7.1 Abstract

Neonatal anoxia is a global neuropathological condition originated due to the severe deprivation of oxygen in the developing brain during the time of birth. We have recently reported the progressive loss of mitochondrial function from d-1 to d-7 after anoxia. Interestingly, we have recently reported that treatment with 2,4-dinitrophenol (2,4-DNP) or tempol preserved mitochndrial function and linked cell death by rescuing the insult progression on d-7. Moreover, the present study is aimed to investigated the combination effects of 2,4-DNP and Tempol (D+T) in targeting two distinct mitochondrial fractions for any synergistic effect after anoxia. This study is the first to assess both synaptosomal (neuronal)- and non-synaptosomal (glial and neuronal soma)-derived mitochondria d-7 after the second anoxic episode in rat pups. Studies have shown that mitochondrial Ca^{2+} overload and ROS are two major determinants in mitochondrial functioning and the two populations differ in their Ca^{2+} handling capacity. We observed no significant ($p<0.05$) differences in both mitochondrial populations in terms of various biochemical (s-3,s-4,RCR, LPO, NO, SOD,CAT, Ca^{2+}, MPT) and molecular (cytochrome-C, mito-Bax,Bcl-2, cyto-Bax,Bcl-2, caspase-9/3) parameters. Further, we observed no synergistic effect in combination D+T as compared to their individual effects.

Keywords: Ca^{2+} overload, Synaptic mitochondria, Non-synaptic mitochondria, Cytochrome-C, caspase-9/3, Bax, Bcl-2.

7.2 Introduction

Neonatal anoxia is a common obstetric complication resulting due to lack of oxygen supply to the fetus or newborn to a small period at the time of birth [127]. Anoxia results in a battery of primary (deprivation of oxygen and glucose, the release of excitatory neurotransmitters) and subsequent events (oxidative stress, mitochondrial Ca^{2+} overloads, and mitochondrial dysfunction linked apoptosis) in developing brain tissue [28, 120]. The estimated incidence is 1/1000 live births, being five- to ten-fold higher in less developed countries [151]. Targeting mitochondrial dysfunction is known to be a prime goal for neuroprotective strategies following anoxia [33]. In our previous findings we have shown compromised cortical mitochondrial bioenergetics (in the crude fraction) in a time-dependent manner after second anoxia episode using a global model of anoxia, and further, a mitochondrial dysfunction linked insult progression and associated neurobehavioral impairments [76, 120]. Soon after anoxia, there is an existence of excitotoxic insult, which in turn increases Ca^{2+} overload in the mitochondria and increase reactive oxygen species (ROS) production, inhibit adenosine triphosphate (ATP) synthesis, and induce mitochondrial permeability transition. Previous reports have shown that by reducing mitochondrial membrane potential (MMP), their uptake of Ca^{2+} can be inhibited [138, 142]. Moreover, it has been reported that a substantial mitochondrial heterogeneity exists among organs and within the CNS. Mitochondria in the non-synaptic fraction originate from neurons and other cell types including glia, whereas mitochondria-enriched from a synaptosomal fraction are predominantly neuronal and presynaptic in origin [45]. Within the CNS, there are regional differences in mitochondrial populations about Ca^{2+}-induced MPT threshold and reactive oxygen species (ROS) production. However, there are no reports in neonates which can

evaluate the variations in Ca^{2+} handling capacity and permeability pore opening in different mitochondrial fractions and also the treatment strategies which are selective to a particular population of mitochondria in CNS to improve their function after anoxia. Therefore, pharmacological compounds that maintain mitochondrial function through reducing Ca^{2+} overloads and oxidative stress may prove beneficial in improving long-term neurobehavioral function after anoxia. Anoxia-induced mitochondrial dysfunction has been implemented as a prime cause of acute sensorimotor deficits [76, 120]. However, using different models, previous studies has considered anoxia to be a prime cause of long-term neurodevelopmental delays [65, 152-154]. Moreover, a continuous development of hypothalamic–pituitary–adrenal (HPA) axis is crucial for the proper function of the developing brain. Unluckily, the perinatal events alter the subsequent phenotypical response to stress by changing the set-point of the HPA axis [155, 156]. As a result, an alteration in corticosterone levels takes place [157]. Formerly, in our recent study, it has been demonstrated that treatment with uncoupler 2,4 DNP or antioxidant tempol within 5 minutes of second anoxic episode causes improvement in mitochondrial function as well as in sensorimotor performance in neonates.

The purpose of the present study was to compare the ability of isolated synaptic versus nonsynaptic cortical brain mitochondria regarding Ca^{2+} handling and other biochemical and biomolecular studies on rat pups (P10) after anoxia. Therefore the present study is based on the individual as well as combined effect of 2,4 DNP and tempol for any synergistic effect in the two mitochondrial populations in terms of mitochondrial respiration, (s-3,s-4 and RCR), oxidative stress, antioxidant enzyme, maintaining Ca^{2+} homeostasis, pro-apoptotic Bcl-2 family proteins and apoptotic proteins (cytochrome-C, caspase-9/3). Further

we observed the effect of combination in long term (d-21 up to d-150) neurobehavioral outcome like changes in spontaneous locomotor activity as a measure of hyperactivity, spatial recognition memory impairments as a measure of cognitive impairments and further anxiety and depression-like behavior and further plasma corticosterone on d-150 after anoxia.

7.3 Materials and methods

7.3.1 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.

7.3.2 Anoxia model

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

7.3.3 Drug preparation and dosing

The minimal effective dose of 2,4 DNP (2.5 mg/kg) and tempol (75 mg/kg) determined from the previous studies. 2, 4 DNP (Himedia) and tempol was freshly prepared in DMSO before intraperitoneal injection (i.p.). The rats were randomly divided into 6 groups (n=5 per group): (1) Control; (2) Anoxia; (3) Anoxia + DNP 2.5 mg/kg; (4) Anoxia + Tempol 75 mg/kg and (5) Anoxia + DNP + Tempol. The drugs were administered within 5 min of second anoxia episode as previously described [158].

7.3.4 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.

7.3.5 Synaptic and non-synaptic mitochondria isolation

Mitochondrial isolation was performed as described previously [68] with some slight modifications [76]. Rat cortex was placed in an all glass dounce homogenizer with 4 mL of isolation buffer with EGTA (consisting of 215 mM mannitol, 75 mM sucrose, 0.1% w/v bovine serum albumin, 20 mM HEPES buffer and 1 mM of EGTA in 100 ml of distilled water and pH adjusted to 7.2 with KOH) and first centrifuged at 1300 × g for 5 min at 4°C. Each supernatant was then topped off with isolation buffer with EGTA and centrifuged at 14,000 × g for 10 min at 4°C to get a tighter mitochondrial pellet. The crude mitochondrial fraction so obtained was resuspended in isolation buffer with EGTA and placed on a discontinuous Ficoll gradient (F5415 Ficoll solution Type 400, 20% in H2O, Sigma, St. Louis, MO), composed of two layers (2 mL of a 7.5% on top of 2 mL of 10% Ficoll cut with isolation buffer with EGTA) and centrifuged at 100,000 × g for 30 min. The gradient produced two separate mitochondrial fractions: the extrasynaptic mitochondrial pellet (bottom) and synaptic mitochondria trapped in synaptosomes located at the interphase of the two Ficoll layers. Synaptosomes were cosiously collected in 2.5 mL tubes, resuspended, and washed in isolation buffer with EGTA by centrifugation at 14,000 × g for 10 min to remove

Ficoll from sample and were subjected to nitrogen decompression in a cell disruption bomb made by Parr Instrument Company (Moline, IL) to release mitochondria and cooled to 4°C under a pressure of 1200 psi for 10 min.The extrasynaptic mitochondrial pellet was collected in 500 µL tubes and also resuspended and washed using isolation buffer with EGTA. The disrupted synaptosomes were placed on a new Ficoll gradient and returned to the ultracentrifuge and spun at 100,000 × g for 30 min at 4 °C. Both mitochondrial fractions (synaptic and extrasynaptic) were resuspended with isolation buffer without EGTA and centrifuged at 14,000 × g for 10 min to wash out the calcium chelator (EGTA). The final mitochondrial pellets were resuspended in isolation buffer without EGTA to yield a concentration of 10 mg/mL or higher. Mitochondrial protein was estimated colorimetrically [67] with a microplate reader (Biotek, USA).

7.3.6 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.

7.3.7 Mitochondrial calcium measurements

Mitochondrial Ca^{2+} cycling/overloading in situ was measured as per [142]. Mitochondria were prepared as described above with the exception that the isolation buffer was Ca^{2+}-free and contained (i) 0.6 µm ruthenium red to block the mitochondrial inward transport of Ca^{2+} via the uniporter and extrusion via the sodium-independent anti-porter, (ii) 10 µm CGP-37157 to block outward flux of Ca^{2+} via the sodium-dependent antiporter, and (iii) 5 µm Cyclosporin A to prevent loss of Ca^{2+} via induction of the permeability transition. Additionally, the isolation buffer did include calcium chelators. Channel blockers were

made up in at least 100 × stock solutions in DMSO. Mitochondria (50 µg) were placed in the wells of 96-well plates to which was added 5 µm Calcium Green 5-N in a final volume of 100 µL 'locking buffer'. Calcium levels before and following solubilization with 100 µm Triton X-100 for 15 min were quantified by measuring the fluorescence using a BIO-teck Synergy HT fluorometer with excitation wavelength 485 nm and emission wavelength 532 nm. A standard Ca^{2+} curve was generated (range of 100 nM to 100 µM) to extrapolate our fluorescence values to concentrations molar $[Ca^{2+}]$/mg mitochondrial protein. All assays and standards were performed using identical total volumes.Mitochondrial protein was estimated colorimetrically [67] with a microplate reader (Biotek, USA).Values are presented as fluorescent units.

7.3.8 Mitochondrial permeability transition (MPT)

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

7.3.9 Mitochondrial oxidative stress

7.3.9.1 Estimation of LPO and NO levels

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

7.3.9.2 Estimation of mitochondrial SOD and CAT activity

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

7.3.10 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 previous [120]. Detailed in chapter 3. Page no.41.

7.3.11 Behavioral Studies

7.3.11.1 Open Field Test (OFT)

The OFT was used to investigate the changes in spontaneous locomotor activity (ambulation), time spent in central grid and rearing behavior. The experimental device consists of square open-field (55 × 44 × 50 cm). The floor of the apparatus was painted dark grey and divided into 12 fields [159]. Each rat was placed individually in the center of the open-field apparatus. The testing session lasted 5 min. The apparatus was cleaned with ethanol after each test.

