5.1 Abstract

Anoxia lead to a rapid and marked mitochondrial-linked cell death occurs in the cerebral cortex of newborn rats which leads to insult advancement within a couple of days and causes lifelong neurobehavioral abnormalities. The present study investigated the pharmacological role of the mitochondrial uncoupler, 2, 4 dinitrophenol (2,4 DNP) in the dose 1, 2.5 and 5 mg/kg on anoxia-induced time-dependent mitochondrial dysfunction and associated neurobehavioral abnormalities in global model of anoxia. Briefly, rat pups of 30 hr age (P2) were subjected to two episodes of anoxia (10 min each) at 24 hr. of the time interval in an enclosed chamber supplied with 100 % N_2 and immersed in a water bath (35-37°C) to avoid hypothermia. Results demonstrated that the uncoupler 2, 4 DNP, in the dose 2.5 and 5 mg/kg injected i.p within 5 min. of second anoxic episode significantly ($P<0.05$) preserved mitochondrial function on day-7 preferentially by maintaining mitochondrial membrane potential (MMP) and inhibiting transition pore opening (MPT). Further, 2,4 DNP improved different states of mitochondrial respiration (s-2, s-3, s-4, s-5), respiratory control ratio (RCR), antioxidant enzyme system (SOD and CAT), mitochondrial complex enzyme (I, II, IV, V) after anoxia. Furthermore, a marked decrease in the levels of expression of cytochrome-C and pro-apoptotic (Bcl-2 family) and apoptotic (caspase-9/3) proteins were observed on day-7 indicating that the treatment prevents mitochondrial dysfunction and further rescued from insult progression (day-1 to day-7). Moreover, 2,4 DNP improves the neurobehavioral outcomes like reflex latency and hanging latency which suggests its role in treating neonatal anoxia.

Keywords: Anoxia; Mitochondrial function; 2, 4 dinitrophenol; Neurobehavioral alteration; animal model.

5.2 Introduction

Neonatal anoxia is one of the leading causes of neonatal mortality and morbidity with an incident of 20 per 1000 live births worldwide. It has been associated with a broad spectrum of short term or long-term sensorimotor deficits. At present no pharmacological treatment is clinically approved for treatment of anoxia. However, some pharmacological interventions like the use of modulators of glutamate, monoamines (dopamine, norepinephrine and serotonin), and GABA, adenosine, erythropoietin and growth factors has been utilized for the treatment to improve neurological outcomes after anoxia [21, 127]. Non-pharmacological strategies like hypothermia have been considered more desirable which are not completely neuroprotective, and 40-50% newborns still suffer from the major neurological problem. Thus, pharmacological intervention is urgently required for inhibiting progression of brain damage following anoxia [24].

Several studies suggest the role of mitochondria as organelle mediating cellular injury in anoxia [62, 63, 128]. We have reported a time-dependent pathological changes in mitochondrial bioenergetics and loss of sensorimotor behavior following anoxia [76] indicating the potential use of mitochondria as a target for pharmacological intervention. Further, apart from the temporal loss of mitochondrial bioenergetics and increase in oxidative stress, there was activation of the intrinsic pathway of apoptosis in the global model of anoxia [120]. Following the two episodes of anoxia pathological changes in mitochondrial membrane potential (MMP) was observed with the opening of mitochondrial

transition pore (MPT) regarding mitochondrial swelling which is the early sign of mitochondrial Ca^{2+} cycling/overloading. Previous findings show that during hypoxia/ischemia there is a release of excitatory amino acid (EAA) this can increase mitochondrial Ca^{2+}sequestration and can result in alterations in MMP and may lead to mitochondrial permeability transition pore opening and ultimately cell death [28, 129]. These events indicate mitochondrial stress-induced release of cytochrome-C, which activates expression of pro-apoptotic proteins (Bax) and simultaneously decreases the levels of anti-apoptotic proteins (Bcl-2). This ultimately leads to mitochondrial-linked apoptotic cell death as observed from up-regulation in the expression of caspase-9 and caspase-3.

Therefore, maintenance of mitochondrial membrane potential and inhibition of mitochondrial swelling could lead to inhibition of reactive oxygen species/nitrogen species (ROS/RNS) formation which is directly or indirectly involved in activation of downstream activation of pro-apoptotic proteins processes (Bax and caspases). In the present study, we investigated the effect of 2, 4-DNP a proton ionophore after second anoxia episode, which causes mild mitochondrial uncoupling by decreasing the proton motive force across the mitochondrial inner membrane and uncouples electron transport chain (ETC) to oxidative phosphorylation. This leads to a reduction in MMP and could further prevent sequestration of Ca^{2+} out of the cytosol into mitochondria (Ca^{2+}cycling/overloading) and production of reactive oxygen species [50, 51]. Previous experiments demonstrated that endogenous mitochondrial uncouplers (UPCs) UPC2 [130], UPC4 [131] decreased cell death by reducing excitotoxicity mediated free radical generation. Therefore, mild uncoupling could serve as a pharmacological intervention to interfere with progression of cortical

mitochondrial dysfunction and further activation of pro-apoptotic proteins and ensuing cell death during neonatal anoxia.

Hence, the present study investigated the effect of 2, 4-DNP on cortical mitochondrial function following primary insult (day-1) and secondary insult (day-7) by examining mitochondrial respiration (state-2, state-3, state-4, state-5 via complex-I and state-5 via complex-II), Respiratory control ratio (RCR), mitochondrial complex enzyme system, oxidative stress, MMP and MPT opening. Furthermore, we studied the upstream intrinsic apoptotic regulator proteins like Bcl-2, Bax and their ratio (Bax/Bcl-2) in both cytosolic and mitochondrial fractions and downstream intrinsic apoptotic proteins like cytochrome-C, caspase-9 and caspase-3 in the cortical brain region.

5.3 Materials and methods

5.3.1 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 °C 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 h 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.

5.3.2 Anoxia model

The anoxia procedure was carried out as validated and defined previously [76]. Chapter 2 Page no.22.

5.3.3 2, 4-DNP preparation and dosing

2, 4- DNP (Himedia) was freshly prepared in 10% DMSO before intraperitoneal injection (i.p.). The rat pups were randomly divided into 5 groups (n=5 per group): (1) Control; (2) Anoxia; (3) Anoxia + DNP 1 mg/kg; (4) Anoxia + DNP 2.5 mg/kg; and (5) Anoxia + DNP 5 mg/kg. The drug was administered within 5 min of second anoxia episode as previously described [120]. The volumes administered of the compound solution i.p. did not exceed 0.3 ml. The control group received an equal volume of 10% DMSO at the same time.

