutic intervention to prevent further degeneration of brain tissue and preserve the healthy one. Therefore, the current study evaluates the long-term changes in mitochondrial dysfunction-linked apoptosis to understand time-dependent progression of insult and to find a suitable pharmacological target for treatment of anoxia.

In the present study, using non-invasive animal model of anoxia, neonates (rat pups) of postnatal day 2 (P2) were subjected to anoxia of two episodes (10 min in each) at a time interval of 24 hr. by passing 100 % N_2 into an enclosed chamber which showed cortical mitochondrial dysfunction and apoptosis as early as 30 min after the last anoxia episode and progressed up to d-7. In our previous study, we demonstrated acute pathological changes in mitochondrial bioenergetics that leads to primary insult in the whole brain after

second anoxia episode and results in sensorimotor deficits in neonates [76]. The present study was focused on anoxia-induced secondary insult (d-7) and its comparison to severity of primary insult (30 min and 24 hr.) in the cortical brain region of neonates which is involved in sensorimotor activity coordination [77]. Secondary insult mechanisms are the result of a number of factors, including ischemia–reperfusion injury, oxidative stress, mitochondrial calcium overload, activation of proteases and caspases [28, 78].

The marked pathological changes in cortical blood flow after anoxia is another aspect of insult progression. Previous reports have stated that the decreased regional cerebral blood flow (CBF) is the prime factor that determines the topography of tissue injury in the developing rodent brain [79]. However, CBF is rarely monitored during and after hypoxic-ischemia (HI) insult in immature rodent models, mainly because of the technical difficulties and invasiveness involved in measuring it. Therefore, little is known about the spatial and temporal extent of CBF response during the reperfusion period after an HI insult. The autoradiographic techniques for analyzing CBF in small animals entail sacrificing the animals at the time of measurement. The non-invasive laser speckle method for imaging vascular structure in tissue has been available since the 1980s. Recently, it has been used to measure CBF [80]. The speckle imaging technique is capable to accurately image the cortical blood flow response over an area ranging from a few millimeters to the whole rodent brain [80].

Apoptosis is regulated by several protein families, including the upstream pro-apoptotic Bax and anti-apoptotic Bcl-2 family proteins and the downstream caspase family like caspase-9 and caspase-3 [81]. It is proposed that the mitochondria can be the regulator of both energy metabolism and apoptotic pathways. Their dysfunction can result in impairment of brain

mitochondrial electron transfer and energy transduction [82, 83]. MMP component determines calcium sequestration and the generation of reactive oxygen species in mitochondria [84, 85]. This further leads to opening of MPT and release of cytochrome-C and triggers intrinsic pathway of apoptosis through activation of caspases-9/3 and finally result in apoptotic cell death [86, 87].

In the present study, a detailed time-dependent mitochondrial function was examined by evaluating mitochondrial respiratory chain enzyme activity, oxidative damage, MMP, MPT, expression of Bax, Bcl-2 and their ratio (Bax/Bcl-2), release of cytochrome-C and further activation of apoptotic markers like caspase-9, and caspase-3 and confirmation of apoptotic cell death by flow cytometry in cortical region at different primary (30 min, 24 hr.) and secondary (d-7) insult time points.

3.3 Materials and Methods

3.3.1 Animals and groups

All experiments were performed by the principles 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. The experimental procedures were approved by the Institutional Animal Ethical Committee of BHU (Protocol No. Dean/11-12/CAEC/328). Rat pups of approximate 30 hr. (P2) Age was used for the experiment. Six groups of five neonates each were assigned to either the anoxia or the similar control treatments for different time points [30 min (P4), 24 hr. (P4) moreover, d-7 (P10) respectively] in such a way that each of the resulting group contained rat pups from at least two different litters.

3.3.2 Anoxia procedure

The anoxic procedure was carried out as validated and defined previously [76]. After exposure to anoxia, the rat pups were returned to the dams and sacrificed at different time points [30 min (P4), 24 hr. (P4) and d-7 (P10) respectively]. The brains were rapidly dissected out, and the cortical portion was immediately used for mitochondrial isolation. Mitochondrial respiration studies were performed immediately after mitochondrial isolation. Further, mitochondrial pellets were stored at -80º C for further biochemical estimations, flow cytometry and western blot analysis.

3.3.3 Materials

Reagents—Tetramethylrhodamine methyl ester (TMRM) and Griess reagent were procured from Sigma-Aldrich (St. Louis, MO, USA). Antibodies such as cytochrome-c, caspase-9, caspase-3, Bax, Bcl-2 and beta-actin (Santa Cruz Biotechnology Inc.; Santa Cruz, California, USA) were purchased.

3.3.4 Behavioral parameter assessment

Behavioral parameters like righting reflex and wire hanging maneuver were performed 24 hr. (P4) moreover, d-7 (P10) after second anoxic episode.

3.3.4.1 Righting reflex

As described previously [65] and detailed in chapter 2.Page no. 23.

3.3.4.2 Wire hanging maneuver

This maneuver tests neuromuscular and locomotor development. Pups suspended by their forelimbs from a horizontal rod (5 mm × 5 mm area, 35 cm long, between two poles 50 cm

high) tend to support themselves with their hind limbs, preventing them from falling and aiding in progression along the rod. Suspension latencies were recorded [65, 88].

3.3.5 Measurement of peripheral oxygen saturation

Measurement of peripheral oxygen saturation was performed for control and anoxia group animals. A pulse oximeter (Physiosuite/Kent scientific corporation, U.S.A) along with a pediatric Y-sensor was coupled with colorless tape near the proximal region of the animal's tail at the dorsal and ventral faces of the abdomen [89]. After positioning the sensor, the apparatus was turned on, and a value between 97% and 99% of oxygen saturation was observed when the sensor was correctly positioned. Rat pups were placed into the chamber with the connected oximetry apparatus, and the values of oxygen saturation were collected by an observer every minute during the anoxia or control procedures [64].

3.3.6 Laser speckle blood-flow imaging

The protocol followed was as previously described [90]. In 12 rat pups, the cortical surface CBF was sequentially measured by a laser speckle flowmetry (LSF) imaging system (Omegazone, Omegawave Inc., Tokyo, Japan) at 30 min, 24 hr. and d-7 time points post-anoxia: In brief, the animals were placed in a prone position and spontaneously breathed under isoflurane anesthesia. The animal's skull was exposed by a midline scalp incision, and the skull surface was diffusely illuminated by a 780 nm laser light. The penetration depth of the laser is approximately 500 μm. The scattered light was filtered and detected by a charge coupled device (CCD) camera positioned above the animal's head. Raw speckle images were used to compute the speckle contrast, which is a measure of speckle visibility that is related to the number and velocity of moving particles (in this case, CBF). Color-coded blood-flow

images were obtained in the high-resolution mode (638 pixels × 480 pixels; 1 image/s). Five consecutive raw speckle images were acquired at 1 Hz and then averaged. For analytical accuracy in the repositioning of the animal's head and regions of interest (ROIs) between imagings, we set the size and the position of an ROI, based on a line drawn from the bregma to the lambda. The entire measuring procedure took approximately 3 min per pup.

3.3.7 Mitochondrial Isolation

Isolation of mitochondria from the cortical brain region was done by the method previously described [66] with some slight modifications [76]. Discussed in chapter 2. Page no.24

3.3.8 Measurement of Mitochondrial Function

Mitochondrial function was assessed using an Oxytherm Clark-type oxygen electrode as discussed earlier [91]. Discussed in chapter 2. Page no.24

3.3.9 Evaluation of mitochondrial membrane potential (MMP) in cortical brain region

Rhodamine dye taken up by healthy mitochondria was measured fluorimetrically at an excitation λ of 535 ± 10 nm and emission λ of 580 ± 10 nm [92]. The results were expressed as fluorescence intensity value/mg protein.

3.3.10 Estimation of mitochondrial permeability transition pore (MPT) opening

MPT was measured as a function of mitochondrial swelling and contraction measurement by light scattering to test mitochondrial membrane integrity as described earlier [93]. Change in absorbance at 520 nm was normalized to the protein concentration.

