6.1 Abstract

Following anoxia, there is a robust generation of reactive oxygen species/nitrogen species which can lead to mitochondrial dysfunction and associated cell death in the cerebral cortex of neonates. The present study investigated the pharmacological role of tempol, a •NO scavenger on the anoxia-induced progression of mitochondrial dysfunction, i.e., from (P4) d-1 to (P10) d-7 in neonatal rat cortex. For this, rat pups of 30 hr. age (P2) were subjected to two episodes of anoxia (10 min each) at 24 hr. Results demonstrated that tempol in multiple doses (75 mg/kg, 150 mg/kg and 300 mg/kg, i.p within 5 minutes after second anoxic episode) significantly ($P<0.05$) decreased the mitochondria-associated progression of secondary insult (d-1 to d-7) by interfering with nitric oxide free radical (•NO) formation and by SOD and CAT mimetic activity. Further, there was an improvement in mitochondrial respiration, mitochondrial complex enzyme activity and mitochondrial membrane potential (MMP) along with inhibition of transition pore opening (MPT) after anoxia. Furthermore, there was decrease expression of pro-apoptotic mitochondrial Bax which results in mitochondrial outer membrane permeabilization (MOMP), preventing the release of cytochrome-C, inhibiting the regular expression of caspase-9/3 expression after treatment with tempol. Beside the above biochemical and molecular parameters tempol improved the associated neurobehavioral activities like reflex latency and hanging latency which suggests its role in treating neonatal anoxia.

Keywords: Anoxia; Mitochondrial function; Tempol; Neurobehavioral alteration; animal model.

6.2 Introduction

Mitochondria has been known to be the prime target organelle for the action of nitric oxide (•NO). It's excessive biosynthesis after trauma has been reported to damage the immature brain soon after anoxia [76, 143]. Moreover, the crucial role of oxidative/nitrosative stress and oxidative metabolism in mitochondrial dysfunction linked apoptosis has been reported to further aggravate progression of secondary abuse cascade from hours to several days after anoxia. Overall •NO contributes to a battery of intracellular bimolecular events and plays a major role in progression of secondary insult cascade. Further, anoxia leads to a robust increase in •NO and failure of the antioxidant mechanism in the cerebral cortical tissue which further plays an active role in mitochondrial dysfunction [120]. However, it is evident that apoptotic neurons remain viable for a period of time and can be rescued [36].

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) is a member of a family of nitroxide compounds that has been studied extensively in animal models of mitochondrial-linked oxidative stress [41, 43, 52]. Nitric oxide along with the diffusion-limited combination of superoxide ($O_2^{\bullet -}$) forms a reactive species peroxynitrite (ONOO-) by the catalytic activation of mitochondrial nitric oxide synthase (mtNOS) [53]. Studies have shown that tempol possess SOD and CAT mimetic properties which scavenge ROS/RNS [54, 55].

Therefore, the present study investigated the effect of tempol on cortical mitochondrial dysfunction following neonatal anoxic primary insult (d-1) and secondary insult (d-7) by examining mitochondrial respiration (state-2, state-3, state-4, state-5 via complex-I and state-5 via complex-II), RCR, mitochondrial complex enzyme system,

oxidative stress, MMP and transition pore 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.

6.3 Materials and methods

6.3.1 Animals

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.See chapter 2.

6.3.2 Anoxia model

The anoxic procedure was carried out as validated and defined previously [76].

6.3.3 Tempol preparation and dosing

Tempol (Sigma, USA) was freshly prepared in 0.9 % saline before intraperitoneal injection (i.p.). The rat pups were randomly divided into 5 groups (n=5 per group): (1) Control; (2) Anoxia; (3) Anoxia + Tempol 75 mg/kg; (4) Anoxia + Tempol 150 mg/kg; and (5) Anoxia + Tempol 300 mg/kg. The drug was administered after 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 normal saline at the same time.

6.3.4 Chemicals

Sodium pyruvate, malate, ADP, succinate, oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), Rotenone, tetramethylrhodamine methyl ester (TMRM) and Griess reagent were procured from Sigma-Aldrich (St. Louis, MO).Antibodies such as cytochrome-C, caspase-9, caspase-3, Bax, Bcl-2, and beta-actin were purchased from Santa Cruz Biotechnology Inc; Santa Cruz, California, USA.

6.3.5 Behavioral parameter assessment

6.3.5.1 Righting reflex

As described previously [65].Chapter 2. Page no. 24.

6.3.5.2 Wire hanging maneuver

As described previously [65, 88]. Chapter 3. Page no. 38.

6.3.6 Mitochondrial Isolation

Mitochondrial isolation was performed as described previously [66] with some slight modifications [76]. Chapter 2. Page no.24.

6.3.7 Measurement of Mitochondrial Function

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

6.3.8 Estimation of NADH dehydrogenase (Complex-I) activity

NADH dehydrogenase activity was estimated as described previously [132]. detailed in chapter 5 Page no.79.

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

Succinate dehydrogenase (SDH) activity was evaluated as previously stated [133] and discussed in chapter 5. Page no.80.

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

Complex-IV activity was assessed in mitochondrial preparation as per the method of [134] detailed in Chapter 5. Page no.80.

6.3.11 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] detailed in chapter 5. Page no.80.

6.3.12 Evaluating mitochondrial membrane potential (MMP)

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]. Details are given in chapter 3. Page no. 40.

6.3.13 Mitochondrial permeability transition (MPT)

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

6.3.14 Mitochondrial oxidative stress

6.3.14.1 Estimation of LPO and NO levels

Mitochondrial malondialdehyde (MDA) content was measured as per standard protocol [94] whereas Nitrite (NO) levels were determined by [69]. Detailed in chapter 3. Page no.41.

6.3.14.2 Estimation of mitochondrial SOD and CAT activity

Superoxide dismutase (SOD) activity was estimated by [95]. Catalase activity was estimated by [96]. Detailed in chapter 3. Page no.41.

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

Western blot analysis was performed as described in previous [120]. Detailed in chapter 3. Page no.41.

