1.1 Neonatal anoxia

Neonatal anoxia is a condition developed due to the severe deprivation of oxygen in neonates at the time of birth. It is one of the leading causes (80%) of neurologic injury, neurodevelopmental delay and if more severe can lead to death in new-born infants worldwide. The signs and symptoms of injury are unpredictable at the time of birth and mostly take hours to days before they manifested [1]. Early recognition of the injury may help in guiding management during those critical first days of life.

1.2 Causes of anoxia

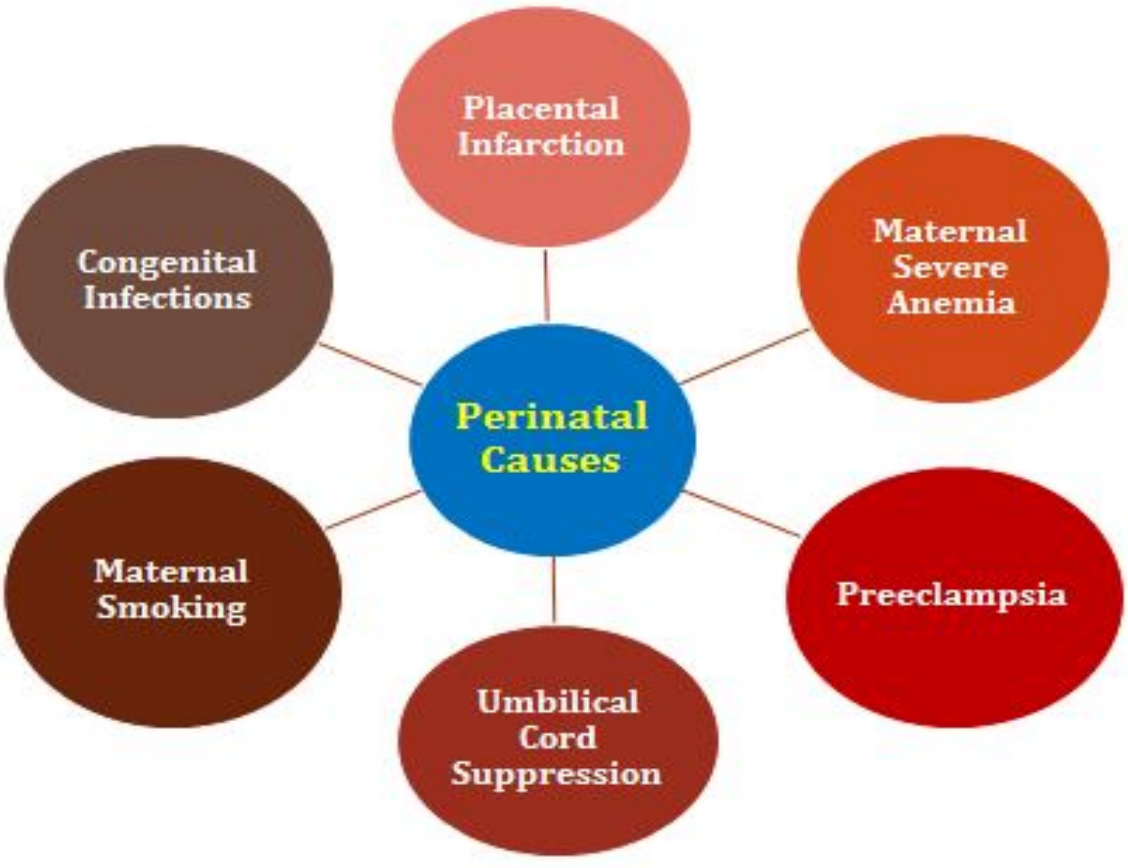


Figure 1.1 Causes of neonatal anoxia

1.3 Epidemiology

As per World Health Organization (WHO) data, between four and nine million newborns develop neonatal anoxia every year. An estimated 1.2 million neonates die and at least the same number develop severe neurological abnormalities like cerebral palsy, autism, epilepsy, as well as problems with cognition, memory, fine motor skills and behavior [1, 2].