3.3.11 Estimation of LPO and NO levels

Mitochondrial malondialdehyde (MDA) content was measured as per standard protocol [94]. The extent of lipid peroxidation (LPO) was expressed as micromoles of MDA/mg protein. Nitrite (NO) levels were determined by a colorimetric assay using Greiss reagent (0.1% at 540nm) [69] and the results were expressed as nanomoles of NO/mg protein.

3.3.12 Assessment of mitochondrial SOD and CAT activity

Superoxide dismutase (SOD) activity was estimated by the reduction of NBT in the presence of phenazine methosulphate and NADH at 560 nm [95]. A single unit of the enzyme was expressed as 50% inhibition of NBT reduction per minute per milligram of protein under the assay conditions. Catalase activity was estimated by the addition of 50 ml H_2O_2 (6%) and decrease in the absorbance was measured at 240 nm for 3 min at 30 s interval [96].

3.3.13 Western blot analysis for cytoplasmic cytochrome-C, caspase-9, caspase-3, Bax, Bcl-2 and mitochondrial Bax and Bcl-2

For western blot analysis, the cortical brain tissues each of control and anoxic (30 min, 24 hr. and d-7) animals was lysed in buffer containing complete protease inhibitor cocktail. Protein concentrations were determined as stated previously [97]. An aliquot of each sample was electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred onto polyvinylidene fluoride membranes (PVDF) and blocked with 5% skim milk powder for one hour. The primary antibodies against cytochrome C (1:2,000), Caspase-9 (1:1,000), Caspase-3 (1:1,000), Bax (1:1,000), Bcl-2 (1:1,000), and β-actin (1:1,000) were diluted according to the manufacturer's instructions and incubated with the membrane overnight at 4°C. After washing three times, the

horseradish peroxidase-conjugated secondary biotinylated antibodies were added (1:2,000) and incubated with the membranes for one hour. After washing three times with PBS, the blots were developed using chemiluminescence (ECL) solution (Amersham Bioscience, USA). Quantification of the results wereperformed by a densitometric scan of films. The bands were quantified using Biovis gel documentation software.

3.3.14 Flow cytometric measurement of cell death using AnnexinV/PI

Flow cytometric assay was performed using the Annexin-V-Fluos Staining Kit (Roche Diagnostics, Penzberg, Germany [98]. In brief, single cell suspensions from the five cortical brain tissue samples were generated in cold PBS buffer, and the final concentrations were adjusted to 5×10^5/ml. Further, 1 ml of suspensions was centrifuged at 300 × *g* for 5 min at 4 °C and washed with 1 ml cold PBS thrice. The pellet so formed was then re-suspended in 200 µl of Annexin-V-Fluos labeling solution and later incubated with 10 µl fluorescein isothiocyanate (FITC)-conjugated Annexin-V and 5 µl of propidium iodide (PI) for 15 min at room temperature in the dark. Samples were kept on the ice and analyzed on a BD LSRFortessa SORP flow cytometer equipped with five lasers. Emission fluorescence was measured with a 525/50 filter for FITC and with a 610/20 filter for red PI. FITC and PI were excited with two different lasers of 488 nm for the first and 561 nm for the second, thus avoiding signal compensation. Data were acquired and analyzed using BD FACSDIVA™ software (BD Biosciences, San Jose, CA). A minimum of 10,000 events was collected for each sample. The apoptosis rate was calculated with the following formula: (number of apoptotic cells/total cells) × 100 %.

3.3.15 Statistical analysis

The data are expressed as means ± SD. For the statistical analysis of peripheral oxygen saturation in both episodes and sensorimotor behavioral activity like hanging latency and reflex latency, a repeated measure two-way analysis of variance (ANOVA) with Bonferroni post hoc analysis was performed. All other statistical analyses of data were done using two-way ANOVA with Bonferroni post hoc analysis to monitor significance among groups. $P < 0.05$ was considered as significant.

3.4 Results

3.4.1 Peripheral arterial oxygen saturation during first and second anoxic exposure

Fig. 3.1 shows a comparison of the peripheral arterial oxygen saturation values of the anoxia and control groups within (A) first and (B) second anoxic exposure using pulse oximetry. A repeated measure two-way ANOVA revealed a significant difference between groups [$F(1, 80) = 49.03$, $P<0.0001$, $\eta^2 = 0.317$], insignificant for time [$F(9, 80) = 1.662$, $P=0.1$, $\eta^2 = 0.096$] during first anoxia episode. Post-hoc analysis revealed that there was a near 30% fall in oxygen saturation for last two minutes (9^{th} and 10^{th}) of first anoxia episode ($P<0.05$) compared to control group. On the other hand there was a significant difference between groups [$F(1, 80) = 8.525$, $P<0.0001$, $\eta^2 = 0.575$] and time [$F(9, 80) = 288.4$, $P<0.0001$, $\eta^2 = 0.152$] along with significant interaction between group and time [$F(9, 80) = 6.220$, $P<0.0001$, $\eta^2 = 0.111$] during second anoxia episode. Post-hoc analysis revealed a significant decrease in oxygen saturation from 3^{rd} minute to 10^{th} minute and an approximate 70% decrease ($P<0.05$) in oxygen saturation till the end of the second episode.

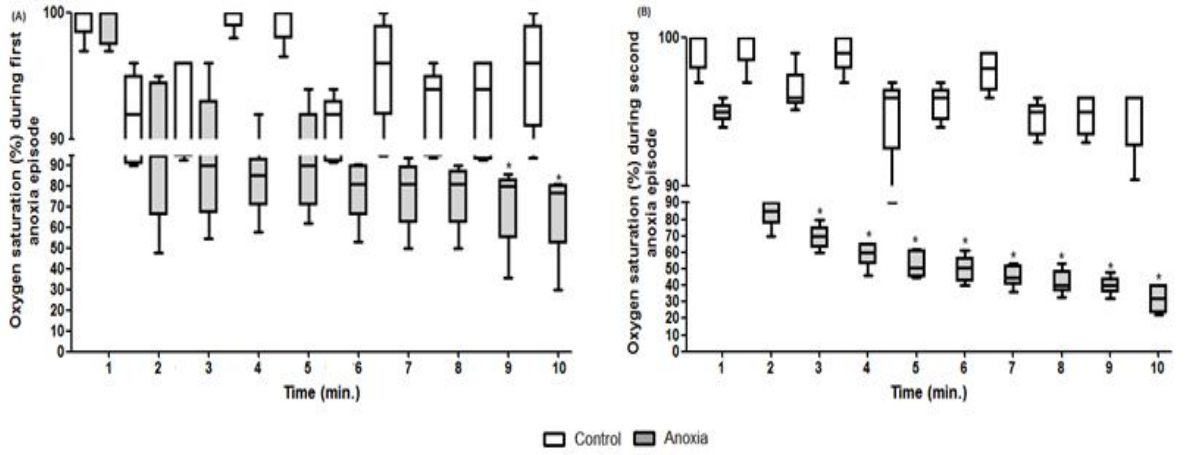

Figure 3.1 Evaluation of peripheral arterial oxygen saturation by pulse oximetry over time within first (A) and second (B) anoxia episode by passing 100% nitrogen in the anoxia chamber. "Box and Whisker" plots indicate center line = median, top of the box = 75th percentile, the bottom of the box = 25th percentile, whiskers = data within 1.5 interquartile ranges. *P<0.05 compared to control and anoxic group respectively [Repeated measure Two-way ANOVA followed by Bonferroni post-test].

3.4.2 Cortical blood flow measurements

Fig 3.2 shows the cortical blood flow in (A) control (B) anoxia 30 min (C) anoxia 24 hr. and (D) anoxia d-7 respectively, and Fig 3.3 shows the mean cortical blood flow at different time points after anoxia. One way ANOVA depicted significant changes in blood flow [F (3, 8) = 147.5; P<0.05] among anoxia group on different days compared to control animals. A Posthoc analysis showed that anoxia caused a marked decrease in cortical blood flow. However, there was a significant difference in the cortical blood flow on d-7 as compared to 24 hr.