5.3.4 Behavioral parameter assessment

5.3.4.1 Righting reflex

As described previously [65] and detailed in chapter 2. Page no. 24.

5.3.4.2 Wire hanging maneuver

As described previously [65, 88] and detailed in chapter 3. Page no. 38.

5.3.5 Mitochondrial Isolation

Mitochondrial isolation was performed as described previously [66] with some slight modifications [76]. Details are given in chapter 2. Page no.24.

5.3.6 Measurement of Mitochondrial Function

Mitochondrial function was assessed using an Oxytherm Clark-type oxygen electrode (OXYT1/ED, Hansatech Instruments, Norfolk UK). Details are given in chapter 2. Page no.24.

5.3.7 Estimation of NADH dehydrogenase (Complex-I) activity

NADH dehydrogenase activity was estimated by catalytic oxidation of NADH with potassium ferricyanide as an artificial electron acceptor at excitation (350 nm) and emission (470 nm) wavelengths for NADH [132]. The reaction mixture consisted of 200 µl of 10 mM potassium ferricyanide, 60 µl of 1 mM NADH in 2 mM potassium phosphate buffer, and 2.64 ml of 0.12 M potassium phosphate buffer. The activity of NADH dehydrogenase was expressed as nmol NADH oxidized/min/mg protein.

5.3.8 Estimation of mitochondrial succinate dehydrogenase (Complex-II) activity

Succinate dehydrogenase (SDH) activity was evaluated by the progressive reduction of nitro blue tetrazolium (NBT) to an insoluble colored compound, formazan (dfz), measured at 570 nm [133]. The SDH activity was expressed as micromole formazan produced/min/mg protein.

5.3.9 Estimation of cytochrome-C oxidase (Complex-IV) activity

Complex-IV activity was assessed in mitochondrial preparation as per the method of [134]. The decrease in absorbance due to reduced cytochrome-C was measured at 550 nm for 3 min. Results were expressed as nmol cytochrome-C oxidized/min/mg protein ($\varepsilon 550 = 19.6$ mmol-1 cm-1).

5.3.10 Estimation of mitochondrial F1F0 ATP synthase (Complex-V) activity

F1F0 synthase activity was measured as described previously [135]. The inorganic phosphate concentration was measured by the method of [136]. Results were expressed as nmol ATP hydrolyzed/min/mg protein.

5.3.11 Evaluating mitochondrial membrane potential (MMP)

As described previously [92] and described in chapter 3. Page no. 40.

5.3.12 Mitochondrial permeability transition (MPT)

MPT was measured as described earlier [93] in chapter 3. Page no. 40.

5.3.13 Mitochondrial oxidative stress

5.3.13.1 Estimation of LPO and NO levels

Mitochondrial malondialdehyde (MDA) content was measured as per standard protocol [94]. Nitrite (NO) levels were determined by a colorimetric assay using Greiss reagent (0.1% at 540nm) [69] as described in chapter 3. Page no.41.

5.3.13.2 Estimation of mitochondrial SOD and CAT activity

Superoxide dismutase (SOD) activity was estimated as per described [95]. Catalase (CAT) activity was estimated as per [96]. Described in chapter 3. Page no.41.

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

As previously described [120]. Described in chapter 3. Page no.41.

5.3.15 Statistical analysis

The data are expressed as means ± SD. $P<0.05$ was considered as significant. All data were analyzed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

5.4 Results

5.4.1 Effect of 2,4-DNP (1, 2.5 mg/kg and 5 mg/kg) on mitochondrial respiration and RCR in cortex on d-1 and d-7 after anoxia.

Table 5.1 depicts the effect of 2,4 DNP in all the three doses on mitochondrial respiration. A two-way ANOVA revealed no significant difference in s-2 respiration among groups [$F(4, 40) = 0.53$; $P>0.05$]. However there was a significant difference in time [$F(1, 40) = 24.86$; $P<0.05$] post-anoxia injury. Post hoc analysis depicted no marked decrease in utilization of mitochondrial complex-I substrate pyruvate (P) and malate (M) in anoxia group compared to control and treatment groups respectively on d-1 and d-7. Further, s-3 respiration was fueled by the addition of ADP in oxytherm chamber which is a substrate for complex-V and phosphorylates ADP to ATP. An ANOVA depicted a significant main effect among groups [$F(4, 40) = 27.69$; $P<0.05$], time [$F(1, 40) = 42.92$; $P<0.05$] and a significant interaction between group and time [$F(4, 40) = 5.18$; $P<0.05$] after anoxia injury. Post hoc analysis showed that 2,4-DNP in the dose 2.5 and 5 mg/kg was markedly effective ($P<0.05$) compared to both anoxia and 2,4-DNP 1mg/kg group on day-7 and inhibited its contribution to insult progression (d-1 to d-7). Oligomycin, a complex-V inhibitor was added in the chamber to initiate s-4 respiration and block the further conversion of ADP to ATP by inhibiting proton gradient through ATPase pump. A two-way ANOVA depicted a significant main effect among groups [$F(4, 40) = 9.68$; $P<0.05$], time [$F(1, 40) = 28.24$; $P<0.05$] and a significant interaction between group and time [$F(4, 40) = 4.69$; $P<0.05$]. Post hoc analysis revealed that 2, 4-DNP in all the three doses attenuated s-4 respiration following anoxia on d-7. The addition of FCCP, an uncoupler causes proton leak through the mitochondrial inner membrane and leads to uncoupling coordination of ETC from oxidative

phosphorylation. An ANOVA revealed a significant difference in s-5 via complex-I among groups [$F(4, 40) = 52.69$; $P<0.05$], time [$F(1, 40) = 89.36$; $P<0.05$] and a significant interaction between group and time [$F(4, 40) = 20.76$; $P<0.05$]. Post hoc analysis revealed that 2,4-DNP in the dose 2.5 mg/kg and 5 mg/kg was significantly effective ($P<0.05$) in correcting s-5 respiration via complex-II compared to anoxia and 2, 4-DNP 1mg/kg group. State-5 via complex-II respiration was initiated by addition of succinate. A two-way ANOVA depicted a significant main effect among groups [$F(4, 40) = 12.56$; $P<0.05$], time [$F(1, 40) = 19.44$; $P<0.05$], however no significant interaction was observed between group and time [$F(4, 40) = 0.35$; $P>0.05$]. It was clear from the post hoc analysis that 2,4-DNP in all the three doses attenuated s-5 respiration via complex-II following anoxia. RCR being the hallmark of mitochondrial integrity denotes the extent of coupling of ETC to oxidative phosphorylation. An ANOVA revealed that there was a significant difference in RCR among groups [$F(4, 40) = 61.22$; $P<0.05$], time [$F(1, 40) = 44.38$; $P<0.05$] and a significant interface between group and time [$F(4, 40) = 11.89$; $P<0.05$]. Post hoc analysis revealed that 2,4-DNP in the dose 2.5 and 5 mg/kg was significantly effective ($P<0.05$) in attenuation of insult progression by inhibiting RCR on d-7.