6.3.16 Statistical analysis

The data are expressed as means ± SD. For the statistical analysis of mitochondrial bioenergetics, mitochondrial RCR, Antioxidant enzyme, mitochondrial complex enzyme system, MMP, MPT, western blot analysis of Bax, Bcl-2, cytochrome-C, caspase9 and caspase-3 Two-way ANOVA followed by Bonferroni post hoc test was used. Repeated measure two-way ANOVA followed by was performed to measure sensorimotor behavioral activities. $P<0.05$ was considered as significant. All data were analyzed using GraphPadPrism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

6.4 Results

6.4.1 Effect of multiple doses of tempol on mitochondrial respiration and RCR in cortex on d-1 and d-7 after anoxia.

A two-way ANOVA revealed no significant difference in s-2 respiration among groups [$F(4, 40) = 0.34$; $P>0.05$] and time [$F(1, 40) = 1.3$; $P>0.05$] 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 day-1 and day-7. Further, s-3 respiration was fueled by the addition of ADP in the oxytherm chamber which is a substrate for complex-V and phosphorylates ADP to ATP. An ANOVA depicted a significant main effect among groups [$F(4, 40) = 21.08$; $P<0.05$], time [$F(1, 40) = 34.31$; $P<0.05$] and a significant interaction between group and time [$F(4, 40) = 5.11$; $P<0.05$] after anoxia injury. Post hoc analysis showed that tempol in all the three doses markedly increased the s-3 respiration and inhibited the progression of insult from d-1 to d-7. Oligomycin, a complex-V inhibitor was added in the chamber to initiate s-4 respiration which blocks 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 [$F(4, 40) = 11.52$; $P<0.05$], time [$F(1, 40) = 52.62$; $P<0.05$] and a significant interaction between group and time [$F(4, 40) = 4.88$; $P<0.05$]. Post hoc analysis revealed that tempol in all the three doses attenuated s-4 respiration following anoxia on day-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 [$F(4, 40) = 36.14$; $P<0.05$], time [$F(1, 40) = 117.8$; $P<0.05$] and a significant interaction between group and time [$F(4, 40) = 14.67$; $P<0.05$]. Post hoc analysis revealed that tempol (75, 150 and 300 mg/kg) was effective in preserving s-5 respiration and inhibited the progression of insult. Succinate was added to fuel s-5 via complex-II respiration. A two-way ANOVA depicted a significant main effect among groups [$F(4, 40) = 9.72$; $P<0.05$], time [$F(1, 40) = 18.03$; $P<0.05$], however no significant interaction was observed between group and time [$F(4, 40) = 1.83$; $P>0.05$]. Post hoc analysis depicted that tempol in all the three doses improved s-5 via complex II respiration after 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 [F (4, 40) = 10.47; P<0.05], time [F (1, 40) = 27.11; P<0.05] and a significant interaction between group and time [F (4, 40) = 3.15; P<0.05]. It is clear from the post hoc analysis that tempol in all the three doses significantly attenuated RCR and prevented insult progression (Table.6.1).

Group	P/M	ADP	Oligomycin	FCCP	Succinate	RCR
Day-1						
Control	30.3 ± 5.1	117.5 ± 6.9	16.7 ± 6.5	111 ± 11	57 ± 5.4	7.0 ± 1.5
Anoxia	26.7 ± 5.8	86.8 ± 11.1*	34 ± 4.3*	70 ± 7.6*	37 ± 7.9*	2.5 ± 1.7*
T-75 mg/kg	25.8 ± 5.0	87.9 ± 4.3*	36 ± 3.2*	68 ± 6.2*	38 ± 9.6*	2.4 ± 1.9*
T-150 mg/kg	23.8 ± 6.9	90.9 ± 7.5*	34 ± 3.3*	71 ± 5.4*	40 ± 8.8*	2.6 ± 1.3*
T-300 mg/kg	25.2 ± 8.5	96.3 ± 7.9*	36 ± 5.6*	77 ± 7.5*	41 ± 7.9*	2.6 ± 1.2*
Day-7						
Control	31.8 ± 4.4	117.2 ± 6.1	15.9 ± 6.3	113 ± 9.0	57.6 ± 5.2	7.3 ± 1.7
Anoxia	30.9 ± 4.6	89.5 ± 4.5*	28.7 ± 8.8*	75 ± 7.0*	40.2 ± 5.8*	3.1 ± 1.3*
T-75 mg/kg	34.8 ± 4.9	108 ± 7.5$^{\#}$	19.0 ± 3.1$^{\#}$	107 ± 6.3$^{\#}$	51.1 ± 6.1$^{\#}$	5.7 ± 1.2$^{\#}$
T-150 mg/kg	32.8 ± 6.7	114 ± 7.6$^{\#}$	20.0 ± 3.0$^{\#}$	109 ± 5.1$^{\#}$	52.3 ± 4.1$^{\#}$	5.7 ± 1.9$^{\#}$
T-300 mg/kg	35.2 ± 8.0	111 ± 7.1$^{\#}$	19.0 ± 5.4$^{\#}$	107 ± 7.3$^{\#}$	53.1 ± 5.7$^{\#}$	5.8 ± 1.1$^{\#}$

Table 6.1 Dose-related effect of tempol (75, 150 and 300 mg/kg) on anoxia-induced alterations in mitochondrial respiration and RCR on day-1 and day-7. Data are group mean ± SD. n = 5/group. *P<0.05 compared to control and $^{\#}$P<0.05 compared to anoxia groups respectively [Two-way ANOVA followed by Bonferroni post-test].

6.4.2 Effect of multiple doses of tempol on oxidative and nitrosative stress in cortex on d-1 and d-7 after anoxia.

A two-way ANOVA revealed a significant difference in levels of NO (Fig.6.1A) among groups [F (4, 40) = 38.17; P<0.05]. However there was no significant changes with time [F (1, 40) = 0.77; P>0.05]. A significant interaction between group and time [F (4, 40) = 3.5;

P<0.05] was observed. Post hoc analysis revealed that tempol in the dose 150 and 300 mg/kg was effective in attenuating NO levels from day-1 and in all the three doses (75, 150 and 300 mg/kg) was effective up to day-7 depicting that tempol was potentially effective against the primary stage of insult following anoxia. LPO (Fig.6.1B) was estimated regarding MDA formed. An ANOVA depicted a significant increase in the levels of MDA among groups [F (4, 40) = 26.54; P<0.05], however no significant change for time [F (1, 40) = 0.81; P>0.05] was observed. Further, there was no significant interaction between group and time F (4, 40) = 0.08; P>0.05 respectively] post-anoxia. Post hoc analysis showed that tempol in the dose 300 mg/kg was effective in attenuating MDA levels and preventing its contribution to insult progression.

