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Neonatal anoxia leads to time dependent progression of mitochondrial linked apoptosis in rat cortex and associated long term sensorimotor deficits

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ABSTRACT

Neonatal anoxia arises due to oxygen deprivation at the time of birth and results in life long neurodevelopmental deficits and sometimes may lead to death. The present study investigated the time dependent cortical mitochondrial dysfunction linked apoptosis and related sensorimotor deficits in neonates. Neonates after 30 h to birth (P2) were subjected to anoxia of two episodes (10 min in each) at a time interval of 24 h by passing 100% N2 into an enclosed chamber as confirmed by pulse oximetry. Sensorimotor activities like reflex latency and hanging latency were carried out 24 h after last anoxic episode i.e. from P4 (day-1) and continued up to P10 (day-7). Mitochondrial dysfunction after anoxia was evident by the decrease in respiration states, respiratory control ratio (RCR), antioxidant enzyme activity but an increase in oxidative stress and lipid peroxidation and alteration in mitochondrial membrane potential (MMP) at different time points (30 min, 24 h and day-7). A change in expression of Bcl-2 family proteins and opening of mitochondrial transition pore (mPTP) in terms of mitochondrial swelling was observed resulting in release of cytochrome-C which further activated intrinsic (mitochondrial) pathway of apoptosis through increased expression of caspase-9/3 as confirmed by flow cytometry. In conclusion, anoxia injury leads to progressive activation of mitochondrial events leading to increase in apoptotic cell death following secondary pathological insult. Therefore, strategies in limiting mitochondrial-linked apoptosis during the secondary insult input process may be useful in treatment of long term sensorimotor deficits following anoxia.

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1. Introduction

Neonatal anoxia is a major cause of acute mortality and chronic neurological morbidity resulting in 0.1–0.3% of full-term infants and nearly 60% of low-weight premature infants (Azra Haider and Bhutta, 2006; Kurinczuk et al., 2010). The mechanisms leading to brain damage after anoxia are complex and related to the developmental stage of the brain and the severity of the insult. It is evident that primary insult occurs more in form of necrosis than apoptosis immediately after the hypoxic/anoxic exposure, resulting from the biomechanical forces directly damaging neuronal tissue. Secondary insult mechanisms are the result of number of factors, including ischemia–reperfusion injury, oxidative stress, mitochondrial calcium overload, activation of proteases and caspases (Badawi et al., 2012; Borutaite et al., 2013). There are several reports to suggest that most of the cell death during secondary insult occurs through apoptosis (Beilharz et al., 1995; Wei et al., 2004; Yue et al., 1997). Therefore, inhibition of this secondary insult processes may be one of the most important means for therapeutic intervention to prevent further degeneration of brain tissue and preserve the healthy one. Therefore the current study evaluates the long term changes in mitochondrial dysfunction-linked apoptosis to understand time dependent progression of anoxic insult.

Apoptosis is regulated by several protein families, including the upstream pro-apoptotic Bax and anti-apoptotic Bcl-2 family proteins and the downstream caspase family like caspase-9 and caspase-3 (Jarskog et al., 2004). It is proposed that the mitochondria are the regulator of both energy metabolism and apoptotic pathways and their dysfunction can result in impairment of brain mitochondrial electron transfer and energy transduction (Seppet et al., 2009; Soane et al., 2007). $\Delta\Psi m$ component determines

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calcium sequestration and the generation of reactive oxygen species in mitochondria (Adam-Vizi and Starkov, 2010; Szydlowska and Tymianski, 2010) which further leads to opening of mitochondrial permeability transition pore (MPTP) and release of cytochrome-C and triggers intrinsic pathway of apoptosis through activation of caspases-9/3 and finally leads to apoptotic cell death (Chowdhury et al., 2008; Kuranaga and Miura, 2007).

The present study was conducted using non-invasive animal model of anoxia in which neonates (rat pups) of post natal day 2 (P2) were subjected to anoxia of two episodes (10 min in each) at a time interval of 24 h by passing 100 % N_2 into an enclosed chamber which showed cortical mitochondrial dysfunction and apoptosis as early as 30 min after the last anoxia episode and progressed up to day-7. In our previous study, we have shown acute pathological changes in mitochondrial bioenergetics that leads to primary insult in whole brain after second anoxia episode and lead to sensorimotor deficits in neonates (Samaiya and Krishnamurthy, 2015). In continuation, in the present study we explored the secondary insult (day-7) and its comparison in terms of severity to primary insult (30 min and 24 h) in cortical brain region of neonates which is involved in coordination of sensorimotor activity (Wei et al., 2015).