Group	P/M	ADP	Oligomycin	FCCP	Succinate	RCR
Day-1						
Control	28.8 ± 4.4	111 ± 6.0	15.7 ± 5.8	114.3 ± 10.7	50.6 ± 5.0	7.0 ± 0.9
Anoxia	24.7 ± 5.8	82.8 ± 11*	30.0 ± 4.2*	67.1 ± 7.6*	33.2 ± 7.8*	2.7 ± 0.4*
2,4DNP 1 mg/kg	22.8 ± 4.9	81.9 ± 8.9*	31.0 ± 3.1*	68.1 ± 6.2*	31.1 ± 10.5*	2.6 ± 0.7*
2,4DNP 2.5mg/kg	22.6 ± 6.7	88.9 ± 7.6*	31.1 ± 2.9*	70.1 ± 5.1*	37.1 ± 8.7*	2.8 ± 0.9*
2,4DNP 5 mg/kg	21.2 ± 8.0	92.3 ± 8.2*	33.0 ± 5.3*	73.2 ± 7.3*	40.8 ± 7.8*	2.7 ± 0.7*
Day-7						
Control	31.8 ± 4.2	117 ± 5.8	16.7 ± 5.3	111.3 ± 10.7	57.6 ± 5.1	7.0 ± 0.7
Anoxia	30.9 ± 4.6	89.1 ± 4.4*	28.7 ± 8.7*	75.1 ± 6.9*	40.2 ± 5.8*	3.1 ± 0.9*
2,4DNP 1 mg/kg	31.1 ± 4.3	87.9 ± 7.6*	19.0 ± 3.3$^{\#}$	77.1 ± 6.2*	38.1 ± 6.0$^{\#}$	3.0 ± 0.9*
2,4DNP 2.5mg/kg	33.8 ± 6.4	115.9 ± 7.3$^{\#,\$}$	20.0 ± 3.1$^{\#}$	114 ± 5.0$^{\#,\$}$	50.1 ± 4.0$^{\#}$	5.7 ± 0.7$^{\#,\$}$
2,4DNP 5 mg/kg	33.2 ± 7.0	117.3 ± 7.1$^{\#,\$}$	19.0 ± 5.1$^{\#}$	116 ± 7.2$^{\#,\$}$	49.8 ± 5.6$^{\#}$	6.1 ± 0.4$^{\#,\$}$

Table 5.1 Dose-related effect of DNP (1, 2.5 and 5 mg/kg) on anoxia-induced changes in mitochondrial respiration on d-1 and d-7. Data represents group mean ± SD. n = 5/group. *P < 0.05 compared to control and $^{\#}$P<0.05 compared to anoxia group and $^{\$}$P<0.05 compared to DNP 1 mg/kg respectively [Two-way ANOVA followed by Bonferroni post-test].

5.4.2 Effect of multiple doses of 2, 4-DNP on oxidative and nitrosative stress in cortex on d-1 and d-7 after anoxia.

A two way ANOVA showed significant differences in levels of NO (Fig.5.1A) and LPO (Fig.5.1B) among groups [F (4, 40) = 33.81; P<0.05 and F (4, 40) = 31.15; P<0.05 respectively], time [F (1, 40) = 22.64; P<0.05 and F (1, 40) = 17.12; P<0.05 respectively] and a significant interaction between group and time [F (4, 40) = 9.73; P<0.05 and F (4, 40) = 6.40; P<0.05 respectively]. Post hoc analysis revealed that 2,4-DNP in the dose 2.5 and 5

mg/kg markedly effective ($P<0.05$) in inhibiting the insult progression by inhibiting the anoxia induced increase in the levels of NO and LPO after second anoxia episode.

5.4.3 Effect of multiple doses of 2,4-DNP on alterations in mitochondrial antioxidant enzyme activities in cortex on d-1 and d-7 after anoxia.

An ANOVA depicted a significant main effect for the changes in the levels of SOD (Fig.5.1C) and CAT (Fig.5.1D) among groups [$F (4, 40) = 15.25$; $P<0.05$ and $F (4, 40) = 15.38$; $P<0.05$ respectively], time [$F (1, 40) = 9.26$; $P<0.05$ and $F (1, 40) = 14.97$; $P<0.05$ respectively], however insignificant interaction between group and time [$F (4, 40) = 2.59$; $P>0.05$and $F (4, 40) = 2.08$; $P>0.05$ respectively] was observed. 2,4-DNP (2.5 and 5mg/kg) treatment significantly improved ($P<0.05$) anoxia-induced decrease in SOD and CAT on d-7 but not on day-1 following anoxic injury. Post hoc analysis revealed that anoxia significantly decreased the levels of mitochondrial antioxidant enzymes SOD and CAT compared to control group animals.