6.4.3 Effect of multiple doses of tempol 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 CAT (Fig.6.1C) and SOD (Fig.6.1D) among groups [F (4, 40) = 27.33; P<0.05 and F (4, 40) = 14.77; P<0.05], time [F (1, 40) = 34.31; P<0.05 and F (1, 40) = 20.75; P<0.05] and a significant interaction between group and time [F (4, 40) = 6.4; P<0.05 and F (4, 40) = 2.8 P<0.05 respectively] after anoxia. Post hoc analysis revealed that treatment with tempol (75, 150 and 300 mg/kg) improved anoxia-induced decrease in CAT and SOD on d-7 and inhibited the advancement of insult.

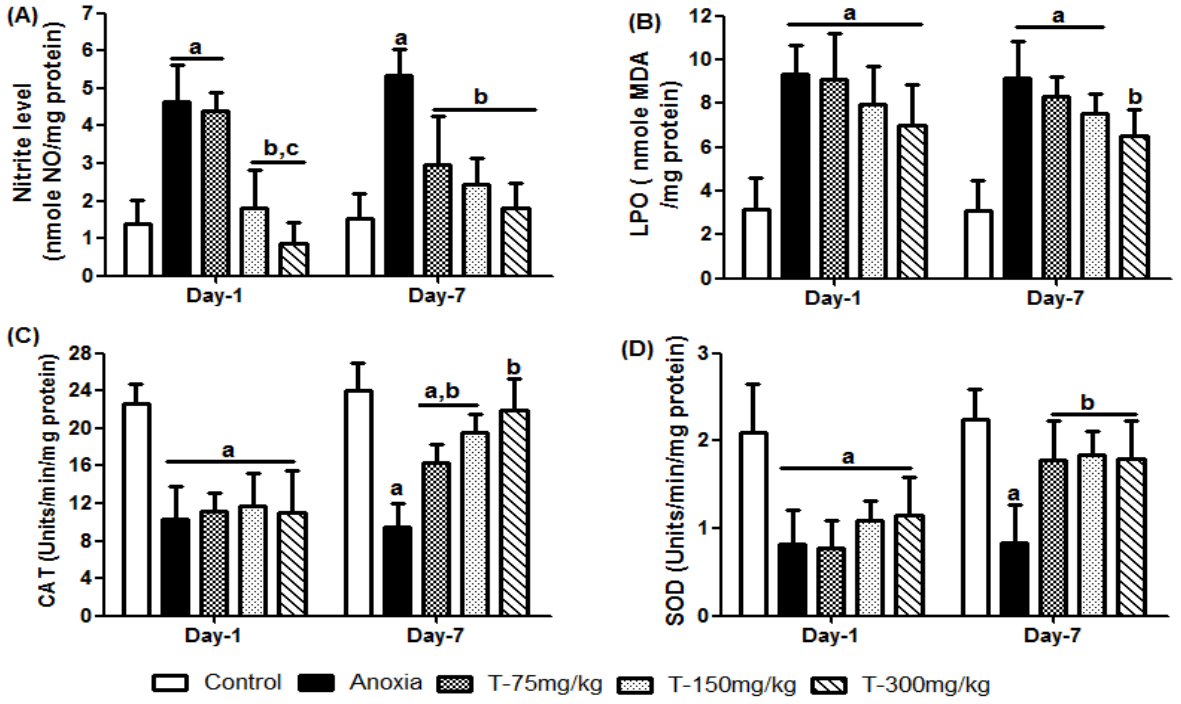

Figure 6.1 Effect of tempol (75, 150 and 300 mg/kg) on anoxia-induced mitochondrial (A) NO, (B) LPO, (C) CAT and (D) SOD levels in cortical brain region on d-1 and d-7. Bars represents group mean ± SD. n = 5/group. [a]P<0.05 compared to control and [b]P<0.05 compared to anoxia groups respectively [Two-way ANOVA followed by Bonferroni post-test].

6.4.4 Effect of multiple doses of tempol 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. 6.2A), complex-II (Fig.6.2B) and complex-IV (Fig. 6.2C) activities among groups [F (4, 40) = 15.21; P<0.05, F (4, 40) = 24.17; P<0.05 and F (4, 40) = 18.56; P<0.05], time [F (1, 40) = 28.41; P<0.05, F (1, 40) = 15.48; P<0.05 and F (1, 40) = 31.31; P<0.05] and a significant interaction between group and time [F (4, 40) = 2.74; P<0.05, F (4, 40) = 2.66; P<0.05 and F (4, 40) = 6.07; P<0.05 respectively]. Similarly an ANOVA depicted significant difference in complex-V (Fig. 6.2D) activity among groups [F (4, 40) = 6.33; P<0.05], time [F (1, 40) = 12.70; P<0.05]. However no significant interaction between group and time [F (4, 40) = 2.38;

P>0.05] was observed. Post hoc analysis revealed that treatment with tempol (75, 150 and 300 mg/kg) markedly improved the activities of all four complex enzyme systems on d-7 and inhibited their contribution to insult progression following anoxia.