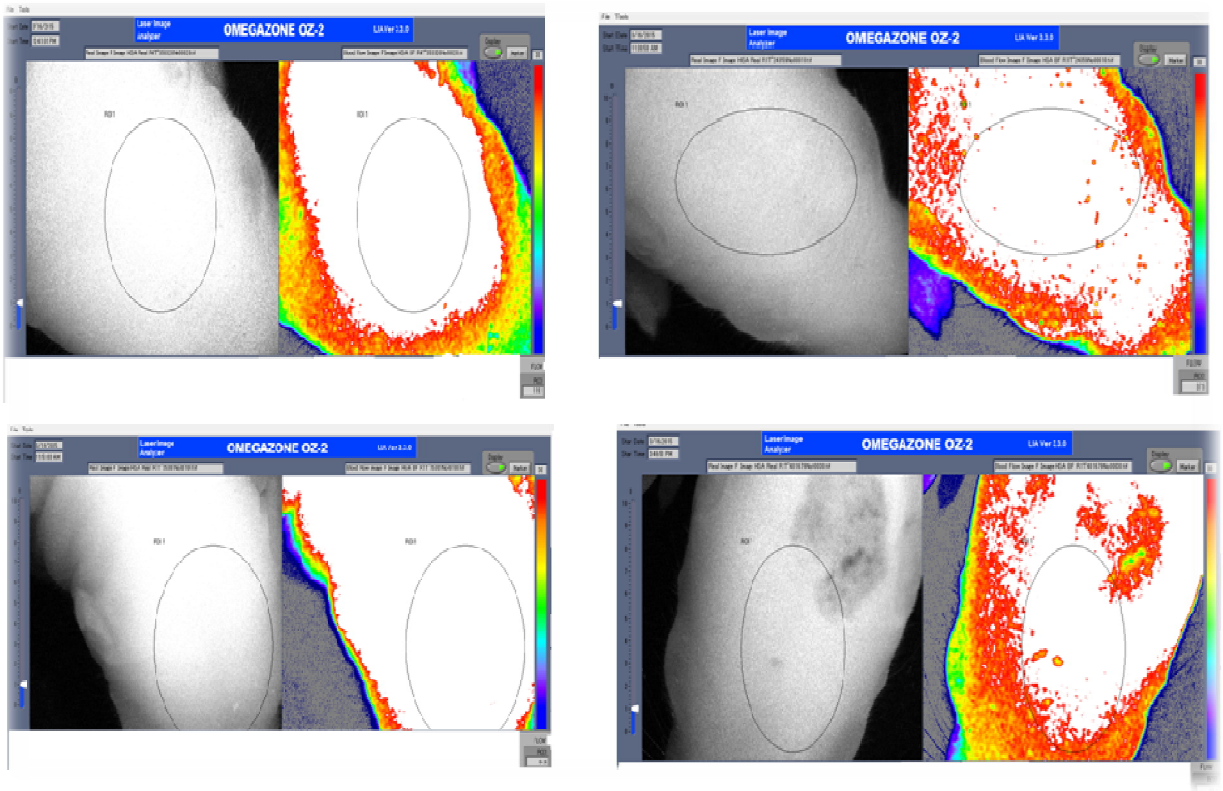

Figure 3.2 Effect of anoxia on cortical blood flow. (A) Regional blood flow (ROI) in control group with a value of 119.1 (B) in 30 min post-anoxia with ROI 37.5 (C) 24 hr. post-anoxia with ROI 84.9 and (D) on d-7 post-anoxia with ROI 35.4 respectively after anoxia.

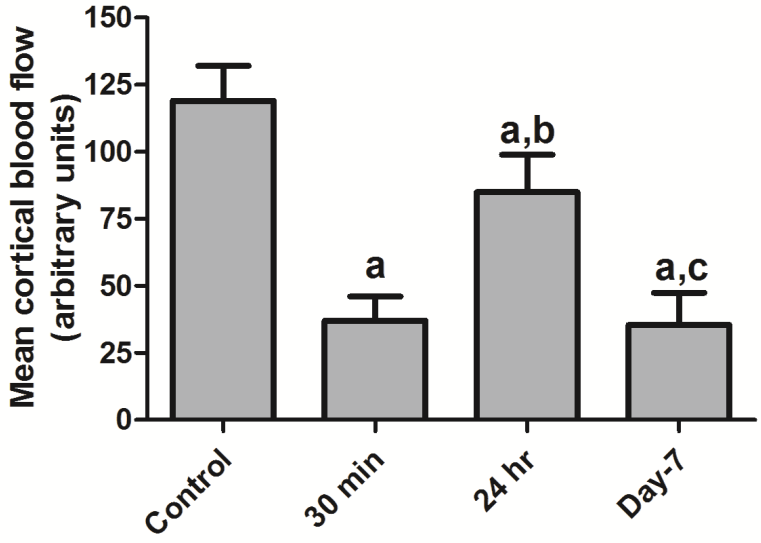

Figure 3.3 The temporal profile of the mean CBF in pups in the cortical brain region. Data are expressed as mean ± SD (n = 3). [a]P<0.05 compared to control, [b]P<0.05 compared to 30 min and [c]P<0.05 compared to 24 hr. anoxia respectively.

3.4.3 Changes in different state of mitochondrial respiration and RCR (state 3/state 4 respiration) on primary (30 min, 24 hr.) and secondary (d-7) insult time points in cortical brain region

3.4.3.1 Pyruvate/malate (state-2 respiration via complex-I)

A two-way ANOVA revealed no significant difference between groups [F (1, 24) = 2.02, *P*=0.16, $\eta^2 = 0.074$] and time [F (2, 24) = 0.37, *P*=0.69, $\eta^2 = 0.027$]. An equal amount of mitochondria was added to the oxytherm chamber, and state-2 respiration via complex-I was used as a baseline that gave all groups equal ability to produce similar respiration traces, regardless of the quantity of mitochondrial protein added, unless damage to components of the ETC existed as a result of the anoxia (Table 3.1).

3.4.3.2 ADP (state-3 respiration)

Statistical analyses showed a significant difference between groups [F (1, 24) = 107, $P<0.0001$, $\eta^2 = 0.801$]. However, the differences were insignificant for time [F (2, 24) = 0.35, $P=0.07$, $\eta^2 = 0.005$] post-anoxia injury (Table 3.1). Post hoc analysis revealed that the magnitude of the deficit following anoxia was in proportionate with mitochondrial ability to utilize ADP with those of control group ($P<0.05$).

3.4.3.3 Oligomycin (state-4 respiration)

Possible time-related changes in state 4 respiration (Table 3.1) in cortical brain region after anoxic injury were assessed by the addition of oligomycin. A two-way ANOVA revealed a significant difference between groups [F (1, 24) = 68.7, $P<0.0001$, $\eta^2 = 0.718$]. However, no significant main effect for time [F (2, 24) = 0.80, $P=0.45$, $\eta^2 = 0.17$] post-anoxia injury (Table 3.1) was observed. Post hoc analysis showed an increase in state-4 respiration following anoxia injury compared to control group animals ($P<0.05$).

3.4.3.4 RCR (State-3/State-4 respiration)

Changes in the overall mitochondrial integrity and the coupling capacity of ETC to ATP production was assessed by comparing RCRs across groups. Two-way ANOVA revealed a significant decrease in RCR between groups [F (1, 24) = 823, $P<0.0001$, $\eta^2 = 0.964$]. However, there was no significant diffeeerence for time [F (2, 24) = 0.44, $P=0.64$, $\eta^2 = 0.001$] post-anoxia injury (Table 3.1). Post hoc analysis revealed a decrease in RCR at different time points following anoxia compared to control group ($P<0.05$).

3.4.3.5 FCCP (State-5 respiration via complex-I)

FCCP, a wholesome uncoupler that acts as a protonophore, allows the protons built up in the inner membrane space to freely pass back into the matrix at a rapid rate. A two-way ANOVA revealed a significant difference between groups [F (1, 24) = 51.01, P<0.0001, η^2 = 0.546], time [F (2, 24) = 5.34, P=0.01, η^2 = 0.113] and a significant interaction of group and time [F (2, 24) = 3.8, P=0.03, η^2 = 0.081] post-anoxia injury (Table 3.1). Post hoc analysis revealed that there was no difference in 30 min post-anoxia injury group compared to sham-operated control group (P>0.05). Whereas, both 24 h and day-7 time points were lower than 30 min post second anoxia episode (P<0.05).