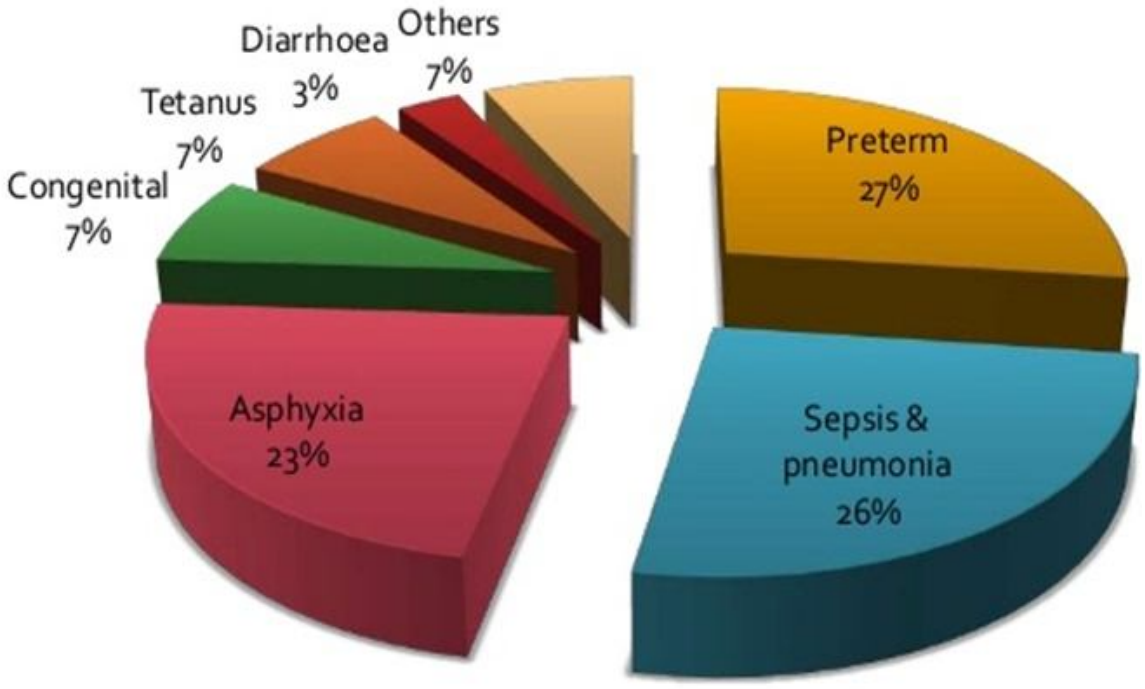


Figure 1.2 Causes of neonatal deaths in India [3]

Anoxia is the second key cause of neonatal deaths after sepsis, contributing to around 30% of neonatal mortality worldwide.

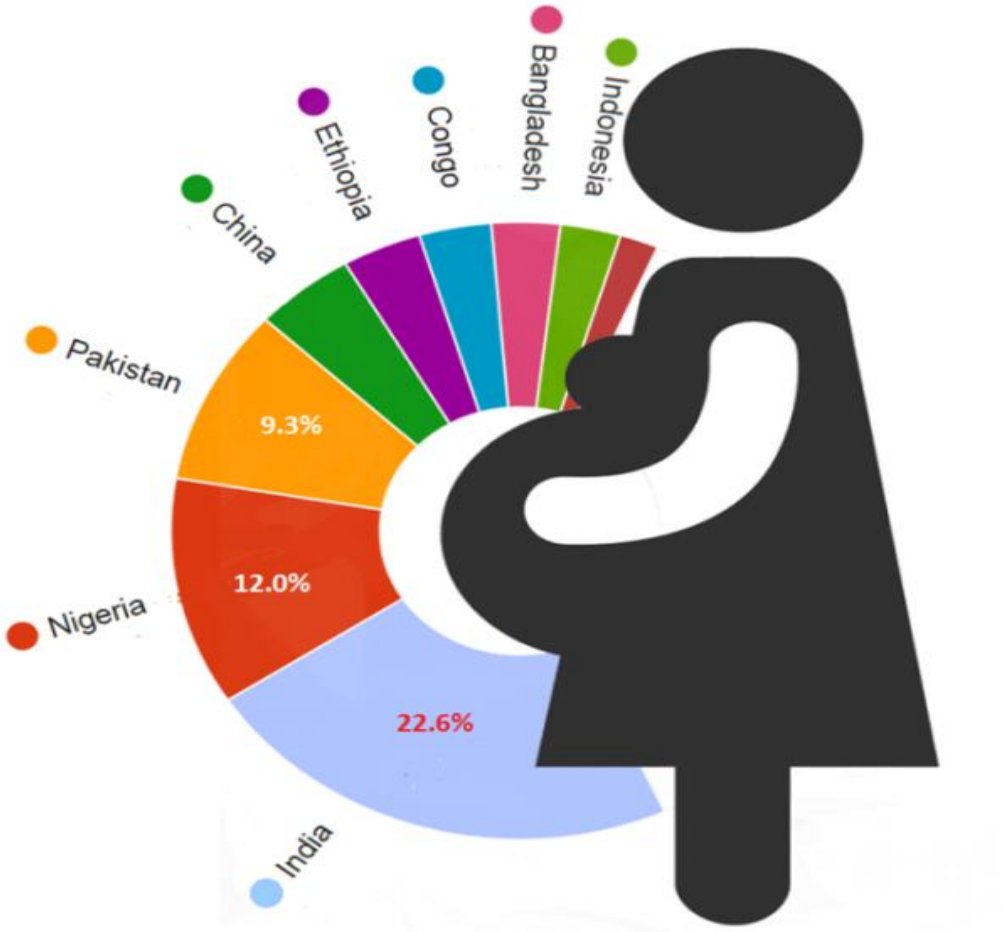


Figure 1.3 Prevalence of neonatal anoxia worldwide [4]

However, India is the tenth country that accounts for more than 65% of all intrapartum-related neonatal deaths (deaths in the first week of life) as a result of birth asphyxia [5-7]. Following an episode of asphyxia, perfusion to vital organs is compromised, primarily due to the diving reflex, which allows a preferential diversion of blood to the brain, adrenals and heart rendering other organs susceptible to ischaemic damage and culminating in multiple organ dysfunctions [8].