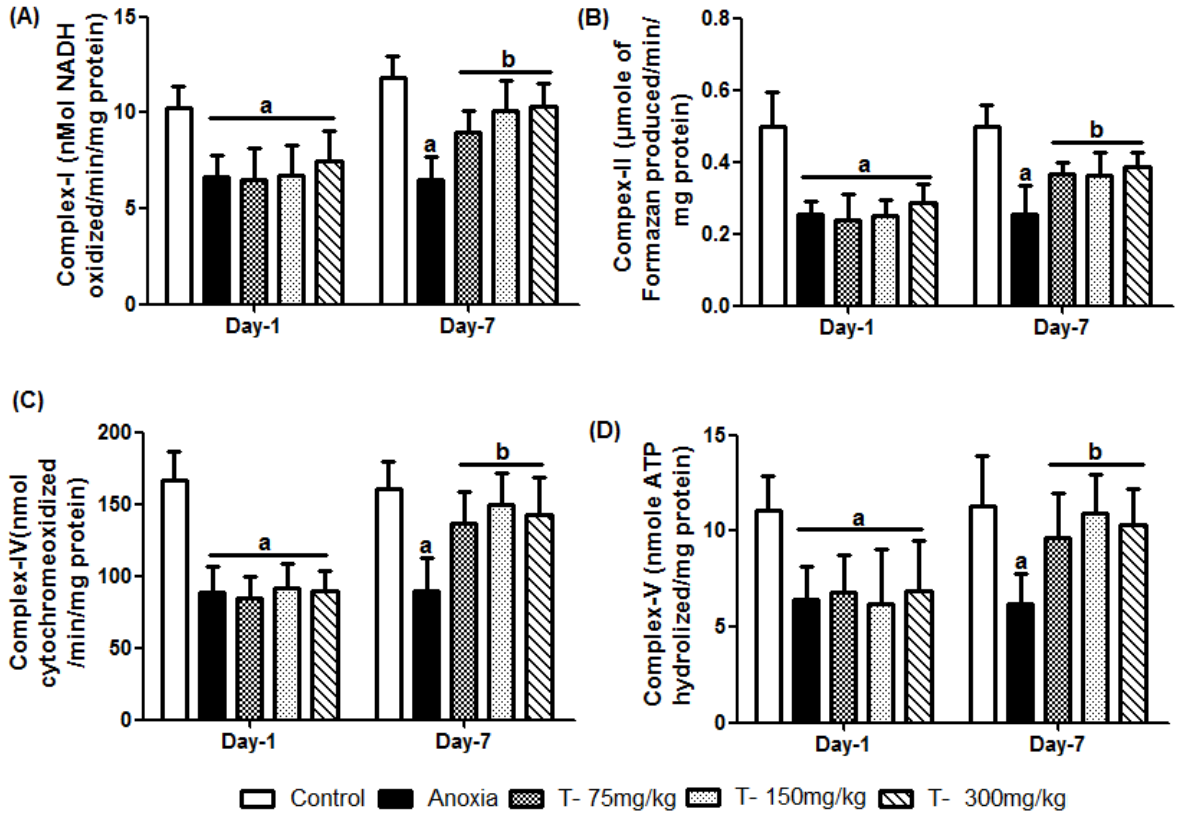

Figure 6.2 Effect of tempol (75, 150 and 300 mg/kg) on anoxia-induced mitochondrial complex I (A), II (B), IV (C) and V (D) activity in cortical brain region on d-1 and d-7. Bars represents group mean ± SD. n = 5/group. [a]P< 0.05 compared to control, [b]P< 0.05 compared to anoxia and [c]P<0.05 compared to tempol 75 mg/kg groups respectively [Two-way ANOVA followed by Bonferroni post-test].

6.4.5 Effect of multiple doses of tempol on altered mitochondrial MMP and MPT on d-1 and d-7 after anoxia.

Table.6.2 represents the effect of tempol on anoxia-induced alterations in MMP and MPT. A Two-way ANOVA revealed a significant main effect for fluorescence intensity among groups [F (4, 40) = 2.87; P<0.05], time [F (1, 40) = 36.69; P<0.05] and a significant

interaction between group and time [F (4, 40) = 14.47; P<0.05] post-anoxia injury. Post hoc analysis revealed that tempol in all the three doses attenuated anoxia-induced hyperpolarization and maintained mitochondrial integrity on day-7. Similarly, ANOVA depicted a significant difference in transition pore opening in terms of mitochondrial swelling among groups [F (4, 40) = 14.50; P<0.05], time [F (1, 40) = 8.62; P<0.05], however there was no significant interaction between group and time [F (4, 40) = 2.31; P<0.05] post-anoxia injury. Post hoc analysis revealed that tempol in the dose 75, 150 and 300 mg/kg was effective in inhibiting mitochondrial swelling compared to anoxia on d-7.

Group	Fluorescence Intensity /mg protein	Mitochondrial swelling (Absorbance at 520 nm) /mg protein
Day-1		
Control	544.3 ± 60.11	0.14 ± 0.022
Anoxia	287.1 ± 31.8*	0.29 ± 0.058*
T-75 mg/kg	345.5 ± 67.3*	0.29 ± 0.050*
T-150 mg/kg	366.0 ± 40.2*	0.27 ± 0.051*
T-300 mg/kg	371.3 ± 110.4*	0.26 ± 0.025*
Day-7		
Control	530.1 ± 71.1	0.14 ± 0.037
Anoxia	820.5 ± 90.5*	0.31 ± 0.057*
T-75 mg/kg	482.7 ± 130.7#	0.21 ± 0.068#
T-150 mg/kg	497.7 ± 116.8#	0.20 ± 0.055#
T-300 mg/kg	538.3 ± 98.0#	0.19 ± 0.038#

Table 6.2 Effect of tempol (75, 150 and 300 mg/kg) on anoxia-induced changes in (A) mitochondrial membrane potential and (B) mitochondrial permeability transition pore activity in cortical brain region on d-1 and d-7. Data are group mean ± SD. n = 5/group. *P<0.05 compared to control, #P<0.05 compared to anoxia groups respectively [Two-way ANOVA followed by Bonferroni post-test].

6.4.6 Effect of multiple doses of tempol 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. 6.3B), caspase-9 (Fig. 6.3C) and caspase-3 (Fig. 6.3D) among groups [$F(4, 20) = 19.77$; $P<0.05$, $F(4, 20) = 27.69$; $P<0.05$ and $F(4, 20) = 11.61$; $P<0.05$], time [$F(1, 20) = 43.26$; $P<0.05$, $F(1, 20) = 167.7$; $P<0.05$ and $F(1, 20) = 67.44$; $P<0.05$] and a significant interaction between group and time [$F(4, 20) = 11.01$; $P<0.05$, $F(4, 20) = 11.64$; $P<0.05$ and $F(4, 20) = 3.39$; $P<0.05$ respectively] after anoxia. Post hoc analysis depicted that tempol in all the three doses were effective in reducing the levels of cytochrome-C, caspase-9 and caspase-3 on d-7.