3.4.3.6 Succinate (State-5 via complex-II)

Rotenone, an inhibitor of complex-I was added to allow succinate driven respiration to be measured independently (Table 3.1). Two-way ANOVA revealed a significant difference between groups [F (1, 24) = 51.69, P <0.0001, η^2 = 0.662], but no significant main effect for time [F (2, 24) = 0.81, P=0.45, η^2 = 0.020] post-anoxia injury. Post hoc analysis showed a decrease in complex-II respiration post-anoxia injury at different time points compared to control group animals (P < 0.05).

Group	P/M (S-2)	ADP (S-3)	Oligomycin (S-4)	FCCP (Com-I)	Succinate (Com-II)	RCR (S-3/S-4)
30 min						
Control	31.8 ± 4.4	117.0 ± 6.1	16.7 ± 6.03	111.3 ± 10.7	57.6 ± 5.2	7.0 ± 0.42
Anoxia	28.7 ± 7.0	82.7 ± 12.7[a]	32.7 ± 4.2[a]	98.1 ± 20.1	41.0 ± 7.6[a]	2.5 ± 0.38[a]
24 hr.						
Control	31.5 ± 4.7	119.0 ± 5.6	16.4 ± 6.4	108.7 ± 8.7	56.9 ± 5.6	7.2 ± 0.40
Anoxia	26.1 ± 5.8	86.8 ± 11.1[a]	34.0 ± 4.3[a]	70.1 ± 7.6[a,b]	37.2 ± 7.9[a]	2.6 ± 0.40[a]
d-7						
Control	31.9 ± 2.6	114.0 ± 5.3	16.3 ± 4.9	110.2 ± 7.8	58.2 ± 6.2	6.8 ± 0.42
Anoxia	30.9 ± 4.8	89.1 ± 2.3[a]	28.7 ± 3.8[a]	75.1 ± 4.8[a,b]	43.2 ± 5.8[a]	3.0 ± 0.42[a]

Table 3.1 Effect of anoxia on different states of mitochondrial respiration State 2. State-3, State-4, State-5 via complex-I and State-5 via complex-II) and respiratory control ratio (RCR) at various time points (30 min, 24 hr. and d-7) in the cortical brain region of rat pups. Data are expressed as mean ± SD (n = 5). [a]P<0.05 compared to control and [b]P < 0.05 compared to 30 min anoxia respectively [Two-way ANOVA followed by Bonferroni post-test].

3.4.4 Increase in mitochondrial nitrosative and oxidative stress on primary (30 min, 24 hr.) and secondary (d-7) insult time points in cortical brain region

Table 3.2 shows the levels of NO, LPO, SOD and CAT at different time points after anoxia. An ANOVA revealed a significant difference in levels of NO between groups [F (1, 24) = 65.87, *P*<0.0001, $\eta^2 = 0.558$], time [F (2, 24) = 9.15, *P*=0.001, $\eta^2 = 0.155$] and a significant interaction of group and time [F (2, 24) = 4.88, *P*=0.01, $\eta^2 = 0.082$]. Post hoc analysis revealed that there was a marked increase (*P*<0.05) in the levels of NO in 24 hr. Compared to 30 min time point and a significant increase (*P*<0.05) in levels of NO in secondary insult time point (d-7) compared to primary insult time point (30 min, 24 hr.). Similarly, there was a significant increase in the levels of MDA between groups [F (1, 24) = 83.7, *P*<0.0001, $\eta^2 = 0.660$], time [F (2, 24) = 4.37, *P*=0.02, $\eta^2 = 0.068$] and a significant interaction of group and time [F (2, 24) = 5.18, *P*=0.01, $\eta^2 = 0.081$] post-anoxia

injury. Post-hoc analysis revealed that there was increased (P<0.05) in the levels of MDA on secondary insult time point (d-7) compared to primary insult time point (30 min, 24 hr.).

3.4.5 Decrease in mitochondrial antioxidant enzymes system following anoxia in different time points in cortical brain region

A significant difference in the levels of CAT (Table 3.2) between groups [F (1, 24) = 54.12, P<0.0001, η^2 = 0.645] was observed. However, the changes in CAT levels were insignificant for time [F (2, 24) = 0.73, P=0.49, η^2 = 0.017]. Post hoc analysis revealed a marked decrease (P<0.05) in the levels of CAT in anoxia group compared to control group. Similarly, an ANOVA depicted a significant decline in the levels of SOD (Table 3.2) between groups [F (1, 24) = 45.07, P<0.0001, η^2 = 0.619]. However, these changes were insignificant with time [F (2, 24) = 0.015, P=0.98, η^2 = 0.004] post-anoxia injury. Post hoc analysis depicted that anoxia caused a marked decrease (P<0.05) in the levels of mitochondrial SOD compared to control group.

Group	Nitrite level (nmole NO/ mg protein)	LPO (nmole MDA/ mg protein)	CAT (Units/min/ mg protein)	SOD (Units/min/ mg protein)
30 min				
Control	1.63 ± 0.8	3.4 ± 1.75	21.1 ± 3.5	1.7 ± 0.48
Anoxia	2.82 ± 0.7[a]	7.0 ± 1.08[a]	12.5 ± 4.3[a]	1.0 ± 0.36[a]
24 hr.				
Control	1.80 ± 0.8	3.3 ± 0.98	22.9 ± 4.8	1.8 ± 0.37
Anoxia	4.07 ± 1.0[a,b]	9.1 ± 3.02[a]	11.7 ± 6.5[a]	0.9 ± 0.39[a]
d-7				
Control	2.0 ± 0.5	3.1 ± 0.92	23.4 ± 4.3	2.0 ± 0.24
Anoxia	5.3 ± 0.6[a,b,c]	12.1 ± 2.20[a,b,c]	6.5 ± 2.9[a]	0.7 ± 0.38[a]

Table 3.2 Effect of anoxia on the levels of Nitrite, LPO, CAT and SOD in the cortical brain region of rat pups at different time points. Data are expressed as mean ± SD (n = 5). [a]P<0.05 compared to control, [b]P<0.05 compared to 30 min and [c]P<0.05 compared to 24 hr. anoxia respectively [Two-way ANOVA followed by Bonferroni post-test].

3.4.6 Change in fluorescence intensity (mitochondrial membrane potential) and mitochondrial swelling on primary (30 min, 24 hr.) and secondary (d-7) insult time points in cortical brain region

An ANOVA depicted a significant difference in MMP (Table 3.3) in terms of fluorescence intensity between groups [F (1,24) = 9.5, *P*=0.005, $\eta^2 = 0.097$], time F (2, 24) = 17.01, *P*<0.001, $\eta^2 = 0.346$] and a significant interaction of group and time [F (2, 24) = 15.26, *P*<0.001, $\eta^2 = 0.311$] post injury at different time points. Post-hoc analysis revealed that there was a significant increase in fluorescence intensity on d-7 (Secondary insult), while a marked decrease in 30 min and 24 hr. (Primary insult) time point posts second anoxia episode. A significant increase in MPT pore opening in terms of mitochondrial swelling between groups [F (1, 24) = 106, *P*<0.01, $\eta^2 = 0.694$], time [F (2, 24) = 4.6, *P*=0.02, $\eta^2 = 0.060$] and a significant interaction of group and time [F (2,

24) = 6.8, *P*=0.004, $\eta^2 = 0.089$] was observed (Table 3.3) post-anoxia injury. Post hoc analysis revealed a significant increase in mitochondrial swelling on secondary insult time point (d-7) compared to primary insult time point (30 min, 24 hr.) groups.

Group	Fluorescence Intensity /mg protein	Mitochondrial swelling (Absorbance at 520 nm) /mg protein
30 min		
Control	466.2 ± 99.1	0.17 ± 0.06
Anoxia	307.4 ± 70.1[a]	0.31 ± 0.04[a]
24 hr.		
Control	477.7 ± 107	0.15 ± 0.03
Anoxia	250.4 ± 55.1[a]	0.40 ± 0.07[a]
d-7		
Control	482.6 ± 47.2	0.15 ± 0.09
Anoxia	610.5 ± 58.3[a,b,c]	0.51 ± 0.08[a,b,c]

Table 3.3 Effect of anoxia on mitochondrial membrane potential and mitochondrial swelling in cortical portion of neonatal at different time points. All data are mean ± SD (n = 5). [a]P <0.05 compared to control, [b]P<0.05 compared to 30 min and [c]P < 0.05 compared to 24 hr. anoxia respectively [Two-way ANOVA followed by Bonferroni post-test].