7.3.11.2 Elevated Plus Maze (EPM)

To measure individual levels of anxiety-like behavior, animals were placed on an EPM as defined previously [160]. The apparatus consists of two opened arms (30 × 5 × 0.25 cm) and two closed arms (30 × 5 × 15 cm) emanating from a common central platform (5 × 5 cm). The entire apparatus was elevated to a height of 40 cm above floor level in a dimly (red light) illuminated room. At the beginning of the session each mouse was placed at the center of the maze, its head facing a closed arm and allowed to explore the maze freely for a 5-min period. An entry was defined as entering into one arm with all four feet. Time spent on open

arms versus enclosed arms is considered to be a metric of anxiety. To calculate a single value that integrates these anxiety-related behaviors, an "anxiety index" value was calculated as follows: {1 − [(open arm time/total time) + (open arm entries/total exploration)]/2[161]. The plus-maze was carefully cleaned with ethanol after each animal test. All training and testing sessions were carried out during the light phase between 08:00 and 14:00 h.

7.3.11.3 Y-Maze test

The spatial recognition memory of animals was investigated using a Y-maze paradigm [162]. The apparatus consisted of three arms (50 × 10 × 20 cm^3 and 120° apart) made of black plastic, placed in a room with visual cues on the walls. Y-maze testing consisted of two trials separated by an interval of 1 h. In the first trial, the animal was placed at the end of one arm and allowed access to that arm and another arm for 5 min. The third arm (the novel arm) was blocked by a guillotine door. The rat was then removed from the maze and returned to its home cage. For the second trial, the rat was placed back into the start arm of the maze and given free access to all three arms for 5 min. The number of entries and the time spent in each arm were recorded. The percentage of entries and time spent in the novel arm was compared to random exploration of the three arms of the maze (i.e., 33%).

7.3.11.4 Forced Swim Test (FST)

Forced swimming testing was divided into two sessions, a pretest session (15min) and a test session (5min), which was administered 24 h later. During testing, each animal was individually placed in a clear Plexiglas cylinder (40 cm height, 18 cm diameter) filled with water (25 °C). Normally, during pretest session naïve rodents exhibit a struggling behavior

in order to escape. However, because of the inescapable nature of the tank, a naïve animal gradually adopts a passive 'despair' behavior (the so-called 'learned helplessness'), which is characterized by a reduction in vigorous activity and an increased occurrence of immobile posture that allows them to float by performing only the necessary movements. Since antidepressant agents reduce immobility time, this measurement is considered an index of depression-like behavior [163]. In order to assess animals' depressive-like behavior, we analysed the immobility time recorded during the test session.

7.3.12 Plasma Corticosterone

The plasma CORT was quantified by a High Performance Liquid Chromatography (HPLC) with Ultraviolet (UV) detector system (Waters, USA), as described earlier [164].

7.3.13 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 Bonferroni post hoc test was performed to measure sensorimotor behavioral activities. $P<0.05$ was considered as significant.All data were analyzed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

7.4 Results

7.4.1 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in synaptic mitochondrial s-III and s-IV respiration and RCR in cortical brain region on d-7

Fig. 7.1 shows the effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in synaptic mitochondrial (A) s-III and (B) s-IV respiration and (C) RCR in cortical brain region on d-7. An ANOVA depicted a significant main effect for s-III [$F(4, 20) = 7.129$; $P<0.05$ (Fig.7.1A)] and s-IV [$F(4, 20) = 9.232$; $P<0.05$ (Fig.7.1B)] respiration and RCR [$F(4, 20) = 8.607$; $P<0.05$ (Fig.7.1C)]. Post hoc analysis revealed that treatment with 2,4 DNP, tempol and their combination (D+T) was significantly effective in treating s-III, s-IV respiration and RCR compared to anoxia group animals. Further, there was no significant difference between the combination (D+T) and individual drug treatment (2,4 DNP or tempol).

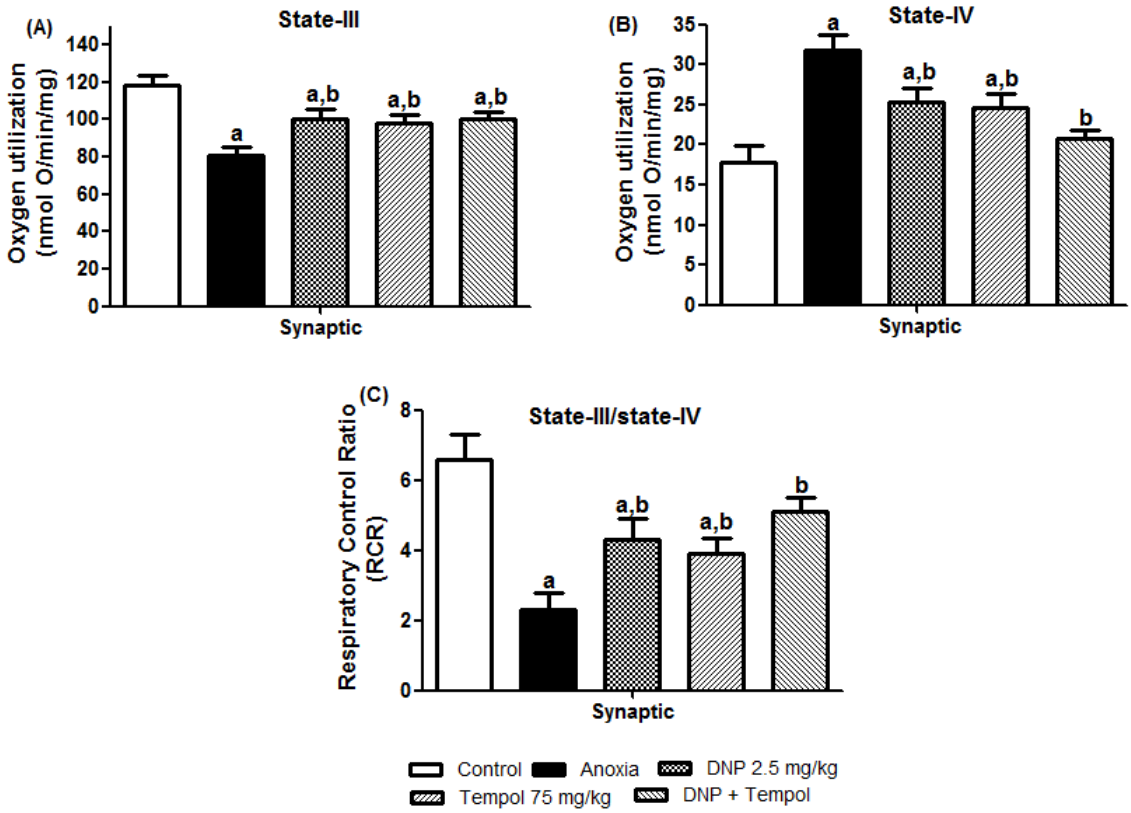

Figure 7.1 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in synaptic mitochondrial (A) s-III and (B) s-IV respiration and (C) RCR in cortical brain region on d-7. Bars represents group mean ± SD. n = 5/group. [a]P<0.05 compared to control and [b]P<0.05 compared to anoxia groups respectively [One-way ANOVA followed by Student–Newman–Keuls test].

7.4.2 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in synaptic mitochondrial NO, LPO, SOD and CAT levels in cortical brain region on d-7

Fig. 7.2 illustrates the effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in synaptic mitochondrial (A) Nitric oxide, (B) LPO, (C) SOD and (D) CAT in the cortical brain region. One way ANOVA revealed a significant difference in the levels of NO [F (4, 20) = 47.92; P<0.05], LPO [F (4, 20) = 18.12; P<0.05], SOD [F (4, 20) = 8.78; P<0.05] and CAT [F (4, 20) = 10.85; P<0.05] post-anoxia injury. Post hoc analysis showed that treatment with 2,4 DNP, tempol and their combination (D+T) was significantly

effective in attenuating NO, SOD and CAT levels compared to anoxia group animals. However, 2,4 DNP, and the combination (D+T) but not tempol reduce the LPO levels. Further, there was no significant difference between the combination (D+T) and individual drug treatment (2,4 DNP or tempol).

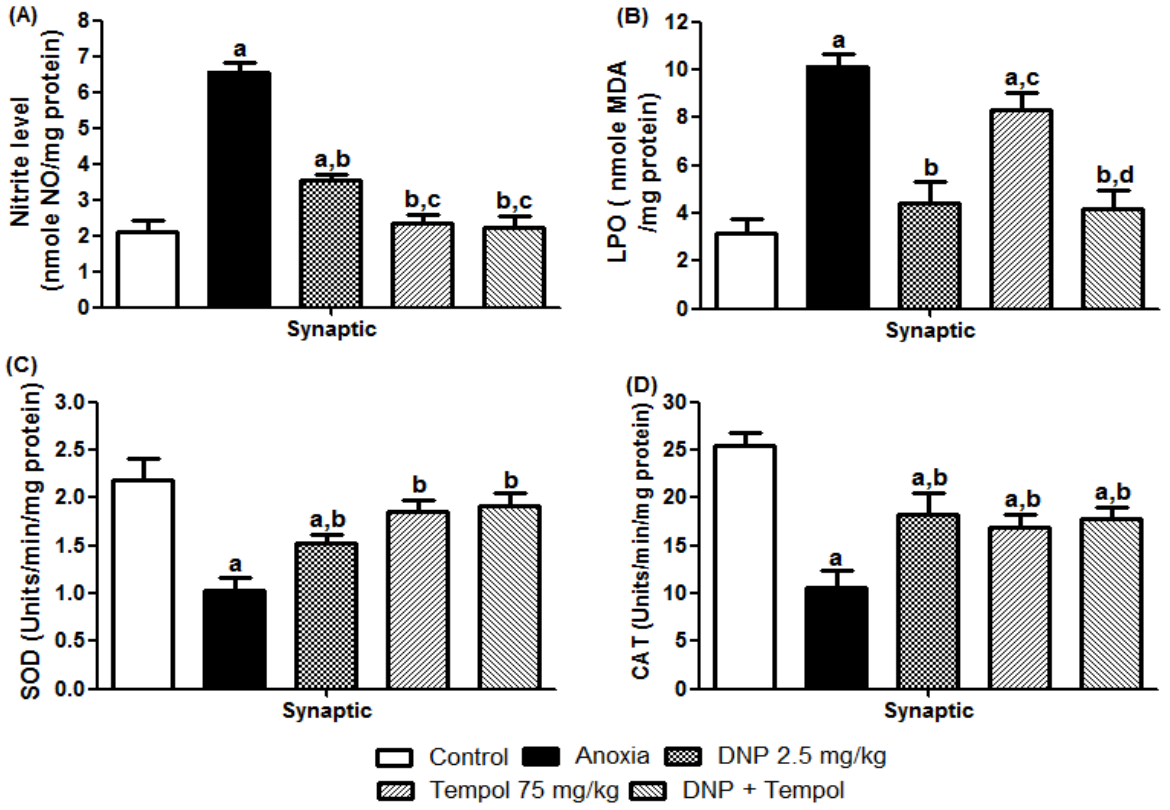

Figure 7.2 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia induced changes in synaptic mitochondrial (A) NO, (B) LPO, (C) SOD and (D) CAT levels in cortical brain region on d-7. Bars represents group mean ± SD. n = 5/group. [a]P<0.05 compared to control, [b]P<0.05 compared to anoxia, [c]P<0.05 compared to 2,4 DNP and [d]P<0.05 compared to tempol groups respectively [One-way ANOVA followed by Student–Newman–Keuls test].