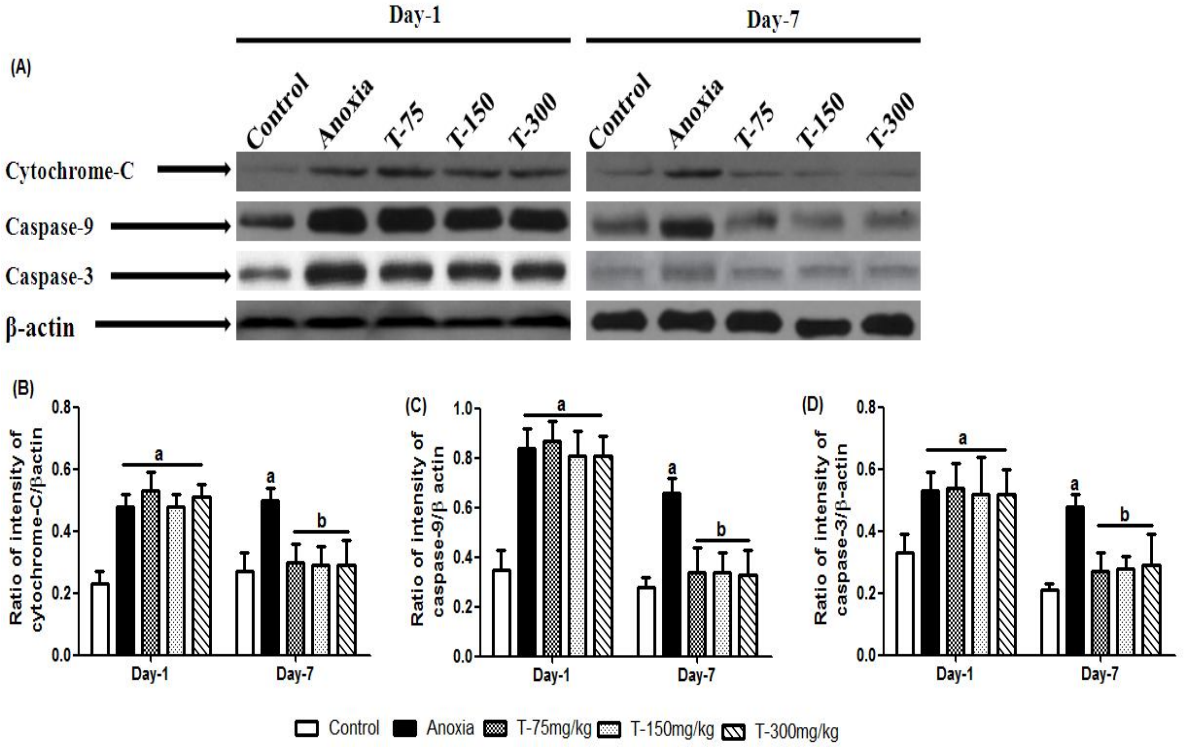

Figure 6.3 The effect of tempol (75, 150 and 300 mg/kg) on anoxia-induced changes in the levels of cytochrome-C (B), caspase-9 (C) and caspase-3 (D), in the cytosol fraction on d-1 and d-7. Blots (A) represents cytochrome-C, caspase-9 and caspase-3 in cortical region. The results in the histogram are expressed as ratio of 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 groups respectively [Two-way ANOVA followed by Bonferroni post-test].

6.4.7 Effect of multiple doses of tempol on alterations in expression of cytoplasmic Bax, Bcl-2, and their ratio in cortex on d-1 and d-7 after anoxia.

Anoxia lead to pathological alteration in expression of cytoplasmic Bax (Fig.6.4B) among groups [F (4, 20) = 15.92; P<0.05], time [F (1, 20) = 50.60; P<0.05] and a significant interaction between group and time [F (4, 20) = 14.09; P < 0.05]. An ANOVA depicted significant changes in Bcl-2 (Fig.6.4C) among groups [F (4, 20) = 35.65; P<0.05]. However, no significant for time [F (1, 20) = 1.61; P>0.05] and no interaction between group and time [F (4, 20) = 0.57; P>0.05] were observed. A significant change in their ratio (Bax/Bcl-2;

Fig.6.4D) among groups [F (4, 20) = 116.9; P<0.05], time [F (1, 20) = 25.29; P<0.05] were observed. Although, there was no significant interaction between group and time [F (4, 20) = 1.82; P>0.05] post anoxia. Post hoc analysis revealed that tempol (75 mg/kg, 150 mg/kg and 300 mg/kg) treatment attenuated increased expression of cytoplasmic Bax on d-7 compared to d-1. However, tempol in all the three doses failed to decrease the expression of cytoplasmic Bcl-2 on day-1 and d-7. Overall Bax/Bcl-2 ratio was increased on d-7 but not d-1 following tempol treatment.