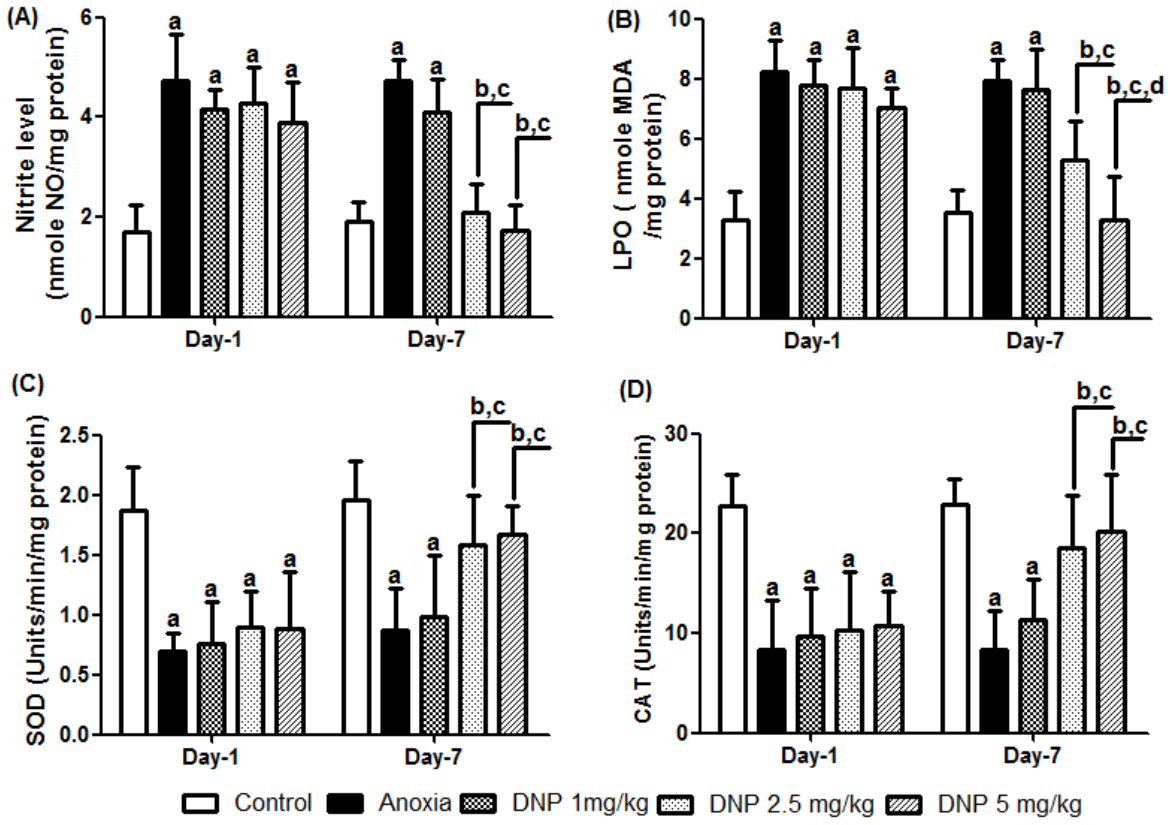

Figure 5.1 Effect of 2,4 DNP (1, 2.5 and 5 mg/kg) on anoxia-induced mitochondrial (A) NO, (B) LPO, (C) SOD and (D) CAT levels in cortical brain region on day-1 and day-7. Bars represents group mean ± SD. n = 5/group. [a]P<0.05 compared to control and [b]P<0.05 compared to anoxia [c]P<0.05 compared to 2,4 DNP 1 mg/kg and [d]P<0.05 compared to 2,4 DNP 2.5 mg/kg groups respectively [Two-way ANOVA followed by Bonferroni post-test].

5.4.4 Effect of multiple doses of 2,4-DNP on mitochondrial complex enzyme system in cortex on d-1 and d-7 after anoxia.

A two way ANOVA depicted significant changes in complex-I (Fig. 5.2A) and complex-II (Fig.5.2B) activities among groups [F (4, 40) = 17.41; P<0.05 and F (4, 40) = 19.42; P<0.05 respectively], time [F (1, 40) = 6.38; P<0.05 and F (1, 40) = 12.16; P<0.05 respectively], However, no significant interaction between group and time [F (4, 40) = 1.45; P>0.05 and F (4, 40) = 1.80; P>0.05 respectively] was observed. Similarly an ANOVA depicted significant difference in complex-IV (Fig. 5.2C) and complex-V (Fig. 5.2D) activities

among groups [F (4, 40) = 15.42; P<0.05 and F (4, 40) = 13.86; P<0.05]. There was no significant interaction for time [F (1, 40) = 3.16; P>0.05 and F (1, 40) = .075; P>0.05] post injury. No significant interaction between group and time [F (4, 40) = 1.65; P>0.05 and F (4, 40) = 0.50; P>0.05 respectively] was observed. 2,4-DNP (2.5 and 5 mg/kg) treatment caused a marked improvement (P<0.05) in the activities of all four complex enzyme systems on day-7 but not day-1 following anoxia. Post hoc analysis revealed that 2,4-DNP (2.5 and 5 mg/kg) attenuated the primary insult progression and improved the mitochondrial complex enzyme system.

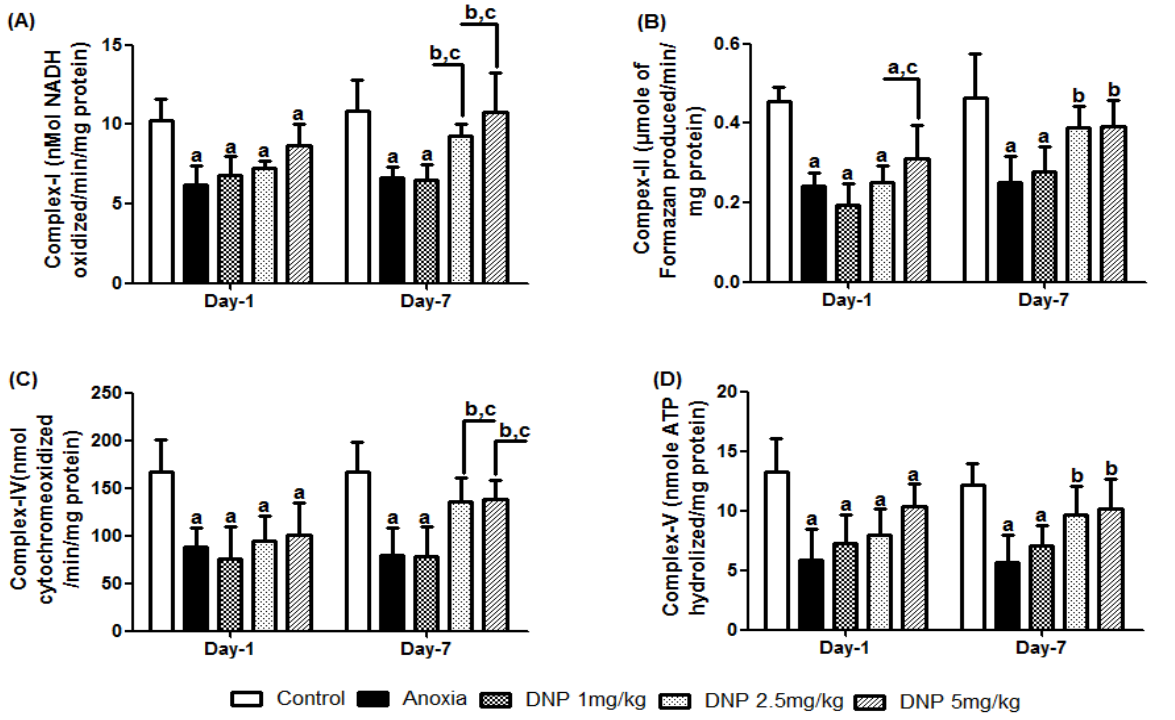

Figure 5.2 Effect of 2,4 DNP (1, 2.5 and 5 mg/kg) on anoxia-induced mitochondrial complex I (A), II (B), IV (C) and V (D) activity in cortical brain region on day-1 and day-7. Bars represents group mean ± SD. n = 5/group. [a]P<0.05 compared to control, [b]P<0.05 compared to anoxia and [c]P<0.05 compared to 2,4 DNP 1 mg/kg groups respectively [Two-way ANOVA followed by Bonferroni post-test].