3.4.7 Changes in level of cytochrome-C, caspase-9 and caspase-3 on primary (30 min, 24 hr.) and secondary (d-7) insult time points in cortical brain region

Fig. 3.4 depicts the change in expression of intrinsic apoptotic markers like cytochrome-C, caspase-9 and caspase-3 after anoxia injury. A two-way ANOVA revealed a significant increase in the levels of expression of cytochrome-C (Fig. 3.4 B) between groups [F (1, 12) = 299.4, *P*<0.0001, $\eta^2 = 0.891$], time [F (2, 12) = 5.56, *P*=0.019, $\eta^2 = 0.033$] and a significant interaction of group and time [F (2, 12) = 6.7, *P*=0.01, $\eta^2 = 0.040$]. Post hoc analysis showed that there was marked increase (*P*<0.05) in the levels of expression of cytochrome-C in secondary insult time point (d-7) compared to primary insult time point

(24 hr.). Similarly there was a significant increase in the levels of expression of caspase-9 (Fig. 3.4 C) between groups [F (1, 12) = 128.4, $P<0.0001$, $\eta^2 = 0.727$], time [F (2, 12) = 7.76, $P=0.007$, $\eta^2 = 0.087$] and an interaction between group and time [F (2, 12) = 10.34, $P=0.002$, $\eta^2 = 0.117$] post-anoxia injury at different time points. Post-hoc analysis revealed a marked increase ($P<0.05$) in the levels of expression of caspase-9 in secondary insult time point (d-7) compared to primary insult time points (30 min and 24 hr.). Further, there was a significant increase in the levels of expression of caspase-3 (Fig. 3.4 D) between groups [F (1, 12) = 206, $P<0.001$, $\eta^2 = 0.906$]. However, these changes were insignificant for time [F (2, 12) = 2.47, $P=0.12$, $\eta^2 = 0.021$] post second anoxic episode. Post hoc analysis revealed that there was a marked increase ($P<0.05$) in the levels of expression of caspase-3 in post-anoxic injury compared to control group animals.

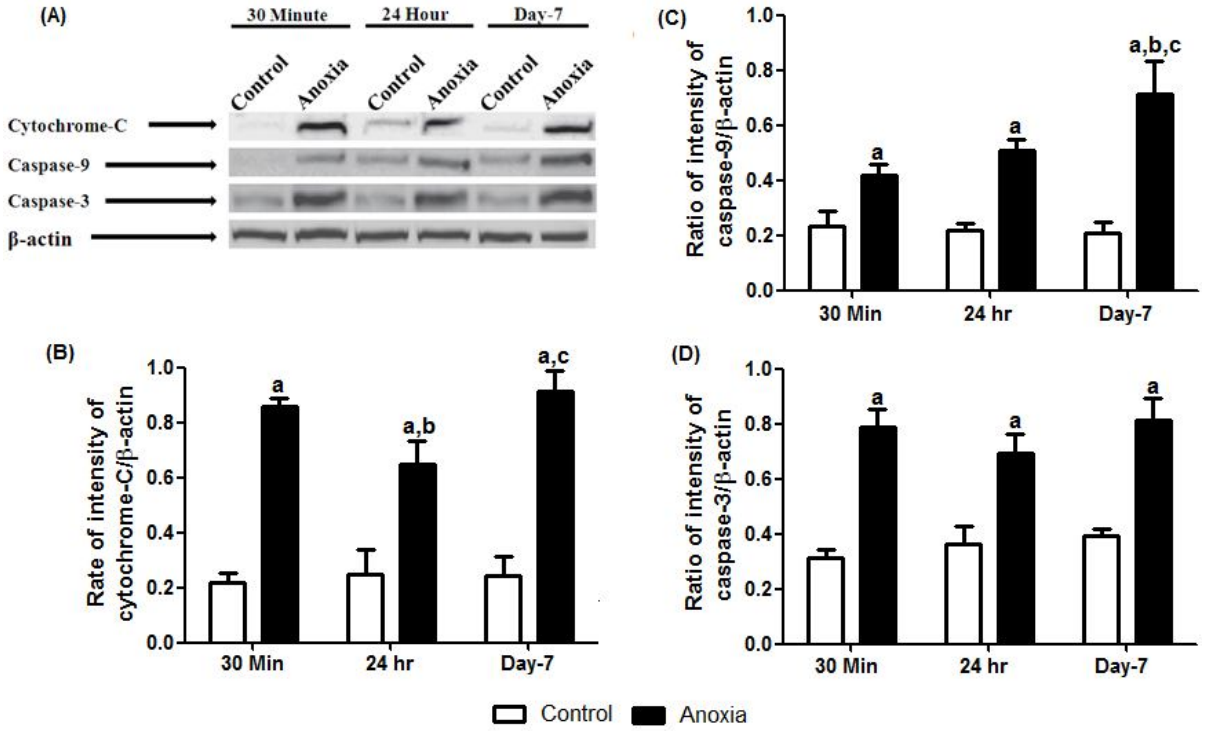

Figure 3.4 The effect of anoxia-induced changes in the levels of cytochrome-C (B), caspase-9 (C) and caspase-3 (D) in the cytosol fraction at different time points. Blot (A) represents cytochrome-C, caspase-9 or caspase-3 in cortical region. The results in the histogram are expressed as the ratio of the relative intensity of levels of protein expression of cytochrome-C, caspase-9 and caspase-3 to β-actin. Data are expressed as mean ± SD of three separate sets of independent experiments. $^{a}P<0.05$ compared to control, $^{b}P<0.05$ compared to 30 min and $^{c}P<0.05$ compared to 24 hr. anoxia respectively [Two-way ANOVA followed by Bonferroni post-test].

3.4.8 Changes in level of cytoplasmic as well as mitochondrial Bax, Bcl-2 and their ratio (Bax/Bcl-2) on primary (30 min, 24 hr.) and secondary (d-7) insult time points in cortical brain region

3.4.8.1 Cytoplasmic Bax, Bcl-2 and Bax/Bcl-2 ratio:

Two-way ANOVA revealed a significant decrease in levels of expression of cytoplasmic Bax (Fig. 3.5 B) between groups [$F(1, 12) = 88.67$, $P<0.001$, $\eta^2 = 0.843$], but the changes were insignificant with time [$F(2, 12) = 1.706$, $P=0.22$, $\eta^2 = 0.032$]. Post hoc analysis depicted that anoxia leads to a marked decrease in the levels of expression of cytoplasmic

Bax on different time points compared to control group animals. Further, a significant increase in the levels of expression of cytoplasmic Bcl-2 (Fig. 3.5 C) between groups [F (1, 12) = 82.94, $P<0.0001$, $\eta^2 = 0.853$] was observed. However, the changes in Bcl-2 (Fig. 3.5 C) were insignificant with time [F (2, 12) = 0.1008, $P=0.90$, $\eta^2 = 0.002$]. Post hoc analysis revealed an increase in levels of expression of cytoplasmic Bcl-2 on 30 min, 24 hr. and d-7 compared to control group. Further, There was a significant decrease in the ratio of cytoplasmic Bax/Bcl-2 (Fig. 3.5 D) between groups [F (1, 12) = 97.61, $P<0.0001$, $\eta^2 = 0.871$]. However, the changes were insignificant with time [F (2, 12) = 0.9333, $P=0.42$, $\eta^2 = 0.016$] post-anoxic injury. Post hoc analysis showed that anoxia leads to a marked decrease in ($P<0.05$) in cytoplasmic Bax/Bcl-2 on different time points compared to control group.