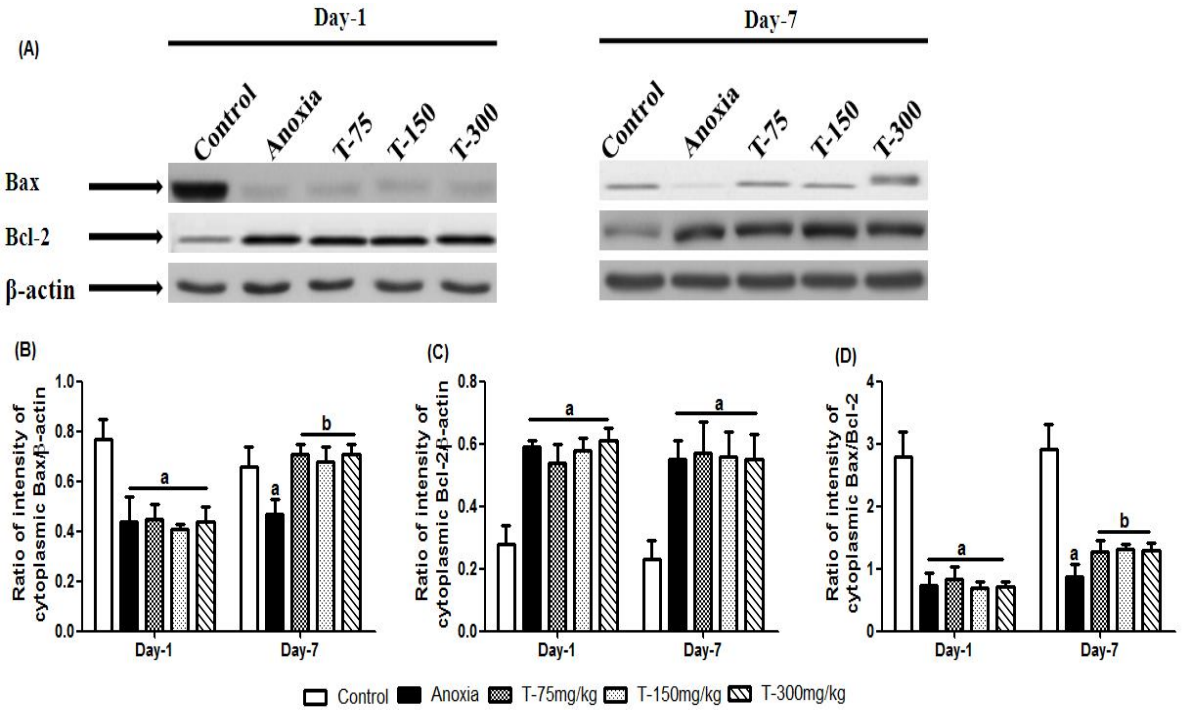

Figure 6.4 The effect of tempol (75, 150 and 300 mg/kg) on anoxia-induced changes in the levels of expression of cytoplasmic Bax (B), Bcl-2 (C) and their ratio (Bax/Bcl-2; D), in the cytoplasmic fraction on d-1 and d-7. Blots (A) represent Bax and Bcl-2 in cortical region. The results in the histogram are expressed as the ratio of the relative intensity of levels of protein expression of cytoplasmic (B) Bax, Bcl-2 (C) and their ratio Bax/Bcl-2 (D), 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 groups respectively [Two-way ANOVA followed by Bonferroni post-test].

6.4.8 Effect of multiple doses of tempol on alterations in expression of mitochondrial Bax, Bcl-2, and their ratio 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.6.5B), among groups [$F(4, 20) = 14.39$; $P<0.05$], time [$F(1, 20) = 61.96$; $P<0.05$] and a significant interaction between group and time [$F(4, 20) = 6.85$; $P<0.05$]. Similarly there was a significant change in levels of expression of Bcl-2 (Fig.6.5C) among groups [$F(4, 20) = 105.7$; $P<0.05$ and]. However, no significant main effect for time [$F(1, 20) = 2.1$; $P>0.05$] and no significant interaction between group and time [$F(4, 20) = 0.78$; $P>0.05$] were observed. Further there was a significant alteration in their ratio (Bax/Bcl-2; Fig.6.5D) among groups [$F(4, 20) = 12.43$; $P<0.05$],time [$F(1, 20) = 24.71$; $P<0.05$], and a significant interaction between group and time [$F(4,20) = 4.52$; $P<0.05$ respectively] post anoxia. Post hoc analysis revealed that tempol in multiple doses decreased the expression of mitochondrial Bax and inhibited insult progression but failed to increase the expression of Bcl-2 on d-7. Overall Bax/Bcl-2 ratio was decreased on d-7 following tempol treatment.