5.4.5 Effect of multiple doses of 2,4-DNP on altered MMP and MPT on d-1 and d-7 after anoxia.

Fig. 5.3 represents the effect of 2,4-DNP on anoxia-induced alterations in MMP and MPT. Statistical analysis using ANOVA showed a significant main effect for fluorescence intensity among groups [$F(4, 40) = 2.65$; $P<0.05$], time [$F(1, 40) = 39.24$; $P<0.05$] and a significant relation between group and time [$F(4, 40) = 4.85$; $P<0.05$] post-anoxia injury. Post hoc analysis revealed that 2,4 DNP in all the three doses significantly attenuated ($P<0.05$) anoxia-induced hyperpolarization and maintained mitochondrial integrity on day-7. Similarly an ANOVA depicted a significant difference in transition pore opening in terms of mitochondrial swelling among groups [$F(4, 40) = 23.04$; $P<0.05$], time [$F(1, 40) = 7.6$; $P<0.05$]. However there was insignificant interaction between group and time [$F(4, 40) = 1.95$; $P<0.05$] post-anoxic injury. Post hoc analysis revealed that 2,4-DNP in the dose 2.5 and 5 mg/kg, but not 1mg/kg was significantly effective ($P<0.05$) in inhibiting mitochondrial swelling compared to anoxia on day-7.

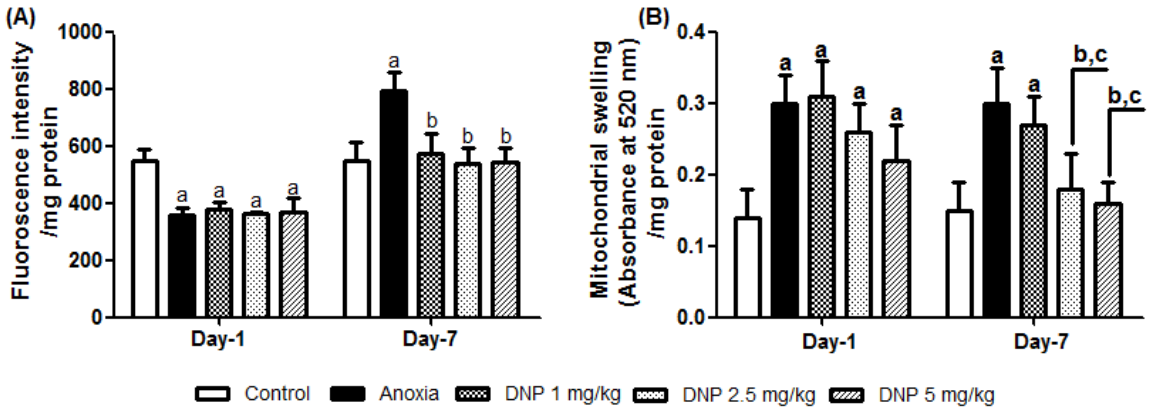

Figure 5.3 Effect of 2,4 DNP (1, 2.5 and 5 mg/kg) on anoxia-induced changes in (A) mitochondrial membrane potential and (B) mitochondrial permeability transition pore activity in cortical brain region on day-1 and day-7. Data represents group mean ± SD. n = 5/group.[a]P<0.05 compared to control, [b]P<0.05 compared to anoxia and [c]P<0.05 compared to 2,4 DNP 1 mg/kg groups respectively [Two-way ANOVA followed by Bonferroni post-test].

5.4.6 Effect of multiple doses of 2,4-DNP on increased expression of cytochrome-C, caspase-9 and caspase-3 in cortex on d-1 and d-7 after anoxia.

A two-way ANOVA depicted a significant main effect for the levels of expression of cytochrome-C (Fig. 5.4B) and caspase-9 (Fig. 5.4C) among groups [F (4, 20) = 60.93; P<0.05 and F (4, 20) = 62.44; P<0.05 respectively], time [F (1, 20) = 56.33; P<0.05 and F (1, 20) = 13.81; P<0.05 respectively] and a significant communication between group and time [F (4, 20) = 19.20; P<0.05 and F (4, 20) = 11.64; P<0.05 respectively] after anoxia. It is clear from post hoc analysis that 2,4-DNP in the dose 2.5 and 5 mg/kg was efficient in reducing the levels of cytochrome-C and caspase-9 on d-7. Similarly, an ANOVA revealed a significant difference in expression of caspase-3 (Fig. 5.4D) among groups [F (4, 20) = 21.90; P<0.05], however there was no significant main effect for time [F (1, 20) = 0.107; P>0.05]. There was a significant interaction between group and time [F (4, 20) = 8.65; P<0.05] post-anoxia. Post hoc analysis showed that treatment with 2,4-DNP (2.5 and 5

mg/kg) significantly (P<0.05) attenuated anoxia-induced increase expression of caspase-3 on d-7 and inhibited insult progression.