7.4.3 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in synaptic mitochondrial Ca^{2+} and MPT in cortical brain region on d-7

Fig. 7.3 represent the effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in synaptic mitochondrial (A) calcium overload (B) MPT in cortical brain

region on day-7. An ANOVA revealed a significant difference in calcium overload [F (4, 20) = 16.98; P<0.05] and synaptic –mitochondrial MPT in terms of mitochondrial swelling [F (4, 20) = 7.34; P<0.05] post-anoxia injury. Post hoc analysis showed that treatment with 2,4 DNP and the combination (D+T) was significantly effective in decreasing synaptic-mitochondrial calcium overloads. However, tempol was not found effective in decreasing the same. Further, 2,4 DNP, tempol and the combination (D+T) was found effective in mitigating mitochondrial swelling compared to anoxia group animals. Furthermore, there was no significant difference between the combination (D+T) and individual drug treatment (2,4 DNP or tempol).

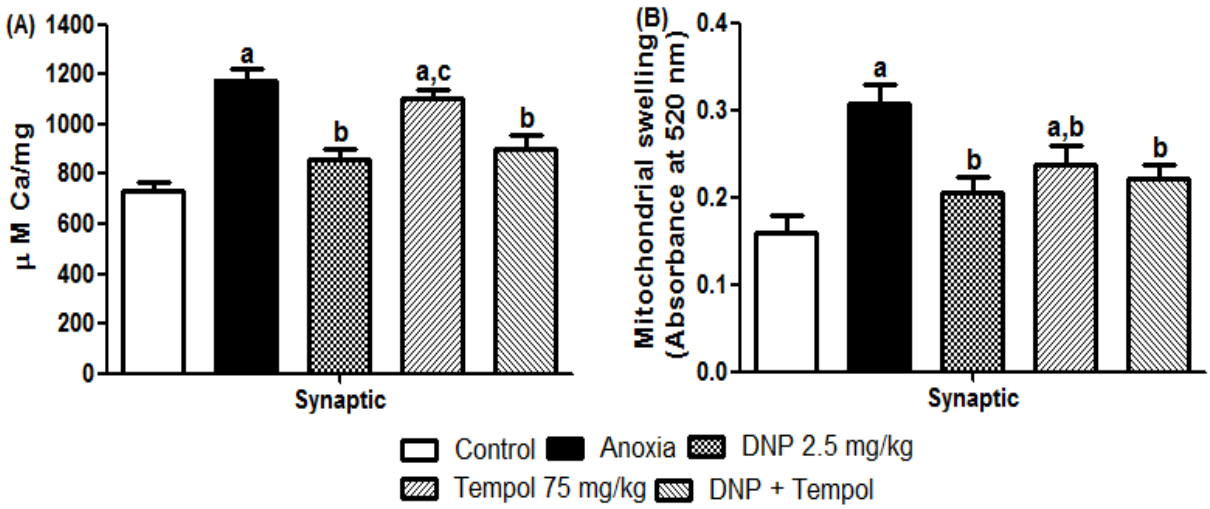

Figure 7.3 Effect of 2,4 DNP (2.5 mg/kg), tempol (75 mg/kg) and their combination (D+T) on anoxia-induced changes in synaptic mitochondrial (A) calcium and (B) MPT regarding mitochondrial swelling in cortical brain region on d-7. Bars represents group mean ± SD. n = 5/group. [a]P<0.05 compared to control, [b]P<0.05 compared to anoxia and [c]P<0.05 compared to DNP (2.5 mg/kg) groups respectively [One-way ANOVA followed by Student–Newman–Keuls test].

7.4.4 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in cytochrome-C, caspase-9 and caspase-3 in cortical brain region on d-7

Fig. 7.4 shows the effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in (B) cytochrome-C, (C) caspase-9 and (D) caspase-3 on day-7. One way ANOVA revealed a significant main effect for cytochrome-C [$F(4, 10) = 29.6$, $P<0.05$], caspase-9 [$F(4, 10) = 18.2$, $P<0.05$] and caspase-3 [$F(4, 10) = 18.2$, $P<0.05$] post-anoxia. Post hoc analysis showed that treatment with 2,4 DNP, tempol and the combination (D+T) was significantly effective in decreasing the levels of cytochrome-C, caspase-9 and caspase-3 compared to anoxia group animals. Furthermore, there was no significant difference between the combination (D+T) and individual drug treatment (2,4 DNP or tempol).

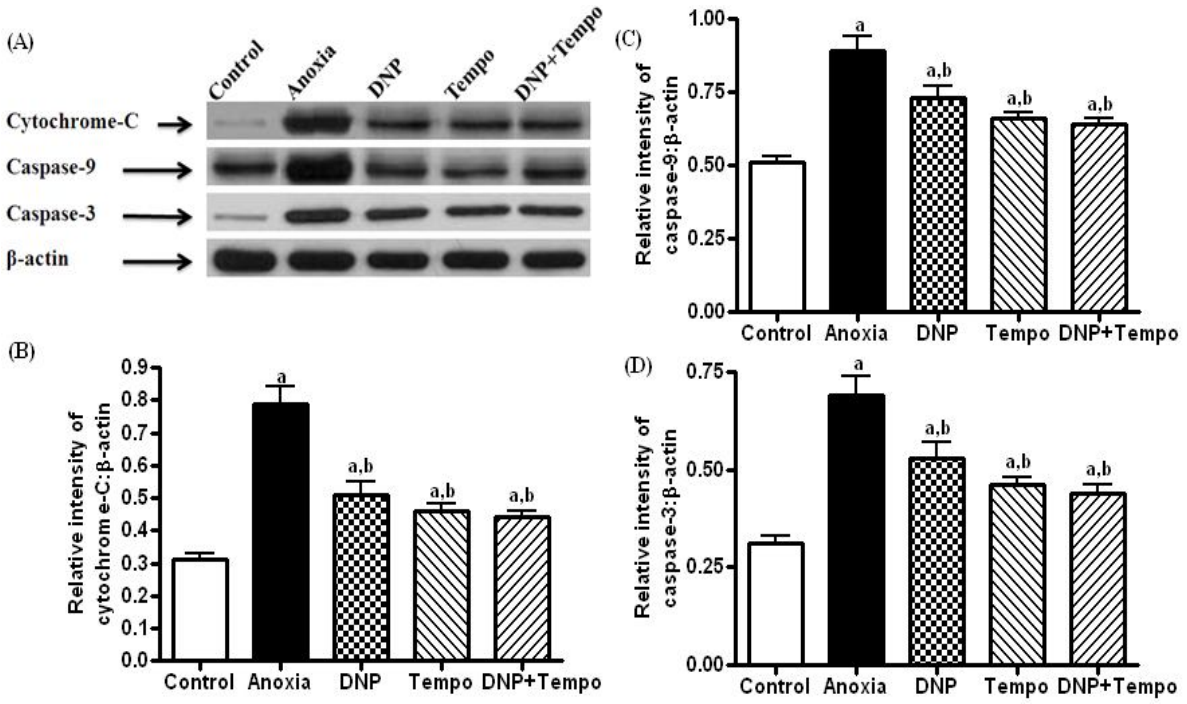

Figure 7.4 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in cytochrome-C (B), caspase-9 (C) and caspase-3 (D) on d-7. Blot (A) represents cytochrome-C, caspase-9 or caspase-3 in cortical tissue. The results in the histogram are expressed as the ratio of the relative 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 anoxia respectively [One-way ANOVA followed by Student Newman-Keuls test].

7.4.5 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in cytoplasmic Bax, Bcl-2 and their ratio (Bax/Bcl-2) in cortical brain region on d-7

Fig. 7.5 shows the effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in cytoplasmic (B) Bax (C) Bcl-2 and ratio (D) Bax/Bcl-2 in cortical brain region on d-7. Statistical analysis with one-way ANOVA showed a significant difference in cytoplasmic Bax [$F(4, 10) = 25.2$, $P<0.05$], Bcl-2 [$F(4, 10) = 10.9$, $P<0.05$] and their ratio (Bax/Bcl-2) [$F(4, 10) = 17.0$, $P<0.05$] post-anoxia. Post hoc analysis showed that treatment with 2,4 DNP and the combination (D+T) was significantly effective in attenuating the levels of cytoplasmic Bax and Bcl-2 whereas tempol was ineffective in decreasing Bcl-2 levels but was effective in attenuating Bax levels compared to anoxia

group animals. Furthermore, there was no significant difference between the combination (D+T) and individual drug treatment (2,4 DNP or tempol).

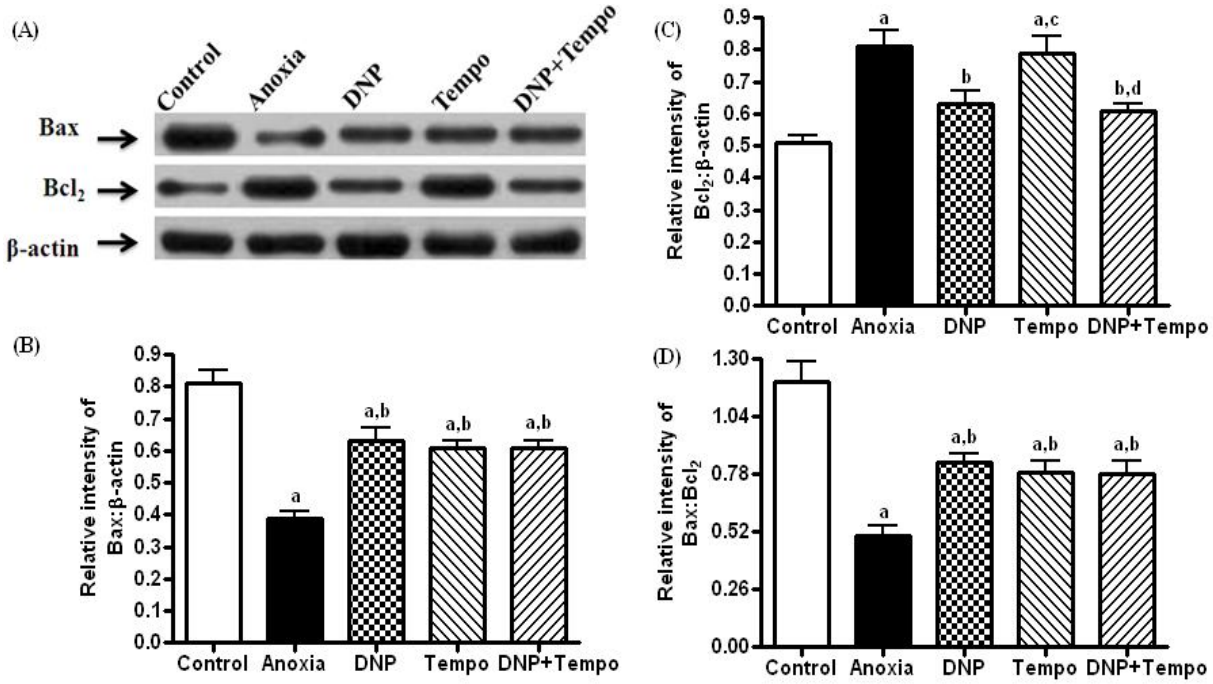

Figure 7.5 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in cytoplasmic (B) Bax, (C) Bcl-2 and (D) Bax/Bcl-2 on d-7. Blot (A) represents Bax, Bcl-2 in cortical brain tissue on d-7. The results in the histogram are expressed as the ratio of the relative intensity of levels of protein expression of cytoplasmic 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 anoxia, [c]P<0.05 compared to 2,4 DNP and [d]P<0.05 compared to tempol respectively [One-way ANOVA followed by Student Newman-Keuls test].