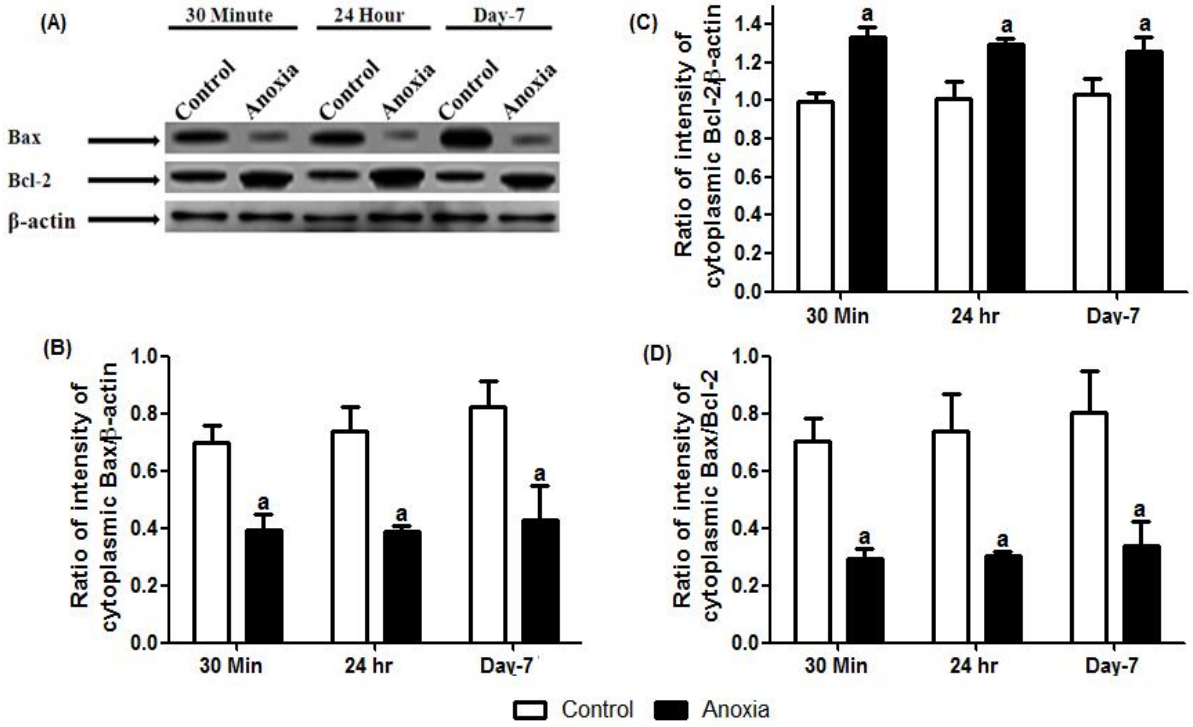

Figure 3.5 The effect of anoxia-induced changes in the levels of cytoplasmic Bax (B), Bcl-2 (C) and their ratio (Bax/Bcl-2) (D)), in the cytoplasmic fraction at different time points. Blot (A) represents Bax, Bcl-2 and their ratio (Bax/Bcl-2) in cortical region. The results in the histogram are expressed as the ratio of the relative intensity of levels of protein expression of cytoplasmic Bax, Bcl-2 and their ratio (Bax/Bcl-2) to β-actin. Data are expressed as mean ± SD of three separate sets of independent experiments. $^{a}P<0.05$ compared to control [Two-way ANOVA followed by Bonferroni post-test].

3.4.8.2 Mitochondrial Bax, Bcl-2 and Bax/Bcl-2 ratio:

An ANOVA depicted a significant increase in the levels of expression of mitochondrial Bax (Fig.3.6B) between groups [$F(1, 12) = 98.72$, $P<0.0001$, $\eta^2 = 0.828$]. However, the changes were insignificant with time [$F(2, 12) = 1.17$, $P=0.34$, $\eta^2 = 0.019$]. Post hoc analysis revealed a marked increase ($P<0.05$) in the levels of expression of mitochondrial Bax post-anoxia compared to control group. Whereas, a significant decrease in the levels of expression of mitochondrial Bcl-2 (Fig.3.6 C) between groups [$F(1, 12) = 169$, $P<0.05$,

$\eta^2 = 0.898$], but changes were insignificant with time [F (2, 12) = 1.22, P=0.32, $\eta^2 = 0.013$]. Post hoc analysis showed a decrease (P<0.05) in levels of expression of mitochondrial Bcl-2 post-anoxia compared to control group. Further, there was a main significant increase in the ratio of mitochondrial Bax/Bcl-2 (Fig.3.6 D) between groups [F (1, 12) = 91, P<0.0001, $\eta^2 = 0.726$], time [F (2, 12) = 5.1, P=0.02, $\eta^2 = 0.081$] and a significant interaction of group and time [F (2, 12) = 6.05, P=0.01, $\eta^2 = 0.092$] post-anoxia injury. Post hoc analysis revealed that the ratio of mitochondrial Bax/Bcl-2 was higher (P<0.05) in the secondary insult time point compared to primary injury time point (24 hr.).

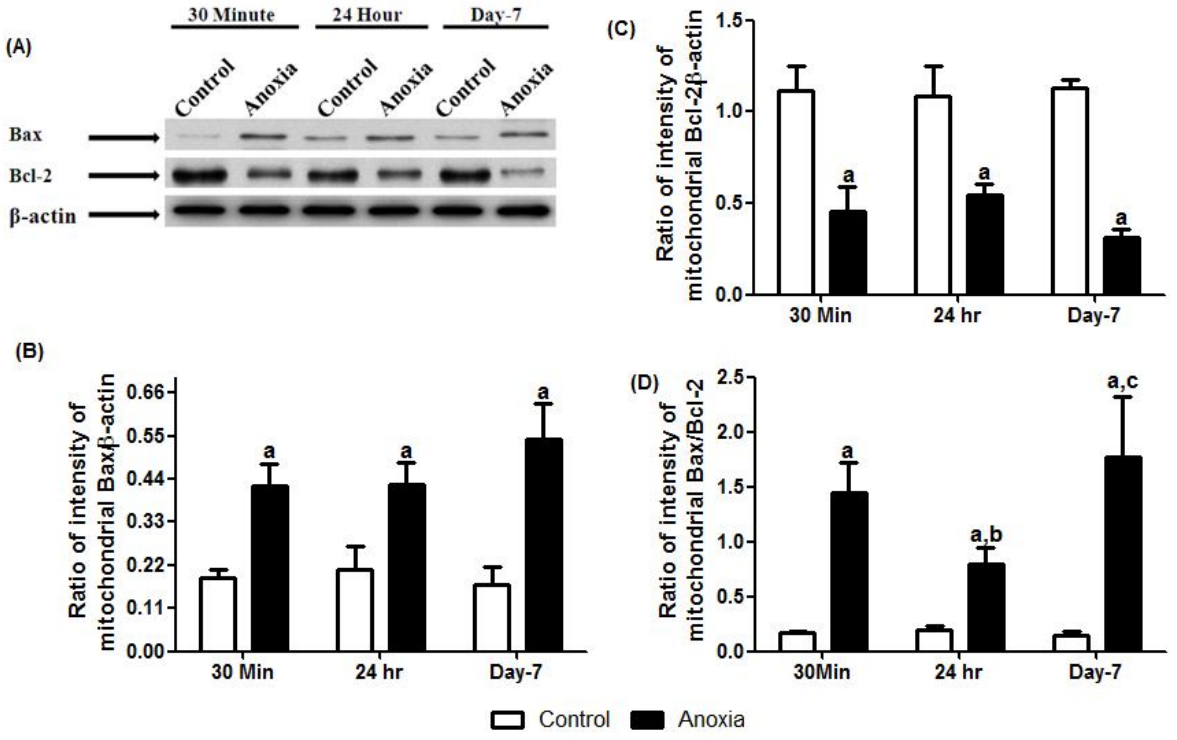

Figure 3.6 The effect of anoxia-induced changes in the levels of mitochondrial Bax (B), Bcl-2 (C) and their ratio (Bax/Bcl-2) (D), in the mitochondrial fraction at different time points. Blot (A) represents Bax, Bcl-2 and their ratio (Bax/Bcl-2) in cortical region. The results in the histogram are expressed as the ratio of the relative intensity of levels of protein expression of mitochondrial Bax, Bcl-2 and their ratio (Bax/Bcl-2) to β-actin. Data are expressed as mean ± SD of three separate sets of independent experiments. $^{a}P<0.05$ compared to control, $^{b}P<0.05$ compared to 30 min and $^{c}P<0.05$ compared to 24 hr anoxia respectively [Two-way ANOVA followed by Bonferroni post-test].