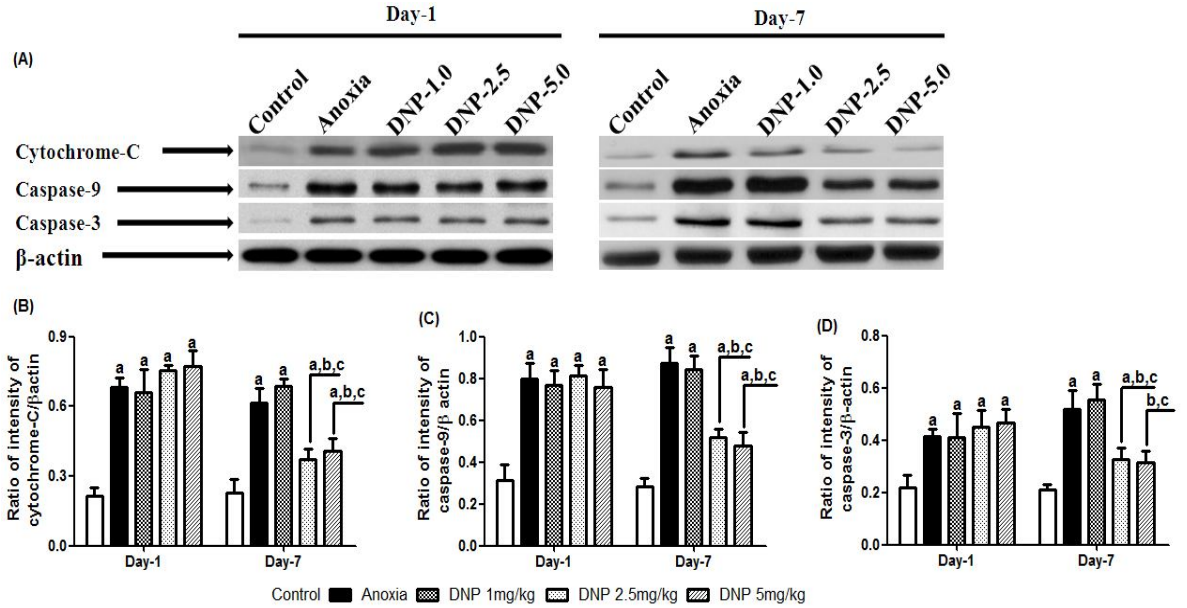

Figure 5.4 Effect of 2,4 DNP (1, 2.5 and 5 mg/kg) on anoxia-induced changes in the levels of expression of cytochrome-C, caspase-9 and caspase-3 on day-1 and day-7. Blots (A) represents cytochrome-C, caspase-9 and caspase-3 in cortical region. The results in the histogram are articulated as the ratio 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 and [b]P<0.05 compared to anoxia and [c]P<0.05 compared to 2,4 DNP 1 mg/kg groups respectively [Two-way ANOVA followed by Bonferroni post-test].

5.4.7 Effect of multiple doses of 2,4-DNP on alterations in expression of cytoplasmic Bax, Bcl-2, and their ratio (Bax/Bcl-2) in cortex on d-1 and d-7 after anoxia.

Anoxia lead to pathological alteration in expression of cytoplasmic Bax (Fig.5.5B), Bcl-2 (Fig.5.5C) and their ratio (Bax/Bcl-2; Fig.5.5D) among groups [F (4, 20) = 40.41; P<0.05, F (4, 20) = 25.89; P<0.05 and F (4, 20) = 60.80; P<0.05 respectively], time [F (1, 20) = 66.96; P<0.05, F (1, 20) = 16.01; P<0.05 and F (1, 20) = 5.2; P<0.05 respectively] and a significant relations between group and time [F (4, 20) = 17.94; P<0.05, F (4, 20) = 2.54; P<0.05 and F(4,20) = 5.43; P<0.05 respectively]. Treatment with 2,4-DNP (2.5 and 5 mg/kg)

significantly (P<0.05) increased expression of cytoplasmic Bax, decreased the expression of Bcl-2 and increased the Bax/Bcl-2 ratio and inhibited the insult progression.

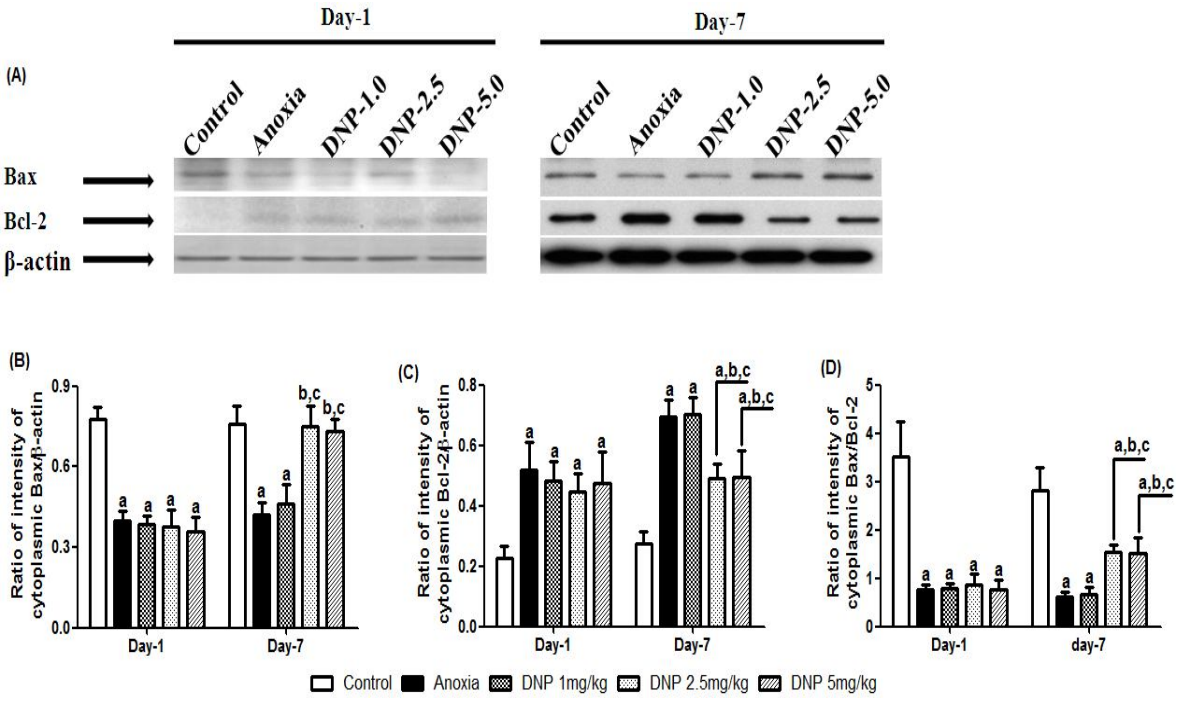

Figure 5.5 Effect of 2,4 DNP (1, 2.5 and 5 mg/kg) on anoxia-induced changes in the expression of cytoplasmic Bax (B), Bcl-2 (C) and their ratio (Bax/Bcl-2; D) in cortical brain region on day-1 and day-7. Blots (A) represent Bax and Bcl-2 in cortical region. The results in the histogram are expressed 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 and ^{b}P<0.05 compared to anoxia and ^{c}P<0.05compared to 2,4 DNP 1 mg/kg groups respectively [Two-way ANOVA followed by Bonferroni post-test].

5.4.8 Effect of multiple doses of 2,4-DNP on alterations in expression of mitochondrial Bax, Bcl-2, and their ratio (Bax/Bcl-2) in cortex on d-1 and d-7 after anoxia.

