4.1 Abstract

Neonatal anoxia is characterized by different degrees of hypoxia-ischemia, manifested by the severity of the underlying brain cell death. The intrinsic pathway of apoptosis has been projected as one mechanism of cell death after asphyxia. We have used enzyme-linked immunosorbent assay to study levels of cytochrome-C, caspase-9 and caspase-3 in the cerebrospinal fluid (CSF) of children with asphyxia (n=42) and control (n=17) at birth. Apgar scores<7 at 5 min were considered as asphyxia. Further, by sarnat scoring the asphyxia neonates were further divided into different stages of HIE. CSF sample was collected as sample-1 on day-1 and sample-2 on day-7 after asphyxia. We observed a significant ($P<0.05$) increase in the levels of cytochrome-C, caspase-9 and caspase-3 in the HIE s-III as compared to s-II and s-I and a marked increase on control group in sample-1. Further, the levels of cytochrome-C, caspase-9, and caspase-3 were markedly elevated in HIE s-III as compared to s-II and s-I in sample-2 which depicts that mitochondrial dysfunction persisted up to our study. Interestingly, we observed a significant ($P<0.05$) elevation in the levels of caspase-9 in the sample-2 as compared to sample-1 which is a principal factor responsible for promoting intrinsic pathway of apoptosis. Therefore, the increase levels of expression of cytochrome-C, caspase-9 and caspase-3 in the CSF of neonates with different stages of HIE after asphyxia are indicative of mitochondrial dysfunction and intrinsic pathway of apoptosis.

Keywords: Cerebrospinal fluid (CSF), Hypoxic ischemic encephalopathy (HIE), cytochrome-C, caspase-9, and caspase-3, apoptosis.

4.2 Introduction

Birth asphyxia with progression to Hypoxic–ischemic (HI) brain injury may result in permanent neurological and cognitive impairment in newborn infants [110]. India is the tenth country that accounts for more than 65% of all intrapartum-related neonatal deaths (deaths in the first week of life) imparted to a result of birth asphyxia [1]. The pathogenesis of cell death after a hypoxic-ischemic injury to the developing brain is complex and still not completely understood.

However, it has been stated that hypoxia-ischemia brain injury followed by resuscitation appears to progress in two distinct phases, as a primary cellular injury during the period of the insult due to necrosis and later to a delayed secondary injury 8-48 hours later after the initial insult by apoptosis [111]. The occurrence of this delayed or secondary energy failure during the recovery period after birth injury is due to lack of perfusion, energy failure, glutamate excitotoxicity and also subsequent reperfusion which serves as the ultimate cause of brain damage [112-115]. Previous studies have reported that after hypoxia-ischemia, the mechanisms of programmed cell death are activated, and a considerable proportion of cells die not by necrosis but by apoptosis [111, 116]. Caspases are a group of cysteine proteases that are essential for initiating and executing apoptosis. Apart from this cytochrome-C is an integral component of the mitochondrial electron transport chain, participating in oxidative phosphorylation and ATP synthesis. In response to apoptotic stimuli, it is released from the mitochondria into the cytosol. Released cytochrome-C results activation of factor (Apaf-1) and caspase-9 leading to the formation of a high-molecular-mass cytoplasmic complex referred to as apoptosome, which later activates Caspase-3 and finally result in apoptotic cell death [85-87]. Thus, the present study hypothesizes that there

is an anoxia-induced release of cytochrome-C in CSF which can initiate mitochondrial dysfunction and ultimately neuronal cell death in newborn.

4.3 Material and methods

4.3.1 Patients

This prospective observational study was conducted at Department of Pediatrics, Institute of Medical Sciences and Department of Pharmaceutics, Indian Institute of Technology, Banaras Hindu University. Informed consent was taken from all parents before inclusion in the study. The study was approved by the Institute Ethics Committee. The study has been endorsed by the Institute Ethics Committee. The group was divided into birth asphyxia (n = 42), and controls (n = 17).

4.3.2 Inclusion Criteria

The study population comprised of full-term newborns with a gestational age ≥ 37 weeks were included in the study group based on the following criteria for asphyxia:

Evidence of fetal distress

Umbilical artery pH < 7

Apgar score <7 at 5th minute

Postnatal resuscitation with positive pressure ventilation

The asphyxia group was further divided into different stages of HIE (s-I,s-II, and s-III) by sarnat and sarnat scoring [10].