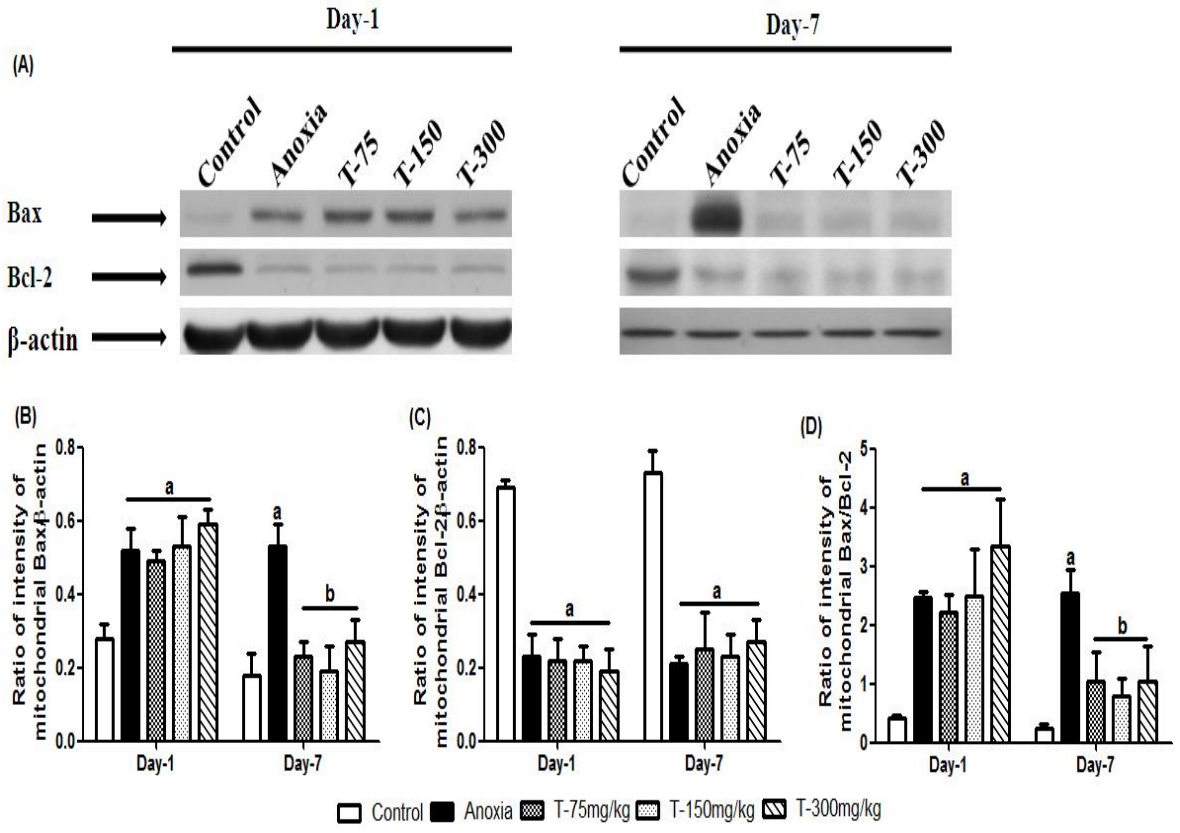

Figure 6.5 The effect of tempol (75, 150 and 300 mg/kg) on anoxia-induced changes in the levels of mitochondrial Bax (B), Bcl-2 (C) and their ratio of Bax/Bcl-2 (D), in the mitochondrial fraction on d-1 and d-7. Blot (A) represents Bax, Bcl-2 and their ratio (Bax/Bcl-2) in cortical region. The results 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 and [b]P<0.05 compared to anoxia groups respectively [Two-way ANOVA followed by Bonferroni post-test].

6.4.9 Effect of multiple doses of tempol on changes in reflex latency and hanging latency on d-1 and d-7 after anoxia.

Fig. 6.6 depicts the sensorimotor activities like a reflex and hanging latency on d-1 and d-7 after anoxia. A repeated measure two-way ANOVA depicted a significant increase in reflex latency (Fig. 6.6A) among groups [F (4, 40) = 19.90; P<0.05], time [F (1, 40) = 483.4; P<0.05] and a significant interaction between group ant time [F (4, 40) = 11.52; P<0.05] post-anoxia. Tempol in all the three doses significantly attenuated reflex latency compared

to anoxia group on d-7. Post hoc analysis showed that there was a significant increase in reflex latency following anoxia compared to control group. However, tempol in all the three doses reduced the reflex latency as compared to anoxia group on d-7 compared to d-1. Similarly a significant decrease in hanging latency (Fig.6.6B**)** was observed among groups [F (4, 40) = 6.570; P<0.05], time [F (1, 40) = 230.6; P<0.05] and a significant interaction between group and time [F (4, 40) = 5.391; P<0.05]. Tempol (75, 150 and 300 mg/kg) significantly (P<0.05) attenuated hanging latency on d-7 as compared to d-1 following anoxic injury. Post hoc analysis showed that there was a marked difference between the sensorimotor activity of control and anoxic group.

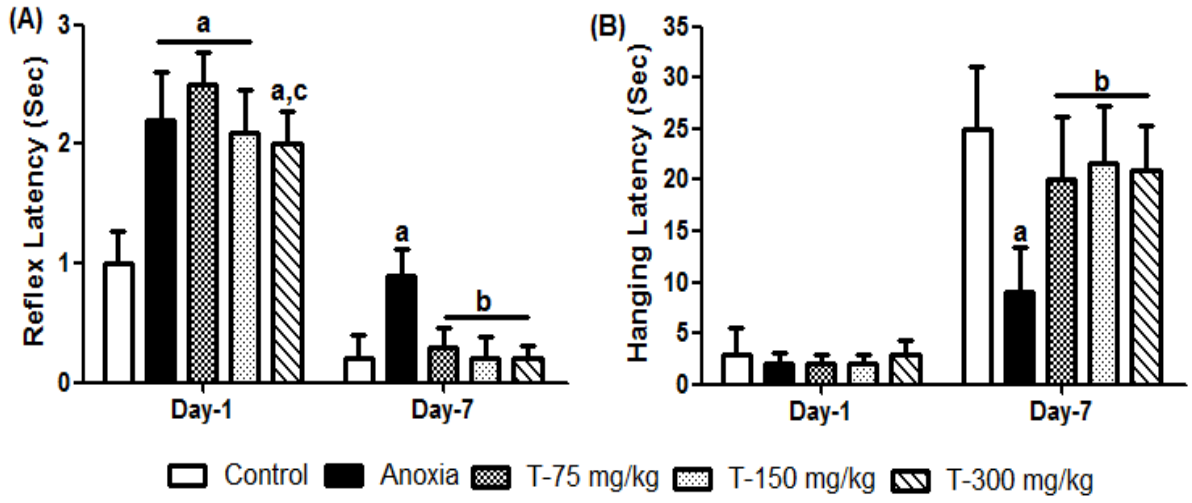

Figure 6.6 Effect of tempol (75, 150 and 300 mg/kg) on anoxia-induced sensorimotor dysfunction [reflex latency (A) and hanging latency (B) on d-1 and d-7. Bars represents group mean ± SD (n=5 in each group). ^{a}P< 0.05 compared to control, ^{b}P<0.05 compared to anoxia and ^{c}P<0.05 compared to tempol 75 mg/kg respectively [Repeated measure two-way ANOVA followed by Bonferroni post-test].

6.5 Discussion

The present study reports the pharmacological effect of antioxidant tempol in a global model of neonatal anoxia. We for the first time report that tempol 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).