1.4 Clinical diagnosis

1.4.1 APGAR score

The Apgar score was developed by Virginia Apgar [9]. APGAR referred to as an acronym for Appearance, Pulse, Grimace, Activity, and Respiration. The Apgar test is usually given to a baby twice: once at 1 minute after birth, and again at 5 minutes after birth.

1.4.2 Sarnat score

Sarnat scale was developed by Sarnat [10]. Clinical signs of hypoxic-ischemic encephelopathy (HIE), by severity, are categorized as mild (stage 1), moderate (stage 2) or severe (stage 3).

1.4.3 Pulse Oximetry

The pulse oximetry technique is employed to measure the degree of hypoxia or anoxia has become a vital tool in pediatrics and neonatology [11]. It is one of the most reliable ways to monitor the blood circulation of a patient and it has the advantage of being a non-invasive procedure. Studies in human fetuses have shown that anoxia manifests when the peripheral oxygen saturation drops below 30% of normal and remains there for more than 10 min.

1.4.4 Analysis of blood lactate & pH

During anoxic injury, fetal blood pH drops below the critical value of 7.2 (a condition is known as metabolic acidosis), which is characteristic of anoxia [12]. A critical deprivation in oxygen and substrate delivery leads to hindrance in aerobic metabolism through Kreb's cycle, and tissues need anaerobic metabolism to meet their energy requirements. This incident refers to an increase in the production and accumulation of blood lactate [13]. A

previous report has demonstrated a correlation between the degree of acidosis and the neonatal neurological outcome [14].

1.5 Models for neonatal anoxia

A previous study has stated that a newborn rat brain is comparable to that of a 24-week-old human fetus and the brain of a ten-day-old rat is comparable to the brain of a newborn human [15]. Therefore, three different methodologies and periods of development have been primarily used to cause oxygen deprivation in neonate rats to mimic the clinical condition during and after anoxia.

1.5.1 Hypoxia–ischemia

Seven-day-old rats undergo occlusion of one of the common carotid arteries and are placed in a chamber containing 8% oxygen and 92% nitrogen for periods ranging from 30 min to 3 h [16, 17].

1.5.2 Perinatal asphyxia

Female about to give birth is decapitated or anesthetized, and its uterus (containing the pups) is removed and immersed in the saline solution for 16–25 min [18].

1.5.3 Neonatal anoxia

Newborns pups (at approximately 30 h of age) are placed in a semi-hermetic chamber with continuous 100 % nitrogen gas flow into the chamber for 10 min each at an interval of 24 h are used. Further, different parameters of temperature, pressure, and flow are used [19, 20]. The third model (neonatal anoxia) is the model of choice for researchers interested in the behavioral outcomes resulting from oxygen deprivation, which includes attention deficit

hyperactivity disorder (ADHD), spatial memory learning deficits and abnormal responses to stress.

1.6 Current treatment for anoxia

At present, there is no US FDA approved treatment available to treat neonatal anoxia. However, treatment involves the use of non-pharmacological and pharmacological interventions [2]. Potential pharmacological interventions include the use of modulators of glutamate, monoamines (dopamine, norepinephrine, and serotonin), GABA, adenosine and growth factors [21]. Moreover, no pharmacological neuroprotective treatment is clinically available for treatment of anoxia. The rarity of effective pharmacological interventions is due, in part to the overall complexity and an incomplete understanding of the fundamental pathways involved in progressive anoxia-induced cell death. Non-pharmacological strategies like hypothermia have been considered more desirable. It has been observed that for every 1°C lowering of the core temperature cerebral metabolism is reduced by approximately 7%, with consequently a lower glucose and oxygen demand [22]. A reduction of 3–4 °C core temperature is associated with a reduction in free radicals and glutamate levels, protecting mitochondrial function and maintaining cerebral high-energy phosphate levels [23]. However, hypothermia has not been considered completely neuroprotective, and 40-50% newborns still suffer from the major neurological problem. Thus, pharmacological intervention is urgently required for inhibiting progression of brain damage following anoxia [24]. However, Xenon being a NMDA receptor antagonist along with therapeutic hypothermia has been shown to protect against nerve cell death after anoxia through inhibiting a key step in the excitotoxic pathway [25]. Therefore, there is need for a potential therapeutic intervention which can minimize the neuronal cell death and can improve

neurobehavioral outcome after anoxia to improve the life span. For this purpose, we need to understand the pathophysiology of anoxia.