7.4.6 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in synaptic mitochondrial Bax, Bcl-2 and their ratio (Bax/Bcl-2) in cortical brain region on d-7

Fig. 7.6 shows the effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in synaptic-mitochondria (B) Bax (C) Bcl-2 and ratio (D) Bax/Bcl-2 in cortical brain region on d-7. Statistical analysis with one-way ANOVA showed a significant difference in Bax [F (4, 10) = 17.2, P<0.05], Bcl-2 [F (4, 10) = 13.4, P<0.05] and their ratio (Bax/Bcl-2) [F (4, 10) = 30.8, P<0.05] post-anoxia. Post hoc analysis showed that treatment with 2,4 DNP and the combination (D+T) was significantly effective in attenuating the

levels of synaptic-mitochondrial Bax and Bcl-2 whereas tempol was ineffective in improving Bcl-2 levels but was effective in attenuating Bax levels compared to anoxia group animals. Furthermore, there was no significant difference between the combination (D+T) and individual drug treatment (2,4 DNP or tempol).

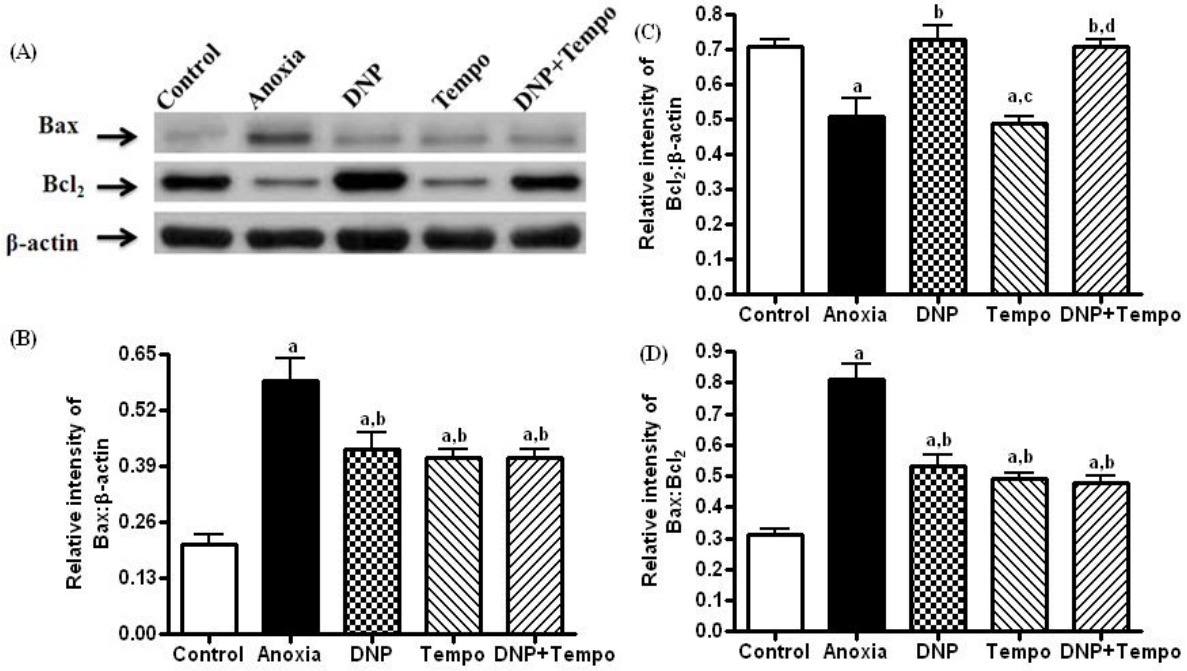

Figure 7.6 Effect of 2, 4 DNP, tempol and their combination (D+T) on anoxia-induced changes in synaptic mitochondrial (B) Bax, (C) Bcl-2 and (D) Bax/Bcl-2 on d-7. Blot (A) represents Bax, Bcl-2 in cortical region synaptic fraction. The results in the histogram are expressed as the ratio of the relative intensity of levels of protein expression of synaptic mitochondrial 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 anoxia, $^{c}P<0.05$ compared to DNP and $^{d}P<0.05$ compared to tempol respectively [One-way ANOVA followed by Student Newman-Keuls test].

7.4.7 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in non-synaptic mitochondrial s-III and s-IV respiration and RCR in cortical brain region on d-7

Fig. 7.7 shows the effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in non-synaptic mitochondrial (A) s-III and (B) s-IV respiration and (C) RCR in cortical brain region on d-7. An ANOVA depicted a significant main effect for s-III [$F(4, 20) = 6.67$; $P<0.05$] and s-IV [$F(4, 20) = 3.48$; $P<0.05$] respiration and RCR [$F(4, 20)$

= 8.49; P<0.05]. Post hoc analysis revealed that treatment with 2,4 DNP, tempol and their combination (D+T) was significantly effective in treating s-III, s-IV respiration, and RCR compared to anoxia group animals. Further, there was no significant difference between the combination (D+T) and individual drug treatment (2,4 DNP or tempol).

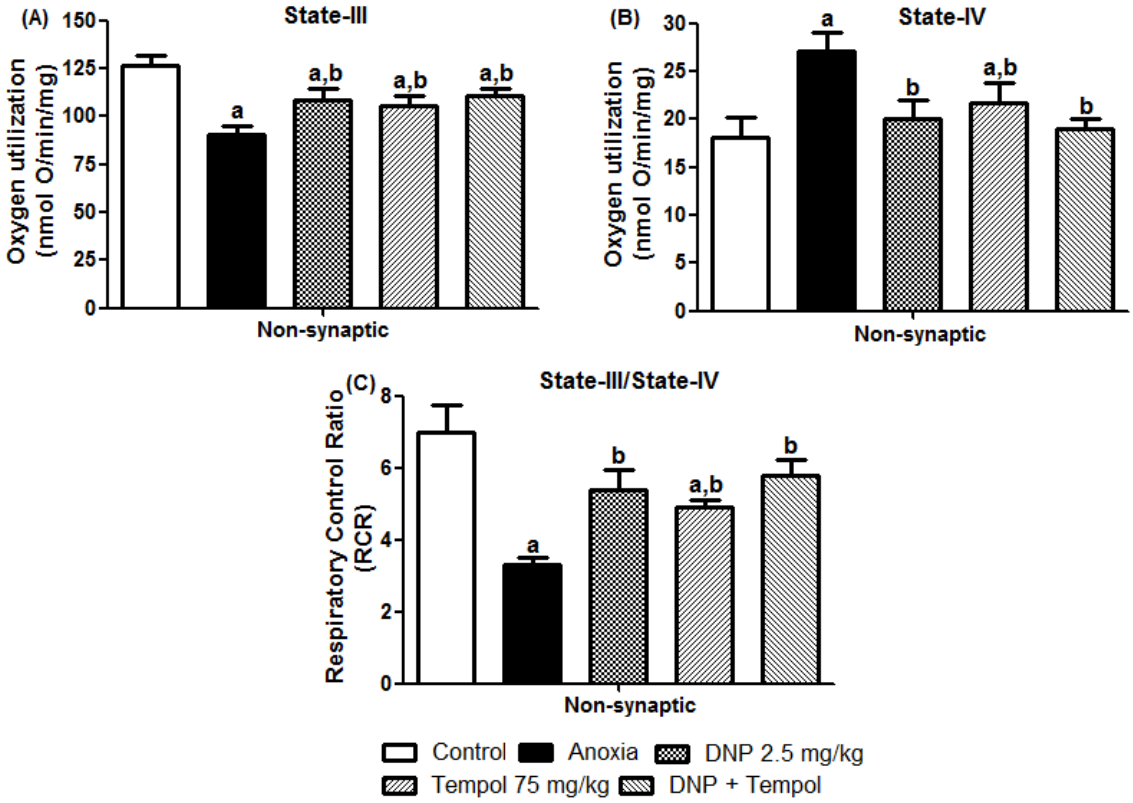

Figure 7.7 Effect of DNP, Tempol and their combination (D+T) on anoxia-induced changes in non-synaptic mitochondrial (A) s-III and (B) s-IV respiration and (C) RCR in cortical brain region on day-7. Bars represents group mean ± SD. n = 5/group. [a]P<0.05 compared to control and [b]P<0.05 compared to anoxia groups respectively [One-way ANOVA followed by Student–Newman–Keuls test].

7.4.8 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in non-synaptic mitochondrial Nitric oxide, LPO, SOD, and CAT in cortical brain region on d-7

Fig. 7.8 depicts the effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in non-synaptic mitochondrial (A) NO, (B) LPO, (C) SOD and (D) CAT in the cortical brain region. One way ANOVA revealed a significant difference in the levels of One-way ANOVA revealed a significant decrease in the levels of NO [$F(4, 20) = 28.10$; $P<0.05$], LPO [$F(4, 20) = 11.20$; $P<0.05$], SOD [$F(4, 20) = 7.32$; $P<0.05$] and CAT [$F(4, 20) = 16.75$; $P<0.05$] following anoxia. Post hoc analysis showed that treatment with 2,4 DNP, tempol and their combination (D+T) was significantly effective in attenuating NO, SOD and CAT levels compared to anoxia group animals. However, 2,4 DNP, and the combination (D+T) but not tempol reduce the LPO levels. Further, there was no significant difference between the combination (D+T) and individual drug treatment (2,4 DNP or

tempol).