Further, a detailed time dependent mitochondrial function was examined by evaluating mitochondrial respiratory chain enzyme activity, oxidative damage, mitochondrial membrane potential (MMP), mitochondrial permeability transition pore opening (mPTP), expression of Bax, Bcl-2 and their ratio (Bax/Bcl-2), release of cytochrome-C and further activation of apoptotic markers like caspase-9, and caspase-3 and confirmation of apoptotic cell death by flow cytometry in cortical region at different primary (30 min, 24 h) and secondary (day-7) insult time points.

2. Materials and methods

2.1. Animals and groups

All experiments were conducted in accordance with the principles of laboratory animal care (National Research Council US Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011) guidelines. The experimental procedures were approved by the Institutional Animal Ethical Committee of BHU (Protocol No. Dean/11-12/CAEC/328). 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 temperature of $25 \pm 1\,^{\circ}C$ and 45–55% relative humidity and a 12: 12 h light/dark cycle, and they were provided with food and water ad libitum. Birth litter count ranged from 8 to 12 pups per rat of weighing 6–8 g. Rat pups of approximate 30 h (P2) age were used for the experiment. Six groups of five neonates each was assigned to either the anoxia or the corresponding control treatments for different time points [30 min (P4), 24 h (P4) and day-7 (P10) respectively] in such a way that each of the resulting group contained rat pups from at least two different litters.

2.2. Anoxia procedure

The anoxic procedure was carried out as validated and defined previously (Samaiya and Krishnamurthy, 2015). Briefly, rat pups were placed inside the chamber with flow rate of 3 l/min of 100% nitrogen with a pressure of approximately 101.7 for 10 min of two anoxia episodes at an interval of 24 h into a non-hermetic chamber (For inducing anoxia). The same experimental procedure was followed for the control group, but the chamber contained air instead of nitrogen (Control group). After exposure to anoxia the rat pups were returned to the dams and sacrificed at different time points [30 min (P4), 24 h (P4) and day-7 (P10) respectively]. The brains were rapidly dissected out and the cortical portion was immediately used for mitochondrial isolation. Mitochondrial respiration studies were performed immediately after mitochondrial isolation. Further, mitochondrial pellets were stored at $-80\,^{\circ}C$ for further biochemical estimations, flow cytometry and western blot analysis.

2.3. Materials

Reagents—Mannitol, sucrose, bovine serum albumin (BSA), EGTA, HEPES potassium salt (St. Louis, MO, USA), potassium phosphate monobasic anhydrous (KH2PO4), MgCl2, malate, pyruvate, 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, USA). Antibodies such as cytochrome-c, caspase-9, caspase-3, Bax, Bcl-2 and beta-actin (Santa Cruz Biotechnology Inc.; Santa Cruz, California, USA) were purchased.

2.4. Behavioral parameter assessment

Behavioral parameters like righting reflex and wire hanging maneuver were performed 24 h (P4) and day-7 (P10) after second anoxic episode.

2.4.1. Righting reflex

The assessment was done by placing the pups on their back and the time taken to turn on to their belly. The time for complete turn on to four limbs touching platform was measured (Fan et al., 2005).

2.4.2. Wire hanging maneuver

This maneuver tests neuromuscular and locomotor development. Pups suspended by their forelimbs from a horizontal rod (5 mm × 5 mm area, 35 cm long, between two poles 50 cm high) tend to support themselves with their hind limbs, preventing them from falling and aiding in progression along the rod. Suspension latencies were recorded (Fan et al., 2005; Hermans et al., 1992).

2.5. Measurement of peripheral oxygen saturation

Measurement of peripheral oxygen saturation was performed for control and anoxia group animals. A pulse oximeter (Physiosuite/Kent scientific corporation, U.S.A) along with a pediatric Y sensor was coupled with colorless tape near the proximal region of the animal's tail at the dorsal and ventral faces of the abdomen (Decker et al., 1989). After positioning the sensor, the apparatus was turned on and a value between 97% and 99% of oxygen saturation was observed when the sensor was correctly positioned. Rat pups were placed into the chamber with the connected oximetry apparatus and the values of oxygen saturation were collected by an observer every minute during the anoxia or control procedures (Takada et al., 2011).

2.6. Evaluation of mitochondrial bioenergetics

2.6.1. Mitochondrial isolation

Isolation of mitochondria from the cortical brain region was done by the method previously described (Berman and Hastings, 1999) with some slight modifications (Samaiya and Krishnamurthy, 2015). Briefly, cortical brain regions of control and anoxic pups (30 min, 24 h (day-1) and day-7 respectively) were homogenized in isolation buffer (consisting of 215 mM mannitol, 75 mM sucrose, 0.1% w/v bovine serum albumin, 20 mM HEPES buffer and 1 mM of EGTA in 100 ml of distilled water and pH adjusted to 7.2 with

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