1.7 Pathophysiology of neonatal anoxia

There has long been a search for therapies that can either prevent injury progression or enhance repair of the immature brain, hopefully improving long-term motor and behavioral outcomes [26]. Therefore, to know the pharmacological targeting strategies a proper understanding of pathophysiology within and after anoxia is necessary.

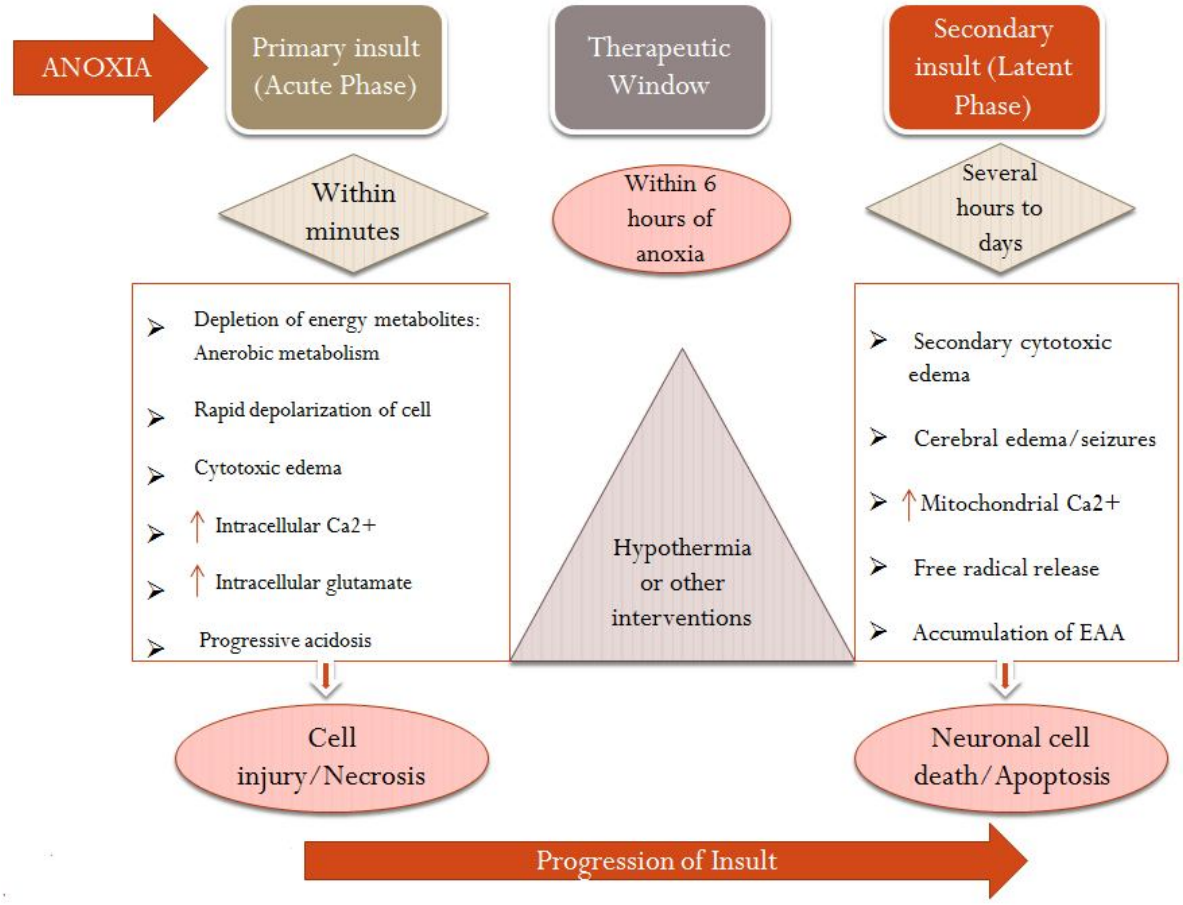


Figure 1.4 Insult progression with cell death transition after neonatal anoxia

Anoxia leads to the occurrence of a first insult (Primary) within minutes manifested by excitotoxicity due to increase in intracellular excitotoxic amino acid (EAA) i.e. glutamate levels leading to rapid depolarization of cell through intracellular Ca^{2+} overload. Further, there is an occurrence of energy failure or drastic decrease in cellular ATP and glucose levels due to a rapid decrease in glucose uptake following anoxia. This declination in ATP levels affects the activity of Na^{+} K^{+} ATPase, which consumes up to 70% of cellular ATP. The rise in cellular Na+ due to a decreased activity of Na^{+} K^{+} ATPase causes Ca^{2+} influx through a Na^{+} Ca^{2+} exchanger and Ca^{2+} Mg^{2+} ATPase leading to cytotoxic edema which ultimately leads to necrotic cell death [27]. Further, after several hours to days, there is a progression of insult to secondary phase in the form further increase in cytotoxic edema mediated through increased mitochondrial Ca^{2+} overload. Furthermore, there is an increased free radical generation which leads to oxidative stress and further accumulation of excitotoxic amino acid (EAA) ultimately leading to apoptotic cell death [28]. Therefore, the essential aspect of this insult progression is the transition of cell death from necrosis to apoptosis. However, the therapeutic intervention of 6 hr. resides within this transitional cell death. So the conclusive remarks on pathophysiology are that:

1. There is a transition of insult from primary to secondary phase.
2. The transition of cell death takes place from necrosis to apoptosis.
3. Intervention resides within primary and secondary insult.
4. Mitochondrial pathophysiology may be principally involved in the insult progression and the process of necrosis to apoptosis.

Therefore the main aim here is to subside this insult progression and ultimately inhibit the transition and attenuate apoptosis. Further, we need to understand the types and mechanism of apoptosis taking place in developing brain after anoxia.

1.8 Apoptosis

Apoptosis is a cell death program that is central to cellular and tissue homeostasis, involved in many physiological and pathological processes [29]. It can take place within two different pathways.

1.8.1 Extrinsic pathway of apoptosis

The extrinsic apoptotic pathway is initiated by the engagement of a transmembrane death receptor (for example, FAS) by an extracellular ligand (For example, FASL), which leads to the assembly of the death-inducing signaling complex (DISC) [30, 31]. The DISC then activates an initiator caspase (caspase-8/10), which triggers the enzymatic cascade that leads to activation of downstream targets such as procaspase-3 to initiate a caspase cascade [31].

1.8.2 Intrinsic pathway of apoptosis

The intrinsic pathway or mitochondrial-linked apoptotic pathway is activated by stimuli that lead to the permeabilization of the outer mitochondrial membrane (OMM) and the release of proteins from the mitochondrial intermembrane space (IMS). Further, numerous proteins that initiate or regulate apoptosis, including cytochrome-C, second mitochondria-derived activator of caspases (Smac/DIABLO), AIF, Bcl-2 family proteins and caspases-9/3 [32]. Previous reports have shown the involvement of mitochondrial-mediated apoptotic cell death after perinatal brain injury [28, 33, 34]. Therefore our main concern is to study the involvement of mitochondria in the pathophysiology of anoxia.

1.9 Role of pro-apoptotic and apoptotic mediators in mitochondrial-linked cell death

1.9.1 Caspases

The name caspase is derived from Cys-dependent Asp-specific protease. Caspases are the central molecules involved in initiation and execution of apoptosis [35]. Caspases are processed through proteolytic cleavage minimum at two sites containing aspartate residues. This leads to the removal of the prodomain as well as the linker region from the proenzymes and results in the formation of a heterodimer containing one small and one large subunit. The apoptotic mammalian caspase is generally divided into two classes: The initiator caspase which includes caspase -2,-8,-9 and -10 and the effector caspase which includes caspase -3,-6,-7 [21].

1.9.2 Bcl-2 family proteins

The proteins of the Bcl-2 family are prime regulators of mitochondrial stress, and their main function is to control mitochondrial permeability and particularly, the release of apoptogenic proteins from this organelle. The Bcl-2 family of proteins can be divided into three groups based on their structure and their role in apoptosis. The anti-apoptotic proteins like Bcl-2, Bcl-xL, Pro-apoptotic proteins such as Bax, Bak, Bcl-Xs and BH3-only proteins (BOP), which include Bid, Bad, Noxa, Puma. These proteins induce apoptosis by activating pro-apoptotic proteins like Bax or by inhibiting anti-apoptotic proteins like Bcl-2 [29]. Bax translocation from cytosol to mitochondria causes the release of apoptogenic factors such as cytochrome-C by permeabilization of the mitochondrial outer membrane while Bcl-2 inhibits MPT opening [36].

1.10 Pathophysiological changes in mitochondrial molecular machinery after anoxia

Mitochondria are marked to be the powerhouse of the cell due to their high efficiency in utilizing O_2 and substrates such as glucose and pyruvate to produce cellular energy in the form of ATP [37]. Mitochondria are composed of two membranes, an intermembrane space, and an internal ‘‘matrix.’’ The molecular machinery for energy production, the electron transport chain (ETC), is organized in an assembly line-like manner within and across the inner mitochondrial membrane. The ETC consists of five protein complexes [38]. Three of the complexes (I, III, and IV) pump protons (H^+) outwardly across the inner membrane to support the proton motive force that leads to generating a membrane potential across the membrane known as mitochondrial membrane potential (MMP). This MMP allows the protonmotive force driven through complex-V for the production of ATP at complex V (ATP synthase) to produce ATP [39]. Mitochondria need respiratory substrates and oxygen, the supply of which stops during hypoxia/ischemia, thus blocking ATP synthesis. In these conditions, the mitochondrial ATP synthase reverses direction and starts working as an ATPase, hydrolysing glycolytic ATP [28], and thus accelerates the depletion of cellular ATP. In the mitochondria, complex-I and complex-III are the primary sites of reactive oxygen species (ROS) [superoxide radical (O^{2-})] generation [40]. Normally, cellular antioxidant systems effectively remove ROS. The superoxide may be converted to hydrogen peroxide (H_2O_2) by MnSOD within the mitochondria or react with NO to generate peroxynitrite (ONOO-) [41]. However, during hypoxic/anoxic injury, ROS production by the mitochondria overwhelms antioxidant capacities, leading to cellular DNA damage, mitochondrial lipid peroxidation, disruption of Ca^{2+} homeostasis and mitochondrial membrane depolarization [42]. Peroxynitrite (PN), formed by the diffusion rate-limited