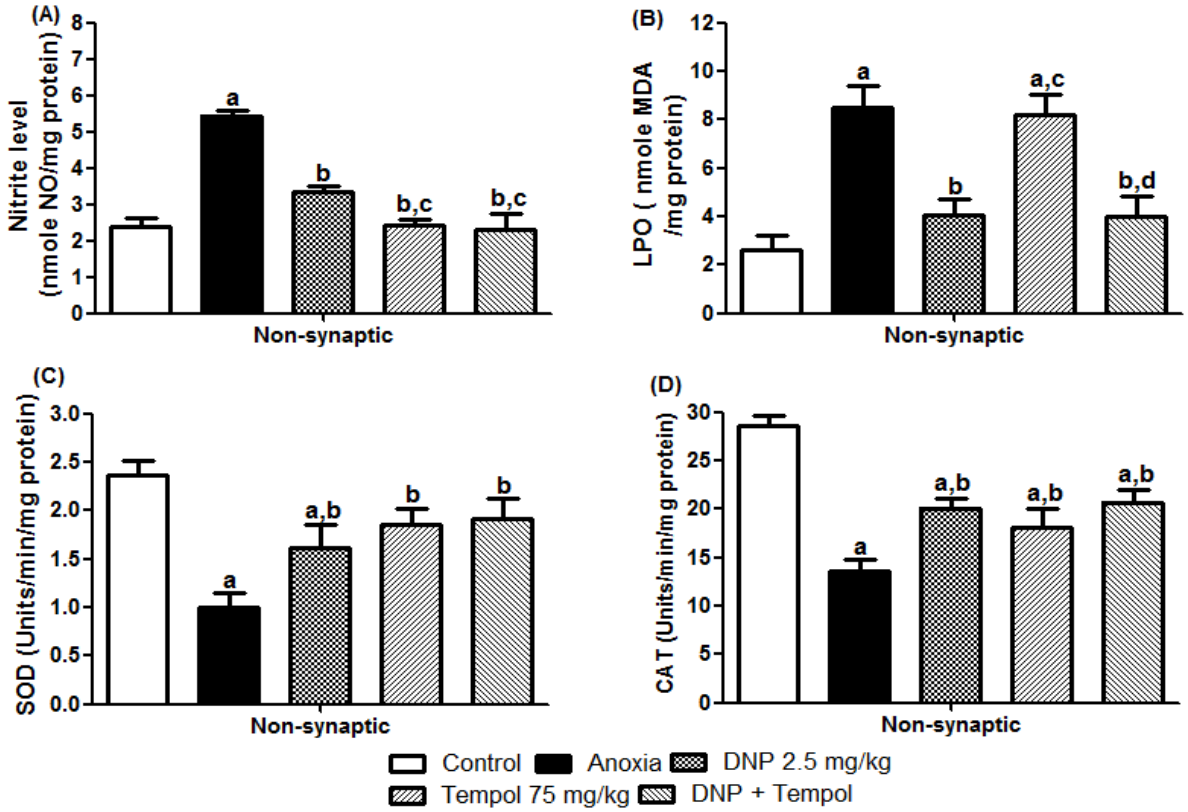

Figure 7.8 Effect of 2,4 DNP, Tempol and their combination (D + T) on anoxia-induced changes in non-synaptic mitochondrial (A) NO, (B) LPO, (C) SOD and (D) CAT levels in cortical brain region on day-7. Bars represents group mean ± SD. n = 5/group. [a]P<0.05 compared to control, [b]P<0.05 compared to anoxia, [c]P<0.05 compared to 2,4 DNP and [d]P<0.05 compared to Tempol groups respectively [One-way ANOVA followed by Student–Newman–Keuls test].

7.4.9 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in non-synaptic mitochondrial calcium and MPT in cortical brain region on d-7

Fig.7.9 represent the effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in synaptic mitochondrial (A) calcium overload (B) MPT in cortical brain region on day-7. An ANOVA revealed a significant anoxia-induced increase in non-synaptic mitochondrial calcium overload [F (4, 20) = 10.98; P<0.05] and increase in non-synaptic

mitochondrial swelling [F (4, 20) = 7.79; P<0.05] post-anoxia injury. Post hoc analysis showed that treatment with 2,4 DNP and the combination (D+T) was significantly effective in decreasing synaptic-mitochondrial calcium overloads. However, tempol was not found effective in decreasing the same. Further, 2,4 DNP, tempol and the combination (D+T) was found effective in mitigating mitochondrial swelling compared to anoxia group animals. Furthermore, there was no significant difference between the combination (D+T) and individual drug treatment (2,4 DNP or tempol).

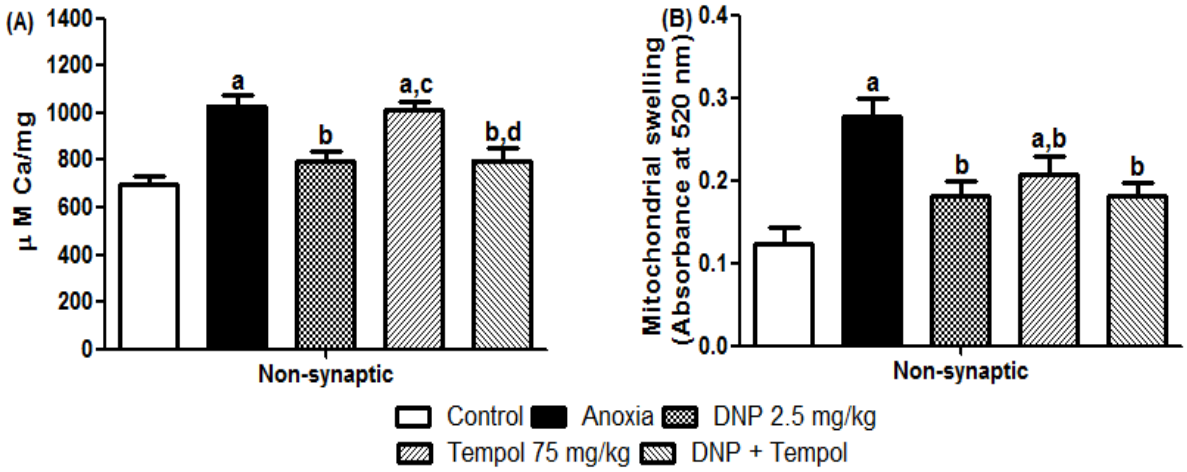

Figure 7.9 Effect of 2,4 DNP, Tempol and their combination (D + T) on anoxia-induced changes in non-synaptic mitochondrial (A) calcium and (B) MPT regarding mitochondrial swelling in cortical brain region on day-7. Bars represents group mean ± SD. n = 5/group. [a]P<0.05 compared to control, [b]P<0.05 compared to anoxia, [c]P<0.05 compared to 2,4 DNP and [d]P<0.05 compared to tempol groups respectively [One-way ANOVA followed by Student–Newman–Keuls test].

7.4.10 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in non-synaptic mitochondrial Bax, Bcl-2 and their ratio (Bax/Bcl-2) in cortical brain region on d-7

Fig.7.10 shows the effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in non- synaptic mitochondrial (B) Bax (C) Bcl-2 and ratio (D) Bax/Bcl-2 in cortical brain region on d-7. Statistical analysis with one-way ANOVA showed a

significant difference in Bax [F (4, 10) = 16.3, P<0.05], Bcl-2 [F (4, 10) = 19.2, P<0.05] and their ratio (Bax/Bcl-2) [F (4, 10) = 19.8, P<0.05] post-anoxia. Post hoc analysis showed that treatment with 2,4 DNP and the combination (D+T) was significantly effective in attenuating the levels of non-synaptic mitochondrial Bax and Bcl-2 whereas tempol was ineffective in improving Bcl-2 levels but was effective in attenuating Bax levels compared to anoxia group animals. Furthermore, there was no significant changes between the combination (D+T) and individual drug treatment (2,4 DNP or tempol).

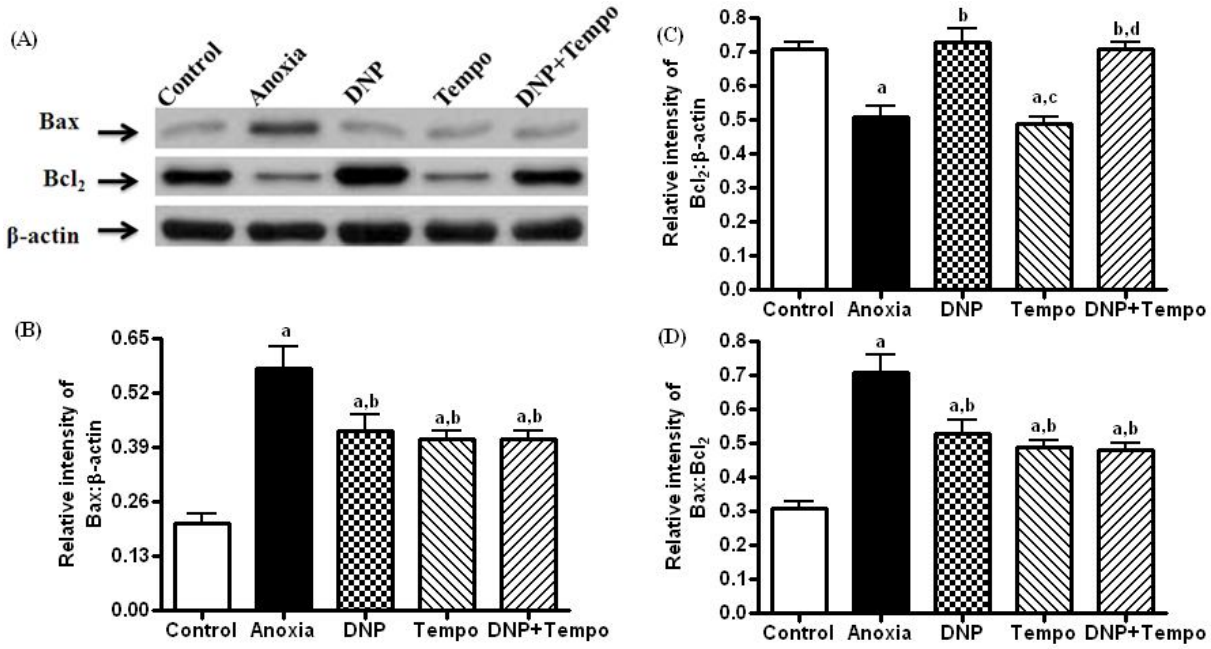

Figure 7.10 Effect of 2,4 DNP, Tempol and their combination (D+T) on anoxia-induced changes in non-synaptic mitochondrial Bax (B), Bcl-2 (C) and their ratio (Bax/Bcl-2) (D) on day-7 post-anoxia. Blot (A) represents Bax, Bcl-2 and their ratio (Bax/Bcl-2) in a cortical region non-synaptic fraction. The results in the histogram are expressed as the ratio of the relative intensity of levels of protein expression of mitochondrial 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 anoxia, $^{c}P<0.05$ compared to DNP and $^{d}P<0.05$ compared to tempol respectively [One-way ANOVA followed by Student Newman-Keuls test].