During a pathological insult like anoxia, there is an immense generation of ROS/RNS which leads to a secondary insult by further promoting mitochondrial dysfunction and associated neuronal cell death [144]. Anoxia caused marked pathological alterations in different states of mitochondrial respiration (s-3, s-4, RCR, s-5 via complex-I and s-5 via complex-II). Tempol in all the three doses attenuated anoxia-induced changes in different states of respiration on d-7 by improving the formation of ATP in s-3, preserving the mitochondrial inner membrane integrity as confirmed by the decrease in respiration after addition of oligomycin (s-4). Oligomycin serves as a metabolic poison which binds and inhibits complex-V and prevents further ADP utilization. By locking the mitochondrial ATP production in s-4, the extent of proton leak can be determined. In healthy mitochondria, protons are allowed to pass via ATPase pump which provides a protonmotive force that converts ADP to ATP. Further tempol significantly improved the s-5 via complex-I respiration in the presence of an uncoupler FCCP which acts as an ionophore and allows the proton to pass other than ATPase pump by proton leak through the inner mitochondrial membrane while the complex-V is blocked by oligomycin. Furthermore, tempol markedly attenuated s-5 complex-II respiration which was fueled by the addition of succinate. This may be because tempol could maintain $FADH_2$ dehydrogenase activity through free radical scavenging mechanism. Overall tempol preserved the mitochondrial bioenergetics by

preserving different states of mitochondrial respiration possibly through its free radical scavenging property and inhibited the progression to secondary insult (d-7). There are no reports on these changes in any other model of anoxia. Excessive ROS formation in the mitochondria is one of the very early symptoms that precedes the collapse of MMP, the release of pro-apoptotic factors and activation of caspases [137]. Nitric oxide functions as an intercellular messenger in physiological conditions in neuronal tissues. However, the excessive production of the same molecule can become highly damaging to the same neurons within a few couple of minutes during pathological insult like cerebral ischemia [145], which is a stage soon after primary insult after anoxia. Our results depicted a robust increase in mitochondrial •NO level up to d-7 after anoxia. In addition, a previous finding has shown that upregulation of •NO can cause nitrosative stress and can cause mitochondrial dysfunction [60], which may further lead to neurodegeneration [146]. Complex-I is one of the main sources of mitochondrial ROS like superoxide ($O_2^{\bullet -}$), and inhibition of complex-I by peroxynitrite stimulates this ROS which in turn can further lead to mitochondrial dysfunction and neurodegeneration [28, 70]. We in the present study report that tempol in the dose 150 and 300 mg/kg significantly inhibited the cortical mitochondrial •NO levels during primary insult (d-1) and in all the three doses to stop progression in secondary insult (d-7), depicting a reduction in nitrosative stress. superoxide may be converted to hydrogen peroxide (H2O2) by tempol which may act as a SOD mimetic agent within the mitochondria or react with •NO to generate peroxynitrite (ONOO-) [147]. Tempol also improved the levels of anoxia-induced decrease in antioxidant enzymes like SOD and CAT on d-7. Previous findings have described tempol to functions as a SOD and CAT mimetic agent [148, 149]. Further, tempol only in the dose 300mg/kg effectively attenuated the levels of

LPO on d-7. Further, we report that tempol significantly attenuated anoxia associated decrease in mitochondrial enzyme system (I, II, IV, and V) and caused the improvement in mitochondrial function. Further, this disturbance in molecular machinery of mitochondria following anoxia further activates the MPT pore in the inner mitochondrial membrane that is freely permeable to small molecules of less than 1500 Dalton, thereby causing swelling of the mitochondria and depletion of cellular ATP [28, 140]. Anoxia-induced pathological alterations in MMP and MPT were markedly overcome by treatment with tempol on d-7 which depicts the role of tempol in maintaining mitochondrial integrity after secondary insult. Studies have shown that hypoxic ischemia in newborns results in activation of pro- and anti-apoptotic proteins and translocation of cytosolic Bcl-2 associated protein (Bax) to mitochondria [33, 123]. As per our knowledge, we are the first to report the effect of tempol on the expression of upstream Bcl-2 proteins and downstream caspases in a global anoxia model of neonates. Interestingly tempol significantly inhibited the anoxia-induced mitochondrial Bax translocation on d-7. However, tempol was ineffective in increasing expression of mitochondrial Bcl-2 on d-7. However, there was an effective decrease in the ratio Bax/Bcl-2 on d-7 in the mitochondrial fraction. Further, tempol effectively increased the expression of cytosolic Bax on d-7, but not cytosolic Bcl-2 on d-7 leading to increased expression of their ratio (Bax/Bcl-2) on day-7. These results denote that overall tempol was effective in maintaining mitochondrial outer membrane integrity through inhibiting the translocation of pro-apoptotic Bax from the cytoplasm to mitochondria and not through anti-apoptotic Bcl-2 following the secondary insult. This is the first such report on in any model of anoxia/hypoxia. The previous finding has shown the expression of these proteins (Bax, Bcl-2 and their ratio) in cytoplasmic as well as a mitochondrial fraction in cortical portion of

neonatal rat brain after anoxia [120]. The translocation of Bax to the mitochondrial outer membrane from cytosol induces mitochondrial outer membrane permeabilization (MOMP) which along with inner membrane lead to the formation of mPTP and causes the leakage of apoptogenic factors such as cytochrome-C through the mitochondrial intermembrane space proteins [36, 123, 150]. It was interesting to note that tempol markedly inhibited the release of cytochrome-C in the cytoplasm and therefore inhibited the progression of insult after anoxia. Cytochrome-C along with caspase-9 engages the apoptotic protease activating factor-1 (APAF1) and forms the apoptosome which leads to the activation of Caspase-3 [48, 49]. Tempol rescued the anoxia-induced mitochondrial-linked apoptosis by inhibiting the expression of both caspase-9 and caspase-3 on d-7.

There was a decrease in hanging latency and increased reflex latency from d-1 to d-7 after the second anoxic episode which indicated an early sign of neurological dysfunction and hindered sensorimotor performance in the early postnatal days as reported earlier [120]. Tempol in all the three doses increased the sensorimotor performance like increased hanging and decreased reflex latency to d-7 (P10) rats, an age when brain development is comparable to the late preterm human infant [16], which indicates that the drug effectively improved the neurobehavioral outcome of developing neonates.

In summary, the effects of tempol (75 mg/kg, 150 mg/kg and 300 mg/kg) on mitochondrial bioenergetics are accompanied by restoring mitochondrial function, complex enzyme activity, MMP and MPT. It probably acts by mimicking antioxidant activity and inhibiting nitric oxide formation which is a major component in peroxynitrite synthesis. Tempol significantly downregulated the expression of pro-apoptotic mitochondrial, Bax which could lead to mitochondrial outer membrane permeabilization (MOMP). It prevented the release of

cytochrome-C and inhibited the expression of caspase-9 and caspase-3 and thereby the progression of apoptosis. Therefore, the present study indicates the preclinical potential tempol for the treatment of anoxia.