4.3.3 Exclusion Criteria

Antenatal Babies with sepsis, respiratory distress syndrome, metabolic disorders and major congenital malformations, were excluded from the study.

4.3.4 CSF Sampling

Approximate 1.5 ml of cerebro-spinal fluid (CSF) samples were collected two times by spinal taps. Firstly, within 24 hours of birth in intramural babies and on the day of admission in extramural infants and second, seven days after the first sample collected. Cytochrome-C, Caspase 9 and Caspase 3 were estimated in CSF. CSF was spun at 4000 rpm for 10 min. Cell counts and CSF proteins were analyzed as routine clinical samples. Samples were stored at − 80 °C until analysis (Cytochrome-C, Caspase-9 and Caspase-3).

4.3.5 Cytochrome-C, caspase-9 and caspase-3 measurement

Levels of human cytochrome-C activated caspase-9 and activated caspase-3 were measured in 70 CSF samples using enzyme-linked immunosorbent assays (ab119521, ab119508, and ab168541; Abcam Plc., Cambridge, MA, USA) according to the manufacturer's instructions. The detection level for cytochrome C was 0.05 ng/ml, for activated caspase-9 was 0.4 ng/ml and 2 µg/ml for caspase-3.

4.3.6 Statistical Analysis

Data are expressed as means ± SD. Groups were compared using the Kruskal – Wallis or the Mann – Whitney U test. $P<0.05$ was considered significant.

4.4 Results

Figure 4.1 depicts that the concentrations of cytochrome-C, caspase-9 and caspase-3 were increased in infants with s-III HIE when compared to control ($^{a}P<0.05$), s-I HIE ($^{b}P<0.05$) and s-II HIE ($^{c}P<0.05$) in sample-1. Within the asphyxia group, infants with s-III HIE had significantly increased concentrations of cytochrome-C, caspase-9, and caspase-3 when compared to s-I ($^{b}P<0.05$) and s-II ($^{c}P<0.05$) HIE groups in sample-2. A post hoc analysis

revealed that there was a significant increase in the levels of caspase-9 in sample-2 compared to sample-1.