7.4.11 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced alterations in spontaneous locomotor activity in OFT

The effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in ambulation, time spent in the central grid and rearing in OFT are depicted in Table.7.1. A repeated measure two-way ANOVA depicted a significant differences in ambulation among groups [$F(4,100) = 106.1$, $P<0.05$], time [$F(4,100) = 11.8$, $P<0.05$]. There was no significant interaction between group and time among groups [$F(16,100) = 0.38$, $P>0.05$]. A post hoc analysis showed that there was a marked increase in ambulatory behavior in anoxia group from d-21 to d-150 compared to control group animals. However, treatment with 2,4 DNP, tempol or their combination (D+T) was significantly ($p<0.05$) effective in decreasing number of ambulations on different days. Similarly, an ANOVA depicted a significant main effect for time spent in central grid and rearing among groups ([$F(4,100) = 9.26$, $P<0.05$ and $F(4,100) = 20.13$, $P<0.05$] respectively), time ([$F(4,100) = 22.0$, $P<0.05$ and $F(4,100) = 10.15$, $P<0.05$] respectively) and a significant interaction between group and time ([$F(16,100) = 4.14$, $P<0.05$ and $F(16,100) = 3.72$, $P<0.05$] respectively). A post hoc analysis showed that there was no significant difference ($P>0.05$) for time spent in the central grid and rearing in all days between control and anoxia groups.

Day	Control	Anoxia	DNP	Tempol	DNP+Tempol
Ambulation (in number)					
21	63.7 ± 7.41	96.5 ± 5.83*	85.6 ± 4.51$^{*,\#}$	82.6 ± 6.81$^{*,\#}$	83.6 ± 4.62$^{*,\#}$
60	65.6 ± 8.24	98.2 ± 6.23*	85.4 ± 6.61$^{*,\#}$	88.4 ± 7.29$^{*,\#}$	80.4 ± 5.21$^{*,\#}$
90	61.9 ± 8.71	97.1 ± 5.37*	84.5 ± 3.26$^{*,\#}$	85.3 ± 6.41$^{*,\#}$	76.5 ± 6.40$^{\#}$
120	55.9 ± 4.45	88.3 ± 6.61*	76.3 ± 7.23$^{*,\#}$	75.5 ± 4.32$^{*,\#}$	70.3 ± 5.33$^{*,\#}$
150	60.5 ± 5.24	94.5 ± 8.38*	82.4 ± 3.62$^{*,\#}$	84.3 ± 7.21$^{*,\#}$	79.4 ± 4.56$^{*,\#}$
Time spent in central grid (in sec)					
21	13.5 ± 4.21	11.5 ± 2.24	12.6 ± 1.93	9.4 ± 1.87*	9.2 ± 2.33*,**
60	12.6 ± 1.40	15.4 ± 1.62	17.3 ± 1.13*	19.6 ± 1.40*	12.9 ± 1.13$^{**,\#\#}$
90	11.8 ± 1.23	13.2 ± 1.13	13.5 ± 2.22	12.9 ± 1.39$^{\#}$	10.5 ± 3.11
120	13.5 ± 1.45	16.5 ± 1.85	11.1 ± 2.36$^{\#}$	13.7 ± 1.15	10.3 ± 2.24$^{\#,\#\#}$
150	11.6 ± 1.12	12.1 ± 2.56	10.5 ± 1.45	9.7 ± 1.23	9.6 ± 3.13
Rearing (in number)					
21	11.5 ± 3.67	15.2 ± 2.25	18.4 ± 1.88*	20.1 ± 3.33$^{*,\#}$	26.7 ± 2.12$^{*,\#,**,\#\#}$
60	12.6 ± 1.32	14.2 ± 1.49	16.1 ± 2.63	15.6 ± 1.47	13.5 ± 3.14
90	14.8 ± 4.11	17.8 ± 4.19	19.5 ± 2.29*	19.9 ± 2.34*	20.5 ± 3.34*
120	15.1 ± 2.47	16.1 ± 1.90	18.8 ± 4.61	20.1 ± 3.25*	18.1 ± 2.26
150	14.2 ± 1.14	12.1 ± 2.44	18.2 ± 2.65$^{\#}$	19.7 ± 1.29$^{*,\#}$	15.8 ± 3.19

Table 7.1 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in ambulation, time spent in central grid and rearing during OFT. Data are mean+SD (n=5) animals in each group *P<0.05 compared to control, $^{\#}$P<0.05 compared to anoxia, **P<0.05 compared to 2,4 DNP and $^{\#\#}$P<0.05 compared to Tempol.

7.4.12 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced anxiety-like behavior in EPM test

Fig. 7.11 shows the effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced alterations in (A) open arm entries (B) time spent in open arms and (C) anxiety index in elevated plus-maze test paradigm. A repeated measure two-way ANOVA depicted a significant differences in open arm entries and time spent in open arms among groups ([F (4,100) = 71.93, P<0.05 and F (4,100) = 9.96, P<0.05] respectively), time ([F (4,100) = 30.38, P<0.05 and F (4,100) =26.13, P<0.05] respectively) and a significant interaction between group and time ([F (16,100) = 16.58, P<0.05 and F (16,100) =6.98, P<0.05] respectively). A post hoc analysis showed that there was a marked decrease in open arm entries and time spent in open arms from d-21 up to d-150. Treatment with 2, 4 DNP, tempol or their combination (D+T) was effective in increasing open arm entries and open

arm residing time. Similarly, a significant main effect for anxiety index among groups [F (4,100) = 16.02, P<0.05], insignificant with time [F (4,100) = 0.388, P>0.05], While, a significant interaction between group and time [F (16,100) = 2.1, P<0.05] was observed. However, there was no effect of anoxia on anxiety index on d-21. A marked increase in anxiety index was observed from d-60 to d-150. 2, 4 DNP, tempol or their combination (D+T) attenuated anxiety-like behavior in EPM task from d-60 to d-150 post-anoxia injury. It is interesting to note that there is no significant difference in D+T as compared to either 2,4 DNP or tempol.

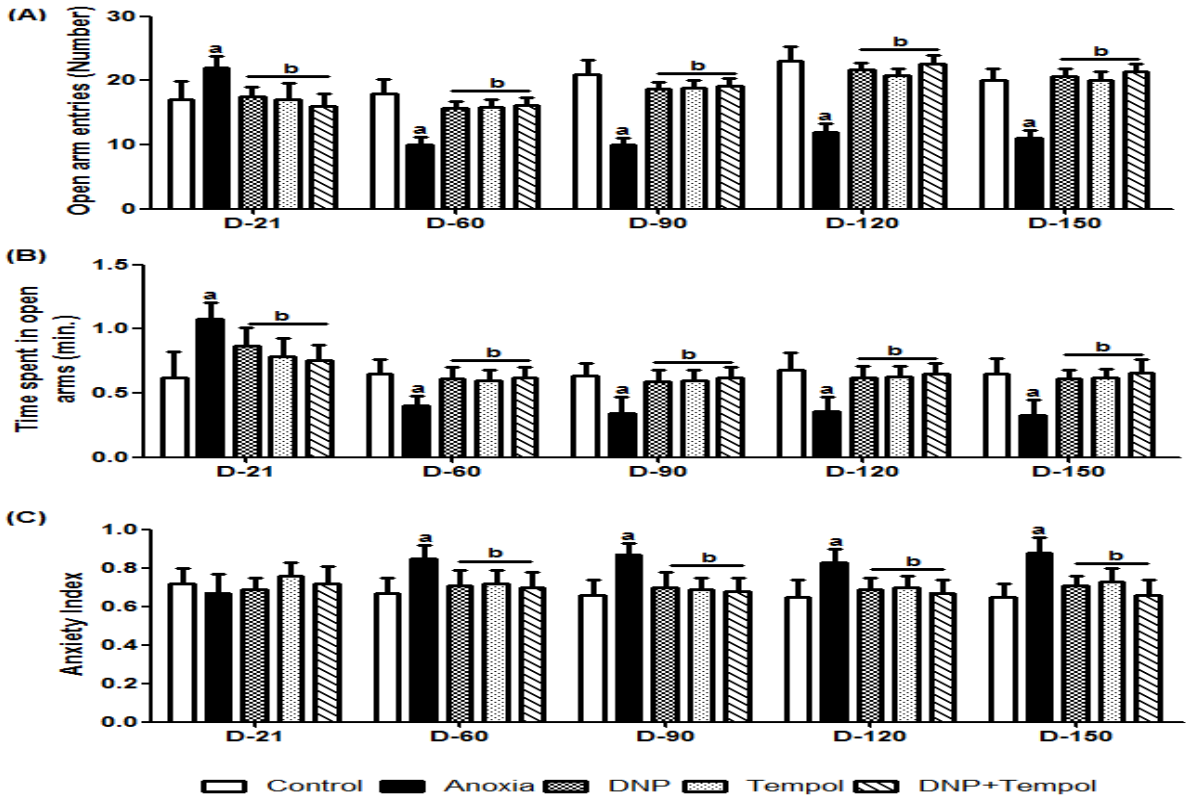

Figure 7.11 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in (A) open arm entries (B) time spent in open arms and (C) anxiety index in elevated plus-maze test paradigm. Data are mean+SD (n=5) animals in each group. [a]P<0.05 compared to control, [b]P<0.05 compared to anoxia group.

7.4.13 Effect of 2,4 DNP, tempol and their combination (D+T) attenuated anoxia-induced behavioral alterations in Y-maze test

The effect of anoxia-induced alterations in general exploratory behavior (curiosity) in trial-1 and trial-2 are depicted in Fig. 7.12 (A) and (B) respectively. Fig. 7.12 (C) shows the coping behavior to novel arm (anxiety-like behavior). A repeated measure two-way ANOVA depicted a significant difference for exploration in trial-1 and trial-2 among groups ([F (4,100) = 80.43; P<0.05] and [F (4,100) = 133.6; P<0.05] respectively), time ([F(4,100) = 1.87; P<0.05] and [F(4,100) = 7.90; P<0.05] respectively). However, no significant interaction between group and time ([F (16,100) = 0.47; P>0.05] and [F (16,100) = 1.27; P>0.05] respectively) was observed. Similarly, there were significant changes in coping behavior among groups [F (4,100) = 104.4; P<0.05], time [F(4,100) = 22.40; P<0.05]. However, There was no significant interaction between group and time [F (16,100) = 0.44; P>0.05]. Post hoc analysis revealed that anoxia leads to a marked increase in exploration regarding hyperactivity both trial-1 and trial-2 and copying behavior from d-21 up to d-150. However, 2,4 DNP, tempol and their combination (D+T) significantly reversed anoxia-induced increase in hyperactiveness both in trial-1 and trial-2 and decreased in coping behavior from d-21 up to d-150. Moreover, there was no synergistic effect observed in the combination (D+T) as compared to individual effects of drugs.