combination of nitric oxide ($NO^{\cdot}$) and superoxide ($O^{2\cdot-}$) free radicals, has been proposed to be a key contributor to posttraumatic oxidative damage. This is mainly because of its highly reactive decomposition products nitrogen dioxide ($^{\cdot}NO_{2)}$ hydroxyl radical ($^{\cdot}OH$) and carbonate radical ($CO_3^{\cdot}$). These PN-derived radicals can oxidize proteins, nitrate tyrosine residues, induce cell membrane lipid peroxidation and cause single-strand DNA break [43].

Mitochondria play the principal role in regulating cellular Ca^{2+} sequestration in physiological conditions. However, neuronal mitochondria take up Ca^{2+} through the so-called Ca^{2+} uniporter and slow efflux via the Ca^{2+}/Na^{+} antiporter or by Na^{+} independent mechanisms [44]. During pathological conditions like anoxia, the increased cellular overload of mitochondria due to overactivation of EAA is handled by mitochondria. As a result, mitochondria become overloaded with Ca^{2+} due to inactivation of Ca^{2+}/Na^{+} antiporter. To exclude this Ca^{2+} a pore is formed known as mitochondrial permeability transition (MPT) pore which allows the molecules of 1500 daltons or smaller to pass through the usually impermeable inner mitochondrial membrane. Further a resultant rupture of the outer mitochondrial membrane caused by osmotic swelling in the influence of Bax takes place [45]. The structure and composition of the MPT pore include both inner membrane proteins, like adenine nucleotide translocator (ANT), and outer membrane proteins, such as porin (voltage-dependent anion channel; VDAC) [46]. However, Bcl-2 suppress pore formation by ANT [47].

MPT causes the leakage of apoptogenic factors such as cytochrome-C through the mitochondrial intermembrane space proteins. 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].