An ANOVA depicted a significant anoxia induced alteration in levels of expression of mitochondrial Bax (Fig.5.6B), Bcl-2 (Fig.5.6C) and their ratio (Bax/Bcl-2; Fig.5.6D) among groups [F (4, 20) = 60.58; P<0.05, F (4, 20) = 92.36; P<0.05 and F (4, 20) = 74.71; P<0.05 respectively], time [F (1, 20) = 26.81; P<0.05, F (1, 20) = 2.9; P<0.05 and F (1, 20) = 3.8;

P<0.05 respectively] and a significant interaction between group and time [F (4, 20) = 13.51; P<0.05, F (4, 20) = 8.9; P<0.05 and F(4,20) = 15.5; P<0.05 respectively]. Treatment with 2,4-DNP (2.5 and 5 mg/kg) significantly (P<0.05) inhibited the insult progression by decreasing the expression of mitochondrial Bax and increased the expression of Bcl-2 on day-7 compared to day-1. Overall Bax/Bcl-2 ratio was decreased on d-7 following 2,4-DNP treatment.

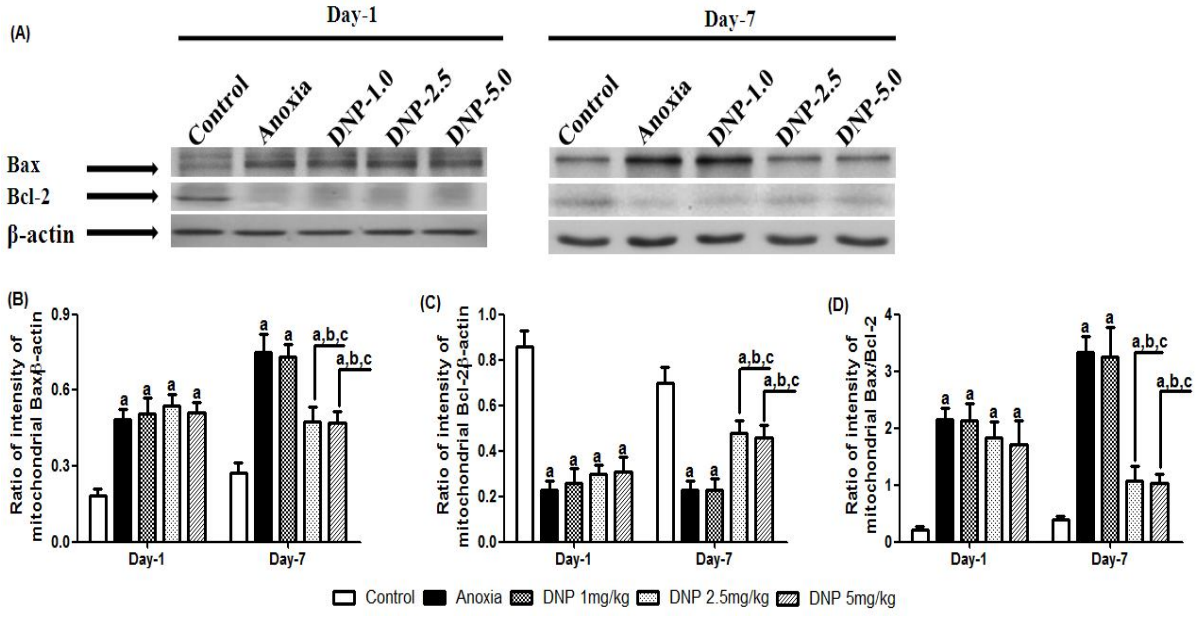

Figure 5.6 Effect of 2,4 DNP (1, 2.5 and 5 mg/kg)on anoxia-induced changes in the expression of mitochondrial Bax (B), Bcl-2 (C) and their ratio (Bax/Bcl-2; D) in cortical brain region on day-1 and day-7. Blots (A) represent Bax and Bcl-2 in cortical region. The results in the histogram are articulated 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 = 3/group. [a]P<0.05 compared to control and [b]P<0.05 compared to anoxia and [c]P<0.05compared to 2,4 DNP 1 mg/kg groups respectively [Two-way ANOVA followed by Bonferroni post-test].

5.4.9 Effect of multiple doses of 2,4-DNP on changes in reflex latency and hanging latency on d-1 and d-7 after anoxia.

A repeated measure two-way ANOVA depicted a significant difference in reflex latency (Fig. 5.7A) among groups [F (4, 40) = 23.23; P<0.05], time [F (1, 40) = 436.3; P<0.05] and

a significant interaction between group and time [F (4, 40) = 8.573; P<0.05] post-anoxia. Post hoc analysis showed that there was a significant increase (P<0.05) in reflex latency in anoxic compared to control group. DNP in the dose 2.5 and 5 mg/kg markedly attenuated (P<0.05) reflex latency compared to anoxia group on day-7. Similarly, An ANOVA depicted a significant changes in hanging latency (Fig. 5.7B) among groups [F (4, 40) = 8.232; P<0.05 respectively], time [F (1, 40) = 209.3; P<0.05] and a significant interaction between group and time [F (4, 40) = 8.082; P<0.05] post-anoxia. Post hoc analysis revealed that there was a significant decrease (P<0.05) in hanging latency in anoxia group compared to control group. However, 2,4-DNP (dose 2.5 and 5 mg/kg) was able to attenuate anoxia-induced decrease in hanging latency.

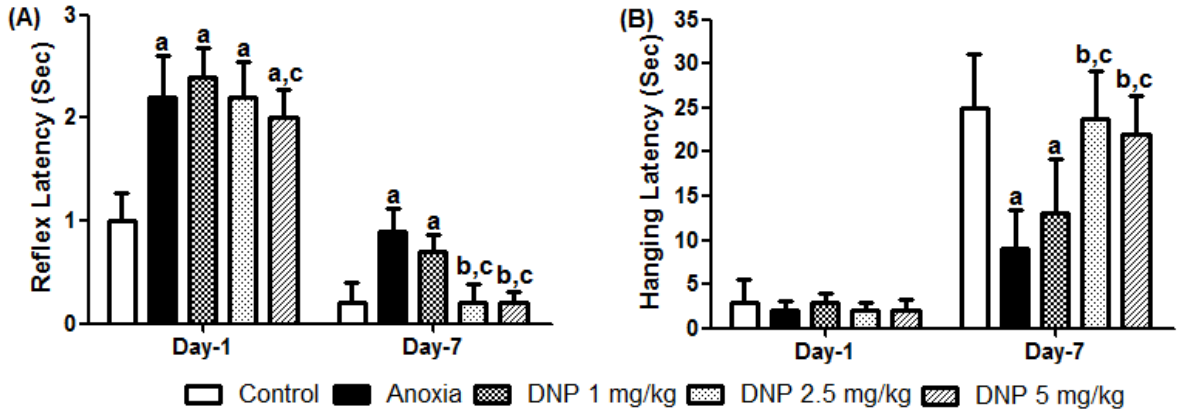

Figure 5.7 The effect of 2,4 DNP (1, 2.5 and 5 mg/kg) on anoxia induced sensorimotor dysfunction [Reflex latency (A) and Hanging latency (B)] on day-1 and day-7. Bars represents group mean±SD (n=5 in each group).[a]P<0.05compared to control, [b]P<0.05compared to anoxia and [c]P<0.05compared to 2,4 DNP 1mg/kg respectively [Repeated measure two-way ANOVA followed by Bonferroni post-test].