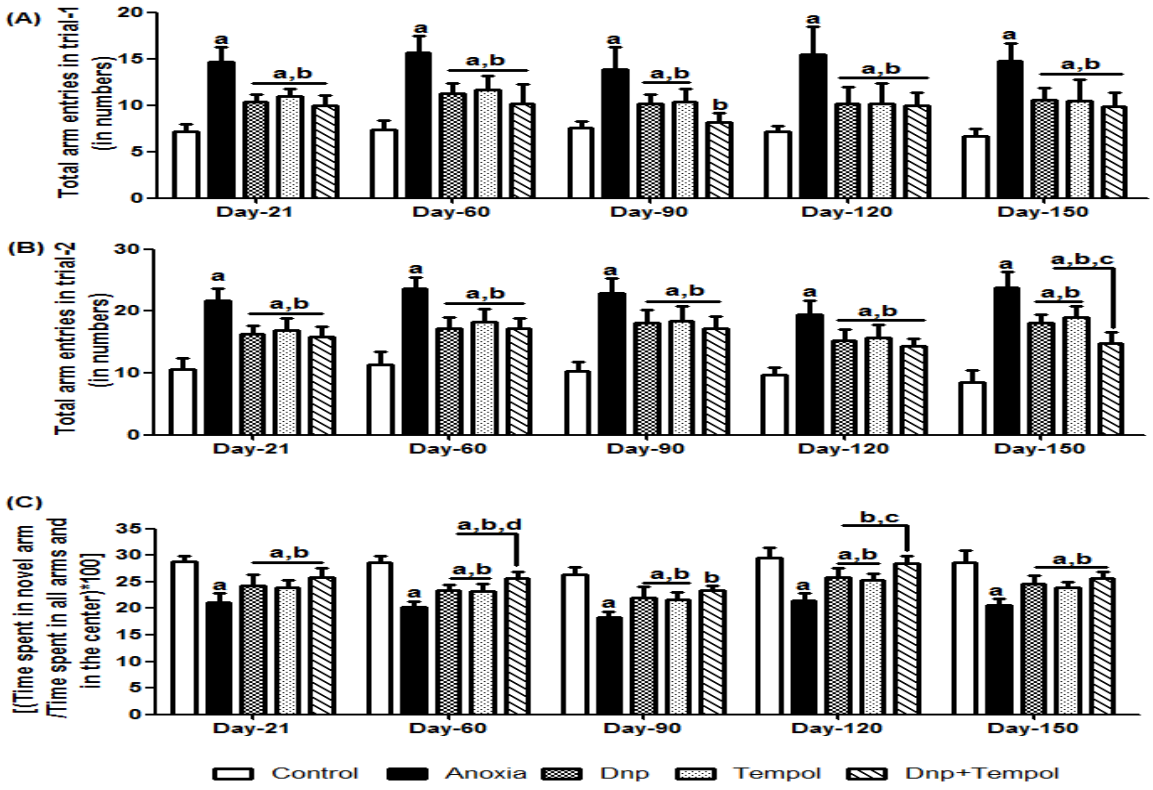

Figure 7.12 Effect of 2,4 DNP, tempol and their combination (D+T) in anoxia-induced changes in total arm entries during trial-1 (A) and trial-2 (B) and coping behavior to novel arm (C) during trial-2 in Y-maze paradigm from d-21 up to d-150. Data are mean+SD (n=5) animals in each group. [a]P<0.05 compared to control, [b]P<0.05 compared to anoxia, [c]P<0.05 compared to 2,4 DNP and [d]P<0.05 compared to tempol group.

Further, the effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced alterations in spatial recognition memory on d-21 (A), d-60 (B), d-90 (C), d-120 (E), d-150 (E) are depicted in Fig.7.13. A repeated measure two-way ANOVA showed significant differences in arm discrimination behavior on d-21, d-60, d-90, d-120 and d-150 during trial-2 among groups [F (4, 40) = 0.41; P<0.05, F (4,40)=0.55; P<0.05, F (4, 40)= 1.64; P<0.05], [F (4, 40) =0.87; P<0.05] and [F (4, 40) =0.33; P<0.05 respectively], significant main effect for known and novel arms ([F (1, 40)= 32.10; P<0.05], [F (1, 40) = 25.95; P<0.05], [F (1, 40)=28.50; P<0.05], [F(1, 40)=86.32; P<0.05] and [F (1, 40)= 9.08; P<0.05 respectively) and a significant interaction between group and arms ([F (4, 40)=28.35;

P<0.05], [F (4, 40)=27.26 ; P<0.05], [F (4, 40)=45.25 ; P<0.05], [F (5, 40)=48.34; P<0.05] and [F (4, 40)=58.09; P<0.05] respectively in Y-maze test paradigm. Post-hoc analysis revealed that there was a marked priority to novel arm entries for control group animals on different days. A within group comparison further depicted that anoxia caused significant decrease in novel arm preference compared to known arm on d-21 to d-150. 2,4 DNP, tempol and their combination (D+T) attenuated the anoxia-induced decrease in novel arm entries from d-21 itself which was further maintained up to d-150. Furthermore, repeated measure two-way ANOVA revealed significant differences for known and novel arm entries among groups ([F (4,100) = 81.59; P<0.05] and [F(4,100) = 128.3; P<0.05] respectively) time ([F (4,100) = 11.89; P<0.05] and [F(4,100) = 3.67; P<0.05] respectively) and a significant interaction between group and time ([F (4,100) = 1.12; P<0.05] and [F(4,100) = 1.38; P<0.05] respectively). Post-hoc analysis revealed that anoxia increased the percentage entries into known arm compared to control group animals on from d-21 to d-150. 2,4 DNP, tempol and their combination (D+T) reversed the anoxia-induced increase in the percentage entries into known arm in all days. However, there was no synergistic effect observed in the combination (D+T) compared to individual effect of 2,4DNP and tempol. Likewise, while considering novel arm entries, anoxia significantly decreased the percentage entries into novel arm compared to control group animals from d-21 up to d-150. 2,4 DNP, tempol and their combination (D+T) markedly reversed the anoxia-induced decrease in the percentage entries into novel arm on different days. Although, there was no significant difference among 2,4 DNP, tempol and their combination (D+T) in both known and novel arm entries on all days.

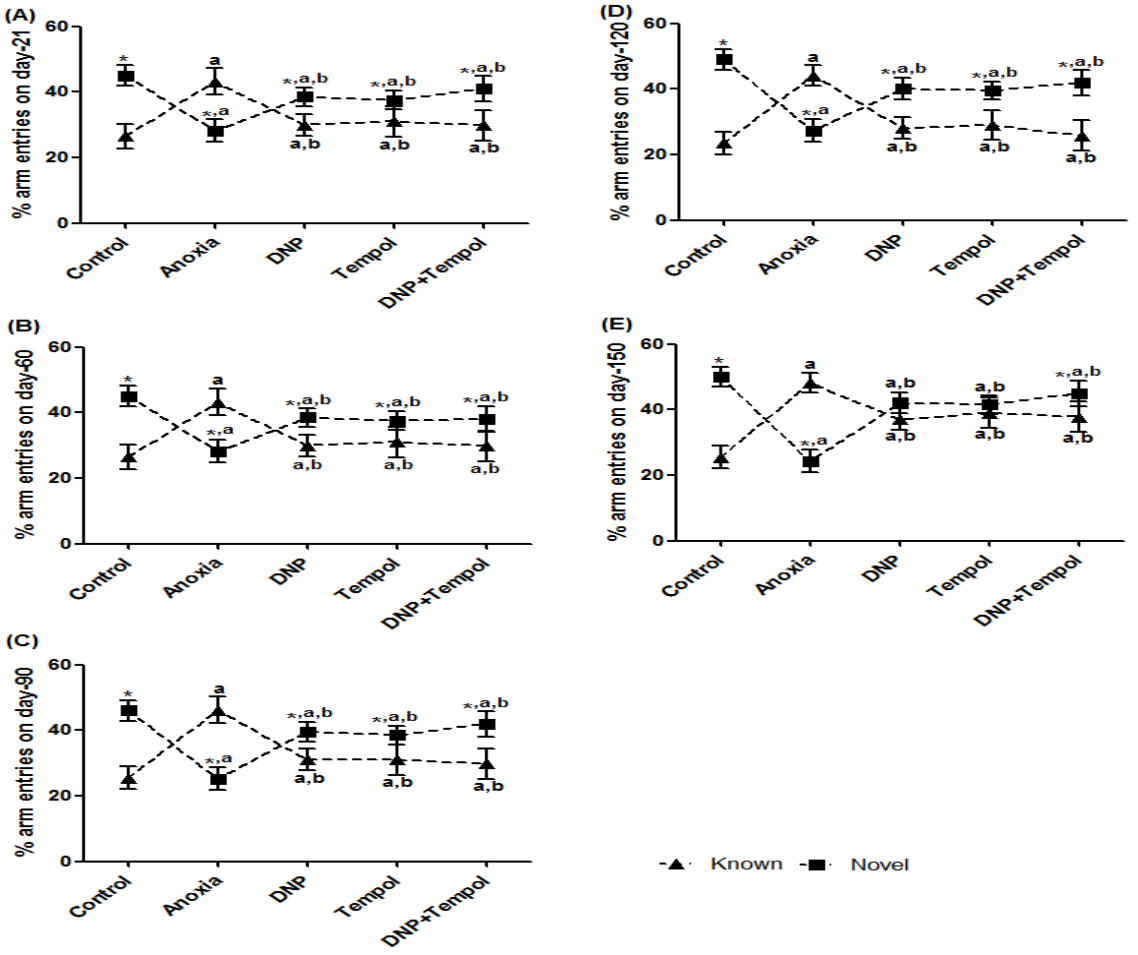

Figure 7.13 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in arm discrimination behavior during Y-maze test paradigm on d-21(A), d-60 (B), d-90 (C), d-120 (D) and d-150 (E). Data are mean+SD (n=5) animals in each group. [a]P<0.05 compared to control, [b]P<0.05 compared to anoxia, [c]P<0.05 compared to 2,4 DNP and [d]P<0.05 compared to tempol group.

7.4.14 2,4 DNP, tempol and their combination (D+T) improved anoxia-induced depression-like symptoms in FST

Fig. 7.14 shows the effect of 2,4 DNP (2.5 mg/kg), tempol (75 mg/kg) and their combination (D+T) on anoxia-induced immobility period in FST on different days of experimental design. Repeated measure two-way ANOVA revealed a significant main effect among groups [$F(4,100) = 374.3$; $P<0.05$], time [$F(4,100) = 4.0$; $P<0.05$] and a significant interaction between group and time [$F(16,100) = 2.09$; $P<0.05$]. Post hoc analysis showed

that anoxia caused a markedly increased the immobility period compared to control animals in all days. Treatment with 2,4 DNP, tempol and their combination (D+T) altered anoxia-induced increase in immobility period on d-21up to d-150. The effectiveness of combination (D+T) was similar to the individual effect of 2,4 DNP and tempol.