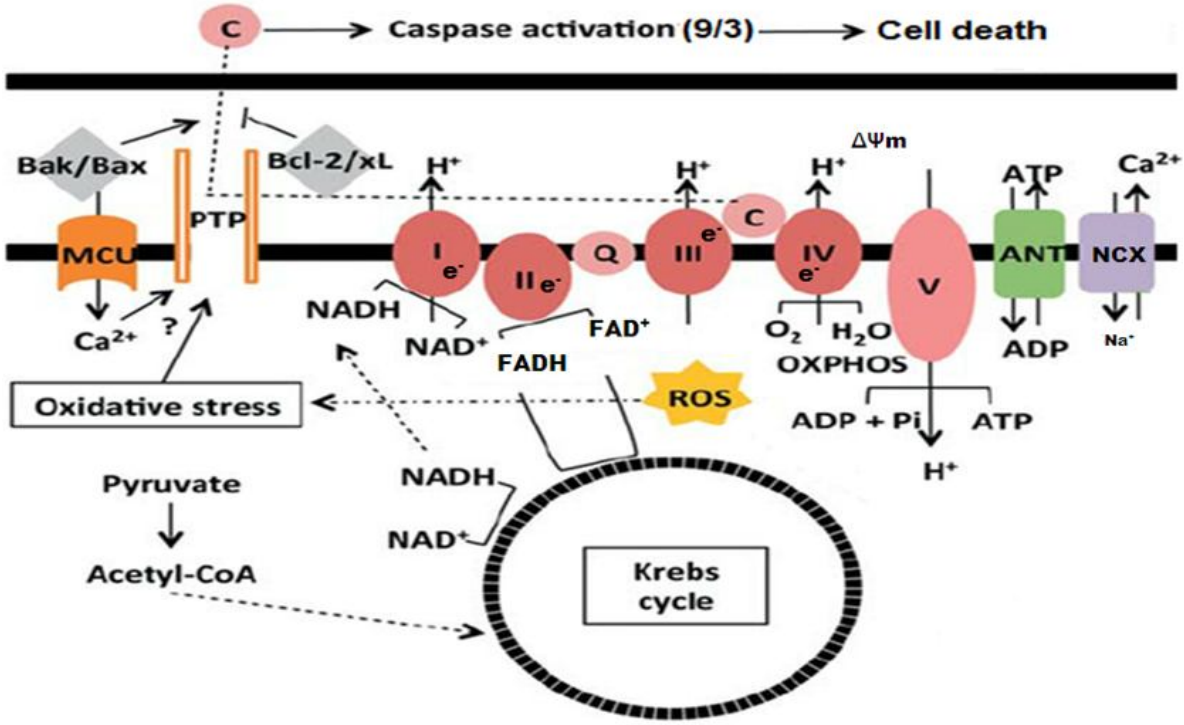


Figure 1.5 Mitochondrial oxidative stress after anoxia

1.11 Hypothesis

The study focuses on directly targeting mitochondrial dysfunction as a novel therapeutic intervention for anoxic injury. The fundamental concept underlying this study is that anoxia leads to a pathological increase in cellular Ca^{2+} under the influence of EAA which ultimately leading to alteration in MMP. This altered MMP leads to mitochondrial Ca^{2+} cycling/overloading and increase in reactive oxygen species/nitrogen species (ROS/RNS) production. Further, in response to the Ca^{2+} cycling/overloading Bax translocation takes place in the mitochondrial outer membrane, and overall there is a formation of transition pore in mitochondrial membrane which ultimately leads to the release of cytochrome-C and mediates mitochondrial dysfunction mediated apoptosis and long-term neurobehavioral impairments.