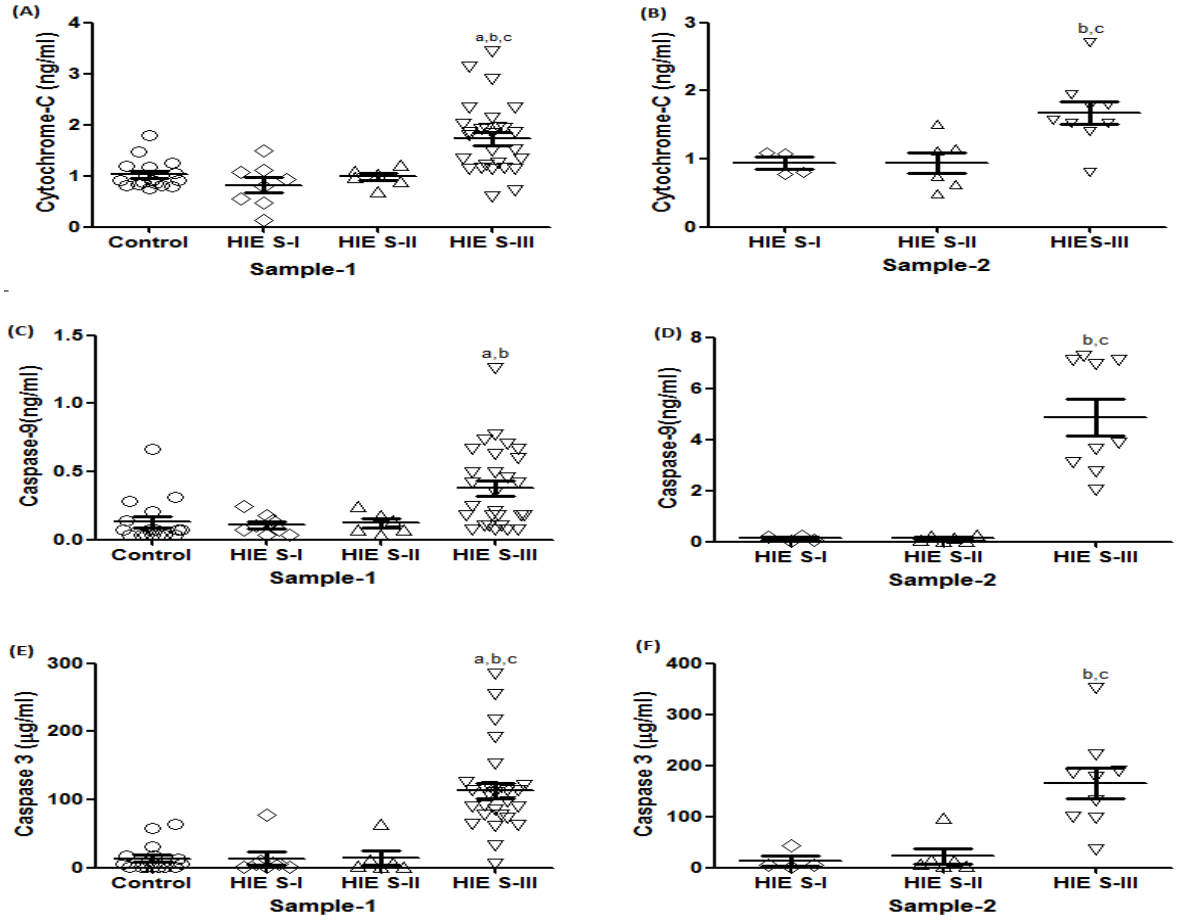

Figure 4.1 Levels of cytochrome-C, caspase-9, and caspase-3 in CSF of control and different stages of HIE in sampe-1 and comparison of the concentration of cytochrome-C, caspase-9, and caspase-3 within HIE group sample-2. All values were expressed as mean ± SD. $^{a}P<0.05$ compared to control, $^{b}P<0.05$ compared to Stage-I HIE and $^{c}P<0.05$ compared to Stage-II HIE groups [Kruskal – Wallis or the Mann – Whitney U test].

4.5 Discussion

We for the first time report that cytochrome-C, caspase-9 and caspase-3 are increased in cerebrospinal fluid of neonates following birth asphyxia. These infants may later develop encephalopathy following asphyxia. Neuronal cell death post HIE is thought to be comprised of primary cell death (necrosis), caused by mechanical destruction, and

subsequent or delayed cell death (apoptosis), caused by several pathologic cascades initiated by trauma [117]. However, the mechanisms that lead to irreversible brain injury and apoptosis in HIE are complex and still partly unknown. In humans, these cascades include ischemia, excitotoxicity and inflammation, each of which can trigger apoptosis [118]. Apoptosis is a tightly regulated homeostatic process necessary in embryologic development and dysregulation of apoptosis can contribute to many human disease processes [119]. Apoptosis has been considered to be the more prominent type of cell death in the pediatric as well as adult patients [117]. We have recently reported the involvement of mitochondrial dysfunction linked apoptosis through cytochrome-C and caspase (caspase-9/3) in insult progression after anoxic injury [120]. However, several other neural and biochemical markers in CSF have been found to play an important role studied in birth asphyxia [111, 121, 122]. Anoxia caused a significant increase in the levels of cytochrome-C, caspase-9 and caspase-3 in the CSF of neonates. Further, previous reports have suggested that secondary brain injury was preceded by impairment of mitochondrial respiration, signs of membrane permeability transition, intramitochondrial accumulation of calcium, changes in the Bcl-2 family proteins and release of proapoptotic proteins (cytochrome c,apoptosis inducing factor), the downstream activation of caspase 9 and caspase-3 occurs after hypoxia-ischaemia in rats [120, 123, 124]. We observed a marked increase in the levels of expression of cytochrome-C, caspase-9 and caspase-3 in sample-1 and sample-2 collected on d-1 and d-7 respectively, post-anoxic injury [125]. It was interesting to note that the levels of cytochrome-C, caspase-9, and caspase-3 were significantly increased in the s-III HIE as compared to s-I and s-II HIE groups in both samples depicting a more severity of insult post-anoxia in s-III HIE. Further, a previous study has reported the involvement of reactive

oxygen species (ROS) in activation of these markers which causes mitochondrial-linked apoptosis after an anoxic injury during HIE [126]. These observations suggest the role of mitochondria being an important therapeutic target for anoxia. Currently, neuroprotective interventions are limited to first six hours of age. Ongoing neuronal injury and beyond the first week of age suggests that window of opportunity for therapeutic neuroprotection may be longer.