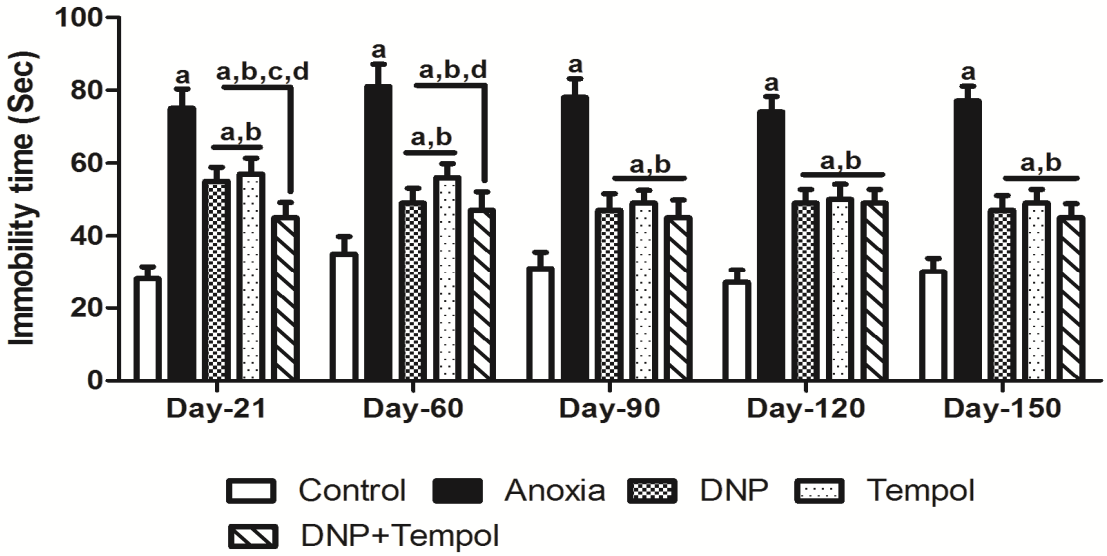

Figure 7.14 Effect of 2,4 DNP, tempol and their combination (D+T) on anoxia-induced changes in immobility in forced-swim test. Data are mean+SD (n=5) animals in each group. [a]P<0.05 compared to control, [b]P<0.05 compared to anoxia, [c]P<0.05 compared to 2,4 DNP and [d]P<0.05 compared to tempol group.

7.4.15 Effect of 2,4 DNP, tempol and their combination (D+T) on plasma corticosterone level

The effect of 2,4 DNP (2.5 mg/kg), tempol (75 mg/kg) and their combination (D+T) on anoxia-induced alterations in plasma CORT level is shown in Fig 7.15. Statistical analysis by one-way ANOVA depicted a significant difference in plasma CORT levels among groups [$F(4,20) = 66.46$; $P<0.05$] on d-150. Post-hoc analysis revealed that anoxia caused a significant decrease in the level of CORT in plasma compared to control animals. 2,4 DNP, tempol and D+T significantly increased anoxia-induced decrease in plasma CORT

level. The combination of both drugs (D+T) was similarly effective as to their individual effects.

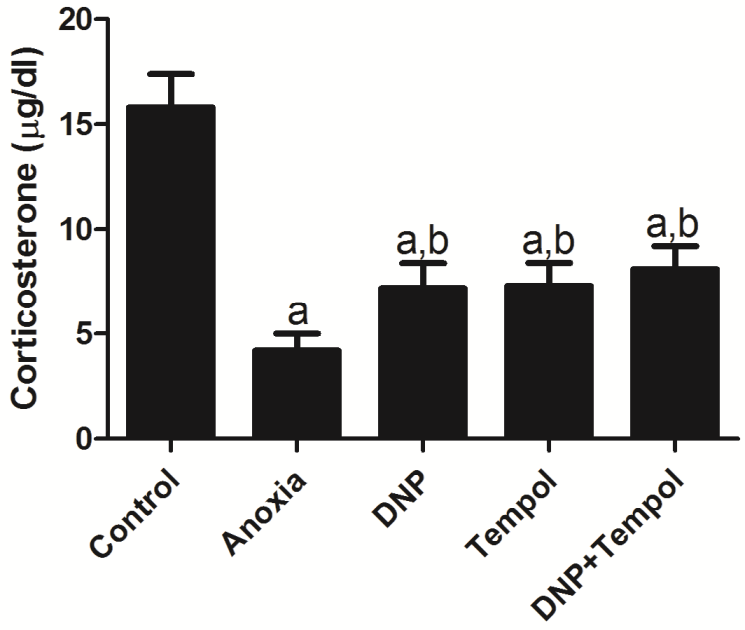

Figure 7.15 Effect of 2,4 DNP (2.5 mg/kg), tempol (75 mg/kg) and their combination (D+T) on plasma CORT level in anoxia exposed rats. Data are mean+SD (n=5) animals in each group. [a]P<0.05 compared to control, [b]P<0.05 compared to anoxia group.

7.5 Discussion

This is the first long-term study showing the combinatorial effect of mitochondrial modulators (2,4 DNP and tempol) on insult progression in developing brain. There was no synergistic effect observed while treatment with the combination of 2,4 DNP and tempol. On the other hand, we did not observe any statistical significance in synaptic and non-synaptic mitochondrial function after anoxia.

Anoxia hampered the mitochondrial respiration in both synaptic and non-synaptic mitochondria regarding altered mitochondrial oxygen consumption in s-3 and s-4. Further, a marked decrease in RCR was also observed in the two different mitochondrial fractions

which depict a compromised mitochondrial bioenergetics. Treatment with the combination of D+T was equally effective to their individual effects in improving s-3, s-4 as well as RCR in both mitochondrial fractions. Further, the combination of D+T was effective as compared to the individual effect of 2,4 DNP and tempol in decreasing anoxia-induced levels of NO and LPO in both the mitochondrial fractions. Further, the levels of antioxidant defence system like SOD and CAT activities were hindered by anoxic injury. The combination of D+T significantly improved the antioxidant defense system in both synaptic and non-synaptic mitochondrial fractions similar to the individual effects of both drugs. In normal physiological conditions, mitochondria act as a sink to maintain Ca^{2+} homeostasis. During pathological conditions like anoxia, mitochondria become overloaded with Ca^{2+} and undergo the cataclysmic mitochondrial permeability transition (MPT) formation within the inner mitochondrial membrane with a resultant rupture of the outer mitochondrial membrane caused by osmotic swelling [45]. We observed a marked increase in the levels of Ca^{2+} concentrations in both synaptic and non-synaptic mitochondria. However, there was no synergistic effect observed on treatment with D+T as compared to 2,4 DNP in mitigating Ca^{2+} overloads in both the mitochondrial fractions. This effect of 2,4 DNP may be due to its mild uncoupling effect. However, tempol failed to elicit any protective effect in reducing the Ca^{2+} loads. Similarly, anoxia caused a significant increase in both synaptic and non-synaptic mitochondrial swelling. The combination of 2,4 DNP, tempol and their combination was equally effective in reducing the swelling in both fractions. 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]. There was a marked decrease in the levels of expression of pro-apoptotic cytoplasmic Bax, increased levels of antiapoptotic Bcl-2 and-and a reduction in their ratio (Bax/Bcl-2). Further, there was an increased expression of synaptic and non-synaptic mitochondrial Bax, decreased expression of Bcl-2 and an increase in their ratio (Bax/Bcl-2) after anoxia. Treatment with 2,4 DNP, tempol and their combination (D+T) significantly attenuated the cytoplasmic, synaptic and non-synaptic Bax, Bcl-2 and their ratio. Furthermore, anoxia leads to a marked increase in the expression of cytochrome-c, caspase-9 and caspase-3.The combination of D+T significantly mitigated the expression of these apoptotic proteins and cytochrome-C.

Anoxic injury in newborns causes delayed behavioral disturbances such as abnormal responses to stress and impaired learning which persists in a lifelong manner [165]. The results of the present investigation confirms the existence of the abnormal responses to stress in adolescent rats previously subjected to neonatal anoxia. OFT is used to evaluate the spontaneous locomotor activity which can be considered as a sign of hyperactivity in animals. Anoxia caused a significant increase in the spontaneous locomotor activity regarding ambulation from d-21 to d-150. A previous report has suggested that the augmented locomotor activity is likely to be due to disturbances in the development of brain regions such as the hippocampus, corpus striatum or amygdala occurring during a specific postnatal time window [156]. However, no marked changes in the time spent in central grid and rearing was observed from d-21 to d-150.However, 2,4 DNP, tempol and their combination (D+T) mitigated the ambulation behavior depicting the attenuation of hyperactiveness in rats from d-21 to d-150. Both experimental and clinical studies suggest that adverse experience during development augments depression and anxiety in

adulthood [166-168]. It has been suggested that the decrease in open arm entries and time spent in open arm and anxiety index are the indicators of anxiety-like activity in EPM [169, 170]. In our study, anoxia caused a marked increase on d-21 and further decrease from d-60 to d-150 in the number of open arm entries and time spent in the open arm on d-21. However, there were no significant changes in the anxiety index on d-21. Further, a marked increase in anxiety index was observed from d-60 to d-150. The increase in open arm entries and open arm time can be predicted as a sign of hyperactivity of rats. The combination of D+T was equally effective in decreasing the open arm entries and open arm time on d-21 and further increasing the same along with decreasing the anxiety index from d-60 to d-150 similarly as 2,4 DNP and tempol. This shows that 2,4 DNP, tempol and D+T decreased the anxiety-like behavior as an effect of anoxia and improved the neurobehavioral outcome.

Anoxia caused marked alterations in the trial-1 and trial-2 regarding exploration (curiosity), time spent in the novel to entry and known arm as a measure of anxiety index (coping) and percentage in known to novel arm entries as a measure of spatial recognition memory in Y- maze paradigm from d-21 to d-150. 2,4 DNP, tempol and D+T combination markedly attenuated the hyperactivity by reducing curiosity in trial-1 and trial-2, reduction in coping behavior and increase in the novel to known arm entries as a measure of improved spatial recognition memory from d-21 to d-150. A previous report has shown that neonatal anoxia leads to disruption of reference spatial memory associated with impairments in the spatial working memory in adulthood [167]. The spatial deficits post neonatal anoxia may be the result of damage to neuronal systems directly involved in cognitive or attentional processes [171]. Another aspect of emotionality that is affected by neonatal anoxia is depression-like behavior, which is typically identified either by expression of gustatory

anhedonia or behavioral despair [163]. There was a marked increase in the immobility time in FST from d-21 up to day-150 as compared to control group animals. We observed a marked attenuation of immobility time on treatment with 2,4 DNP, tempol and D+T from d-21 to d-150. Postnatal stress in rodents caused by neonatal anoxia increases emotionality, an effect that tends to be associated with HPA axis dysregulation [172]. The stress-induced corticosterone hyposecretion was also observed in adult animals born by caesarean section applied after experimental anoxia [156]. We observed marked decrease in the levels of plasma corticosterone. 2,4 DNP, tempol and D+T markedly corrected the corticosterone levels and attenuated the HPA axis activity on day-150.

Therefore, we conclude that 2,4 DNP and tempol imporved long term behavior, mitochondrial function, and stress response. However, the combination of 2,4 DNP and tempol did not show any synergistic effect on individual drugs in improving mitochondrial function as well as neurobehavioral outcomes post-anoxia injury.