3.4.9 Flow cytometric analysis of apoptotic cells on primary and secondary insult time points using Annexin-V/PI

The flow cytogram of different time points post control, and anoxia for the cortical region has been illustrated in Fig. 3.7 (A–F).

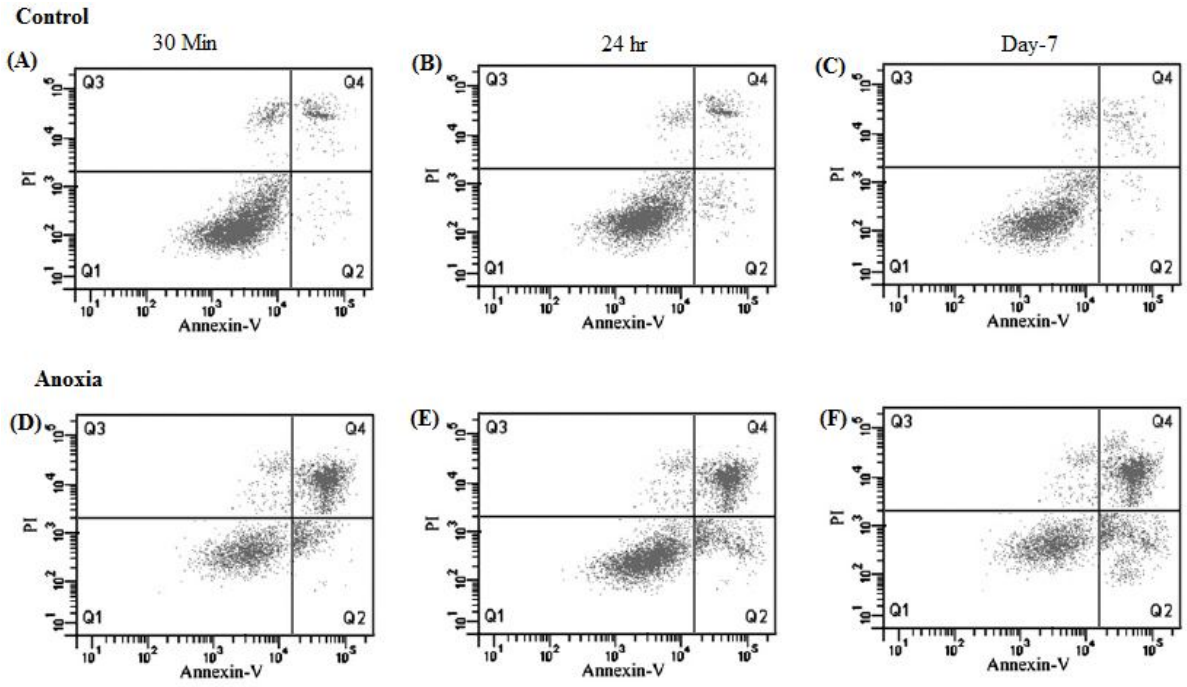

Figure 3.7 Flow cytogram is depicting anoxia-induced cell death in the cortical region assessed by cytometric analysis using annexin-V/PI. Dotted plots represent control 30 min (A), control 24 hr. (B) control day-7 min (C) anoxia 30 min (D) anoxia 24 hr. (E) and anoxia day-7 (F) respectively. Each cytogram has been divided into four quadrants: lower left quadrant (Q1): It represents cells that were negative for both annexin-V and PI and thus regarded as live cells. Lower right quadrant (Q2): It represents those cells that were positive for annexin-V and negative for PI. These cells assumed to be undergoing early stages of apoptosis in which the plasma membrane is still intact and exclude PI. Upper left region (Q3): It represents the population of annexin-V-negative and PI-positive cells. They are regarded as necrotic cells. Upper right region (Q4): It displays both annexin-V-positive and PI-positive cells. It represents the late stages of apoptosis and necrosis when dying cells can no longer exclude PI. The intensity of red fluorescence by PI-stained cells is indicated on the y-axis, whereas the intensity of green fluorescence emerging from cell-bound annexin-VFITC is shown on the x-axis.

A two-way ANOVA revealed a main significant increase in the percentage of apoptotic cells (Fig. 3.8) between groups [$F\ (1,\ 12) = 183.8$, $P<0.0001$, $\eta^2 = 0.844$], time [F (2,

12) = 6.7, *P*=0.01, η^2 = 0.062] and a significant interaction of group and time [F (2, 12) = 4.089, *P*=0.04, η^2 = 0.037]. Post-hoc analysis revealed that anoxia leads to a significant increase (*P*<0.05) in the percentage of apoptotic cells on 24 hr. and day-7 time points compared to 30 min and control group in the cortical brain region.

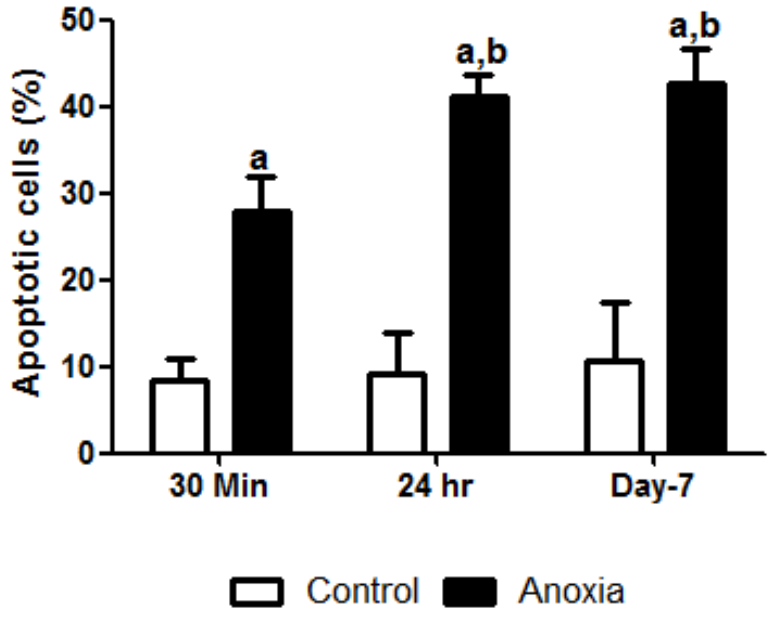

Figure 3.8 Changes in the percentage of apoptotic cells in the cortical region at different time points after second anoxia episode. Data are expressed as mean ± SD of three separate sets of independent experiments. [a]P<0.05 compared to control and [b]P<0.05 compared to 30 min anoxia [Two-way ANOVA followed by Bonferroni post-test].

3.4.10 Sensorimotor changes from P4 (24 hr.) to P10 (day-7) after second anoxic episode

A repeated measure two-way ANOVA revealed that there was a significant decrease in hanging latency (Fig. 3.9A) between groups [F (1, 56) = 105.8, *P*<0.0001, η^2 = 0.313], time [F (6, 56) = 21.17., *P*<0.001, η^2 = 0.411] and a significant interaction of group and time [F (6, 56) = 4.80, *P*<0.001, η^2 = 0.093]. Post hoc analysis depicted that there was a gradual increase in the hanging latency from P4 to P10 in control group animals due to

neurodevelopmental changes with time. Similarly, there was a significant increase in reflex latency (Fig. 3.9B) between groups [F (1, 56) = 50.92, P<0.0001, η^2 = 0.392] and time [F (6, 56) = 3.002, P=0.01, η^2 = 0.138] but no significant interaction of group and time post second anoxia episode [F (6, 56) = 0.79, P=0.57, η^2 = 0.036].