5.5 Discussion

The present study reports the pharmacological effect of mitochondrial uncoupler 2,4-DNP in global model of neonatal anoxia. We for the first time reported that 2,4-DNP attenuated progressive loss of cortical mitochondrial function and associated neurobehavioral deficits during the intervening time of primary insult (d-1) and secondary insult (d-7).

Anoxia caused a decrease in mitochondrial respiration states and RCR starting from d-1 up to d-7 indicating a progressive loss of mitochondrial function. Pathological alterations in mitochondrial bioenergetics play a pivotal role in this biphasic mechanism of neurodegeneration and cell death after anoxia [120]. 2,4-DNP in the dose 2.5 and 5 mg/kg maintained mitochondrial bioenergetics by attenuating the changes in different states of mitochondrial respiration (s-3, s-4, s-5 via complex-I and s-5 via complex-II) from day-1 to day 7. 2,4 DNP also ameliorated mitochondrial function as evident from the reversal of RCR in anoxic pups. Excessive ROS formation in the mitochondria results in a decrease in antioxidant enzymes, the collapse of membrane potential and activation of pro-apoptotic factors like Bax and caspases [137]. Our results depicted a robust increase in mitochondrial LPO and NO levels and decreased activity of antioxidant enzymes such as SOD and CAT up to d-7 after anoxia. However, 2,4-DNP (2.5 and 5 mg/kg) ameliorated the oxidative damage by reducing MDA and excessive NO formation and improving antioxidant enzyme activity following anoxia. In the present experiment, 2,4-DNP significantly reduced the anoxia-induced increase MMP on d-7. Therefore, inhibition of ROS formation linked to mitochondrial dysfunction may be because mild uncoupling reduces the MMP and sequestration of Ca^{2+}into mitochondria [138]. Further, 2,4-DNP in the dose 2.5 and 5 mg/kg improved the activity of complex-I, II, IV, and V and inhibited the insult progression from

(From d-1 to d-7). Further, inactivation of complex enzyme system leads to Ca^{2+}overloading and transition pore opening [139]. A marked depolarization on day-1 and hyperpolarization on d-7 of mitochondrial membrane potential was observed following anoxia as seen in our recent finding [120]. MMP plays a major role in mitochondrial Ca^{2+} cycling/overloading, and alterations in MMP during pathological insult like anoxia leads to opening of mitochondrial permeability transition pore. As discussed earlier, 2,4-DNP in all the three doses attenuated changes in MMP and inhibited the insult progression to d-7.

Up-regulation of pro-apoptotic Bax on the mitochondrial outer membrane and down-regulation of anti-apoptotic Bcl-2 leads to mitochondrial membrane permeabilization and release of free permeability small molecules of less than 1500 Dalton. Anoxia exposure induced activation of mitochondrial pro-apoptotic Bax and decrease in the levels of Bcl-2 both on d-1 and d-7 as reported earlier [120]. The present study for the first time suggests the role of mitochondrial uncoupler 2,4-DNP on the expression of upstream Bcl-2 family proteins (Bax and Bcl-2) following anoxia. It was interesting to note that 2,4-DNP in the dose 2.5 and 5 mg/kg increased the levels of expression of cytoplasmic Bax and decreased in expression of cytoplasmic Bcl-2 and efficiently increased their ratio (Bax/Bcl-2) in cytoplasm on d-7 post-anoxia. Further, 2,4-DNP (2.5 and 5 mg/kg) effectively decreased the levels of expression of mitochondrial Bax while the increase in the levels of expression of mitochondrial Bcl-2 and decreased expression of their ratio (Bax/Bcl-2) on the mitochondrial outer membrane. These results indicate that 2,4-DNP inhibited the progression of insult cascade following anoxia by maintaining mitochondrial outer membrane integrity through inhibiting the translocation of pro-apoptotic Bax from the cytoplasm to mitochondria and relocating Bcl-2 on mitochondrial outer membrane post-

anoxia. Anoxia caused a marked opening of transition pore regarding mitochondrial swelling and its progression from primary to secondary insult.

Treatment with 2,4-DNP in the dose 2.5 and 5 mg/kg inhibited the progression of mitochondrial swelling post-anoxia injury (d-1 to d-7). This may be due to the fact that 2,4-DNP reduces the Ca^{2+} overloading and ROS generation [138]. Furthermore, the activation of MPT pore in the inner mitochondrial membrane causes the release of intrinsic apoptotic factors like SMAC/DIABLO and cytochrome-C [28, 140]. Cytochrome-C along with caspase-9 activates caspase-3 which leads to apoptotic cell death [86]. Anoxia caused a marked increase in the levels of expression of cytochrome-C, caspase-9 and caspase-3 levels in the cortical region of the brain [120]. It was interesting to note that 2,4-DNP (2.5 and 5 mg/kg) attenuated the anoxia-induced mitochondrial-linked apoptosis by inhibiting the release of cytochrome-C, activation of caspase-9 and caspase-3 and inhibited the progression of insult (d-7). Previous reports have suggested the role of 2,4-DNP in inhibition of release of cytochrome-C and the activation of caspase-3 and reduction of neuronal cell death after stroke and cerebral ischemia [141, 142]. A hampered sensorimotor performance viz. decreased hanging latency and increased reflex latency from d-1 to d-7 post second anoxia episode was observed which reflects an early sign of neurological dysfunction in the early postnatal days as depicted in our previous finding [120]. 2,4-DNP in the dose 2.5 and 5 mg/kg increased the time of hanging maneuver and decreased the reflex latency to d-7 (P10) rats, an age when brain development is comparable to the late preterm human infant [16], which shows that the drug effectively improved the sensorimotor performance of developing neonates.

In summary, our results demonstrate that mitochondrial uncouplers such as 2,4-DNP can be potential pharmacological agents in the treatment of anoxia. The pharmacological effects of DNP on mitochondrial bioenergetics was accompanied by a decrease in MMP and inhibition of ROS which resulted in the restoration of mitochondrial function, complex enzyme activity, inhibition of expression of pro-apoptotic mitochondrial Bax. This led to decrease in mitochondrial MPT pore opening and resultant release cytochrome-C. These events ultimately caused inhibition of mitochondrial liked apoptosis by reduced activation of caspase-9 and caspase-3.