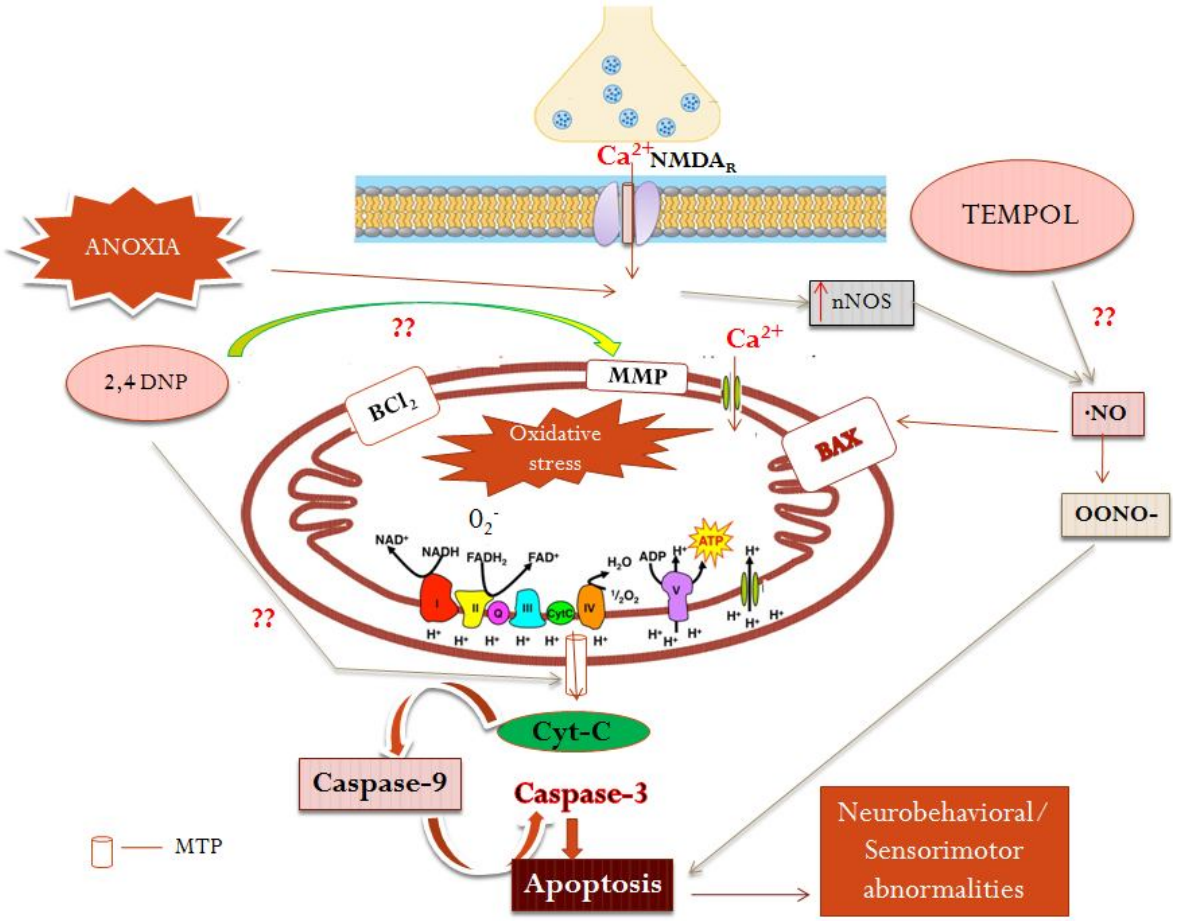


Figure 1.6 Proposed hypothesis

1.12 Proposed treatment strategies for neonatal anoxia

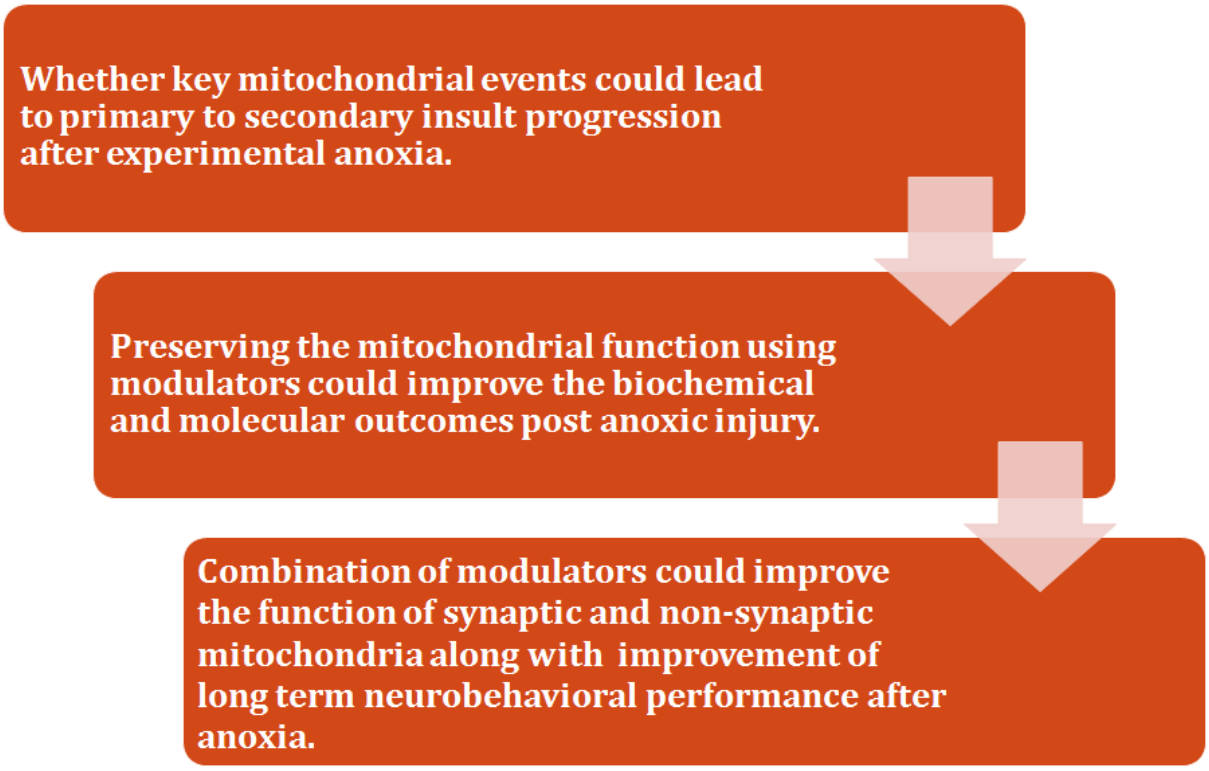
Therefore, we hypothesize that drugs which prevent or reduce mitochondrial Ca^{2+} overload and scavenge $^{\cdot}NO$ may improve the mitochondrial function and can be a suitable candidate for the treatment of anoxia. Our current approach in this context is to use mitochondrial uncouplers as well as nitroxide scavenger to maintain mitochondrial homeostasis following anoxia.

2,4 Dinitrophenol: 2,4-DNP is a proton ionophore, which causes mild mitochondrial uncoupling by decreasing the proton motive force across the mitochondrial inner membrane

and uncouples ETC to oxidative phosphorylation. This in turn leads to a reduction in MMP and could further prevent sequestration of Ca^{2+} out of the cytosol into mitochondria (Ca^{2+}cycling/overloading) and production of reactive oxygen species [50, 51].

Tempol: 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].

1.13 Key interrogations



1.14 Objectives of thesis

Objective 1. Evaluation of temporal pathological changes in mitochondrial bioenergetics after anoxia.

Objective 2. To study the role of mitochondrial-linked apoptosis in insult progression from day-1 up to day-7 (i.e., from primary insult to secondary insult) post experimental anoxia injury.

Objective 3. To study the alterations in mitochondrial activity in the cerebrospinal fluid (CSF) of anoxic neonates.

Objective 4. Evaluation of mitochondrial modulators like 2, 4 DNP or tempol that can maintain mitochondrial function and improve neurobehavioral outcome.

Objective 5. To study the combinatorial effect of 2,4 DNP and tempol in two different mitochondrial fractions (i.e., synaptic and non-synaptic) from the cerebral cortex and to evaluate long-term anoxia-induced neurobehavioral impairments.