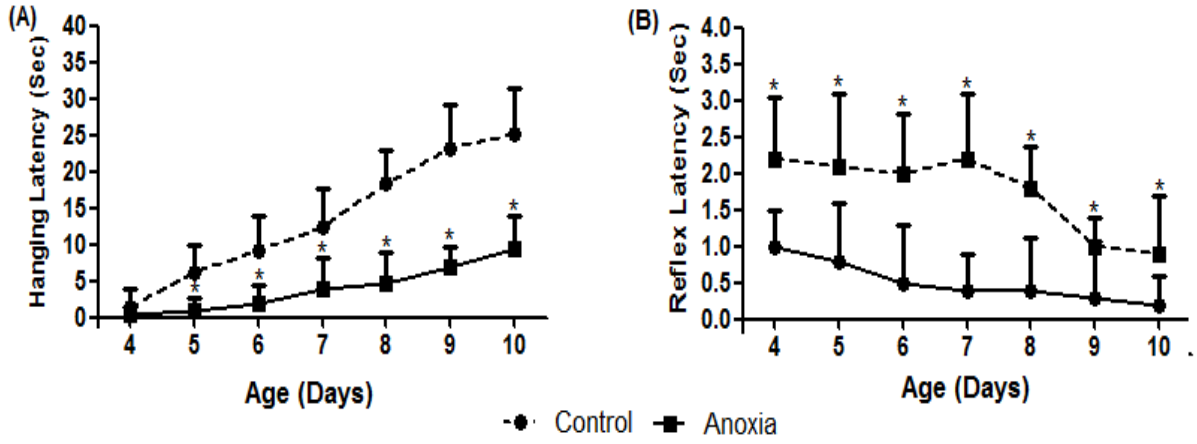

Figure 3.9 Effect of anoxia on hanging latency (A) and reflex latency (B) from day-1 (P4) up to day-7 (P10) after second anoxia episode. Data are expressed as mean ± SD (n = 5). *P<0.05 compared to control [Repeated measure two-way ANOVA followed by Bonferroni post-test].

3.5 Discussion

In this present study, we for the first time investigated anoxia-induced mitochondrial–linked apoptosis during the primary (30 min, 24 hr.) and secondary insult (d-7) in cortical region and its consequences in neonates in the form of sensorimotor deficits. A marked change in the expression of cytoplasmic and mitochondrial Bax, Bcl-2 proteins, cytochrome-C, caspase-9 and caspase-3 was observed in cortical brain region and was more detrimental in secondary insult than in the primary.

In the current study, an approximate 30% drop in oxygen saturation was observed during last two minutes of the first episode (i.e. 9th and 10th minute) which indicates that the rat

pups were resistant to anoxia injury [11]. Peripheral oxygen saturation was below 30% as early as the 3rd minute and remained practically unchanged until the 10th minute of the second anoxic episode. Therefore, from the beginning of the procedure, there was an average 70% drop in peripheral arterial oxygen saturation in a second anoxic episode which confirms the development of anoxic injury in the rat neonates as reported earlier in a different model of anoxia [64]. Similar findings were reported in human fetus [12, 99]. The condition was further confirmed by the significant changes in hanging and reflex latency on P4 (d-1) and P10 (d-7) post second anoxic injury [20].

The speckle imaging method presented in this report provides an attractive alternative to other methods because of its ability to precisely image the cortical blood flow response over an area ranging from a few millimeters to a centimeter over time scales of milliseconds to hours [80]. Since cerebral blood flow is firmly coupled to the underlying neuronal response, it is crucial to monitor the spatial and temporal extent of CBF changes in studies of functional activation [100] and pathophysiologic conditions accompanied by uncoupling. In the present study, we demonstrated a time-dependent cortical blood flow (30 min, 24 hr. and d-7) which depicted a progression of cortical blood flow decrement from 24 hr. i.e., from d-1 to d-7 resembling the progression of hypoxic ischemic encephalopathy (HIE) after anoxia. Overall, there is an increase in neuronal apoptosis with severity of the degree of HIE post-anoxic injury.

Apoptosis is an orderly, energy-dependent process in which mitochondria plays a pivotal role as regulators of cell death. We studied the effect of anoxia on different states of cortical mitochondrial respiration in terms of oxygen consumption at different time points post-injury. Anoxia caused a significant reduction in s-3, s-4, s-5 complex-I, s-5 complex-II

and RCR at all the three-time points after anoxic insult which indicates a compromised mitochondrial bioenergetics up to d-7 (secondary insult). Respiration in the form of oxygen consumption on acute time points (30 min and 24 hr.) in the whole brain are in agreement with our previous findings [76].

Mitochondria are considered as the principal site of oxidative and nitrosative stress [28]. There was an increase in the levels of MDA and NO in all the three-time points, and it was interesting to note that significantly higher levels of MDA and NO were found following secondary insult compared to primary insult time points. Meanwhile, a decrease in the functioning of antioxidant enzymes (CAT and SOD) at different time points was observed depicting a further increase in oxidative and nitrosative stress and causing damage to mitochondrial machinery post-anoxia injury. Mitochondrial membrane potential (MMP) is an important index of the bioenergetics state of the cell, because it is precisely regulated to cope with the energy need of cells. The MMP significantly decreased (mitochondrial depolarization) from 30 min of last anoxia episode and up to 24 hr. (primary insult). It was interesting to note that, a marked increase of fluorescence intensity (mitochondrial hyperpolarization) was observed on d-7 (secondary insult) reflecting a compromised mitochondrial membrane integrity. The reason behind it may be due to reperfusion in the early phase of secondary insult which may result in ROS generation after hypoxia/anoxia injury [101-103].

The mitochondrial pathway of apoptosis is regulated by the anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins which play a principal role in regulating mitochondrial outer membrane integrity [104]. Previous studies have shown that Bax is commonly predominant in the cytosol. The translocation patterns of Bax to mitochondria from cytosol can affect the

resultant cell death phenotype (necrotic or apoptotic) [105, 106]. Our results depicted a decrease in expression of cytosolic Bax, increased expression of cytosolic Bcl-2 and the decreased ratio of cytosolic Bax/Bcl-2. Further, an increased expression of mitochondrial Bax, a decreased expression of mitochondrial Bcl-2 and an increase in mitochondrial Bax/Bcl-2 ratio was observed. It was fascinating to note that there was an increase in mitochondrial Bax/Bcl-2 ratio on d-7 (secondary insult) compared to 30 min (primary insult) indicating decrease of mitochondrial integrity with time post-anoxia injury in cortical region. Further activation of the pro-apoptotic BH3-only proteins (Bid) allows them to bind directly and activate Bax, and may also bind (and inhibit) to the prosurvival Bcl-2-like proteins to indirectly activate Bax. As a result, Bax becomes activated on the mitochondrial outer membrane or translocates from the cytosol and causes mitochondrial outer membrane permeabilization (MOMP) which along with inner membrane leads to the formation of MPT pore [32, 107]. Our results depicted an increase in MPT post-anoxia on d-7 compared to 30 min and 24 hr. time point. Further, there was an increase in expression of cytochrome-C in the cortical region on d-7 compared to 30 min time point post-anoxia. Furthermore, an increased expression of caspase-9 was observed on day-7 compared to 30 min and 24 hr. Indicating that increase in mitochondrial-linked apoptosis in the cortical region following the secondary insult. Previous studies depict that cytochrome-C along with caspase-9 engages the apoptotic protease activating factor-1 (APAF1), and forms the apoptosome which causes the activation of caspase-3 [48]. We found an increase expression of caspase-3 in different primary and secondary insult time points in the cortical region leading to apoptotic cell death in later days of life in neonates. A previous study has shown that immature brain cells are more vulnerable to apoptosis than mature cells [108]. Apoptotic

cell death was reported in a different model of anoxia on day-2, day-8 and day-15 in rat striatum [109]. It was interesting to note that a higher percentage of apoptotic cells in the cortical brain region were observed at secondary insult time point as compared to primary insult time point which shows the progression in cortical mitochondrial-linked cell death post-anoxia injury as analyzed by flow cytometry.

Anoxia significantly decreased the hanging latency and increased the reflex latency from P4 to P10 which shows an early sign of neurological dysfunction and hindered sensorimotor performance in the early postnatal days reciprocating mitochondrial dysfunction linked cell death results. The present temporal study shows progressive loss of neonatal cortical mitochondrial function and activation of the apoptotic pathway with sensorimotor abnormalities. A compromised mitochondrial bioenergetics in the primary insult which later progressed to secondary insult as suggested from our results indicates hampered mitochondrial respiration, RCR, antioxidant system, altered MMP, MPT opening and a further increase in the levels of pro-apoptotic markers suggesting the pivotal role of mitochondrial integrity in the pathophysiology of anoxia. Moreover, by unveiling the time course of mitochondrial bioenergetics failure (30 min) and the factors involved in insult progression and further cell death transition may provide the possibility of novel targets and window of opportunity for pharmacological interventions.