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Research Article

Comparison of cerebrospinal fluid Cytochrome-c and Caspase-9 as biomarkers for newborns with hypoxic ischemic encephalopathy with non-asphyxiated babies and followup of these biomarkers after day 7

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ABSTRACT

Background: There are very less previous study for cytochrome–c and caspase-9, the key players in apoptotic cell death, in human newborns. The objective was to measure the level of cerebrospinal fluid biomarkers cytochrome –c and caspase -9 in newborns with hypoxic ischemic encephalopathy and comparison with clinically suspected sepsis controls and to compare these after 7 days.
Methods: We compared 50 hypoxic babies with 20 newborns with clinically suspected sepsis at median age of day-3 and 9 in cases and day-1 in controls.
Results: In the present study in sample-1 we observed a significant increase in the levels of cases cytochrome c (1.46 ± 0.71 ng⁄mL) and caspase- 9 (0.29 ± 0.27 ng⁄mL) when compared to controls cytochrome-c (1.02+0.27 ng⁄mL) and caspase -9 (0.13+0.16 ng⁄mL) with significant p-value of 0.001 and 0.009 respectively. In sample -1 Cytochrome-c, P-value was significant when compared stage –III (1.74 ± 0.68) with stage-I (0.82 ± 0.43) and stage –II (0.99 ± 0.18). Similarly in Caspas-9 P-value was significant when compared between stage-III (0.38 ± 0.30) with stage-I (0.11 ± 0.07). In sample -2 P- value was significant when compared stage –III (1.68 ± 0.50) with stage-I (1.01 ± 0.14) and stage –II (0.94 ± 0.38). Similarly in Caspas-9 P-value was significant when compared between stage-III (4.84 ± 2.44) with stage-I (0.13 ± 0.10) and stage –II (0.13 ± 0.11).
Conclusions: First time done in human newborns with asphyxia, showing that CSF Cytochrome- c and Caspase 9 increases significantly. In sample-2, the caspase 9 levels showed a further increase, whereas cytochrome c levels decreased from the sample 1 value indicating that neuroprotection time should be increased.

Keywords: Cytochrome-C, Caspase-9, Hypoxic ischemic encephalopathy, Newborns

INTRODUCTION

Birth asphyxia is most common and important preventable cerebral injury in neonatal period. Moderate to severe hypoxic ischemic encephalopathy (HIE) occurs at a rate of approximately 1–2 per 1,000 term live births with a total HIE incidence of 3-5 cases per 1,000 term live births.[1-3] Incidence is up to 10-fold higher in developing countries and globally, 23% of the 4 million annual neonatal deaths are attributed to birth asphyxia.[4] Early prediction of insult, diagnosis, management and proper follow up only can improve asphyxia.

Mechanisms of neuronal injury in perinatal asphyxia include necrosis, autophagia,[5] apoptosis[6] and cell death followed by rapid depletion of high-energy phosphate reserves including adenosine triphosphate.[7] To date, potential biomarkers have been identified in neonates with HIE. These biomarkers were obtained from CSF, serum, and urine and include S100B, neuron specific enolase, umbilical cord Interleukin-6, TNF-α, CPK-BB, glial fibrillary acidic protein, myelin basic protein, caspases and cytochrome c, Ubiquitin carboxyl-terminal hydrolase L1 (UCHL-1).[8-13]

Since caspases and cytochrome-c are the executioners of apoptosis, they are the key players in apoptotic cell death. Caspase-9 is major initiator caspase in this process. There are very less previous study for cytochrome–c and caspase-9 in human newborns. The purpose of this study was to determine the levels of cytochrome-c and caspase -9 as diagnostic markers in different stages of HIE, and their significance as prognostic markers.

METHODS

This prospective observational study was conducted in the Department of Pediatrics, Institute of Medical Sciences and Department of Pharmaceutics, Indian Institute of Technology, Banaras Hindu University. The period of study extended from December 2012 to July 2014. Informed consent was taken from all parents before inclusion in the study. The study was approved by the Institute Ethics Committee.

Inclusion Criteria

The study population comprised of 50 term newborns (37-41weeks) who suffered from perinatal asphyxia. Perinatal asphyxia was defined as the need for delivery room resuscitation and development of clinical manifestations suggestive of hypoxic ischemic encephalopathy (HIE). Both inborn and outborn babies were included. Control group comprised of 20 newborns who were evaluated for possible sepsis but had negative laboratory work up including blood culture.

Exclusion Criteria

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

One ml of Cerebrospinal fluid (CSF) sample under aseptic precautions was collected within 24 hours of birth in intramural neonates and on the day of admission in extramural neonates. The second sample of CSF was collected in cases after 7 days of first collection. Samples were stored immediately at -20^{0}C until further analysis. Newborns were managed as per unit protocol. Progression of HIE into different stages was categorized according to the classification of Fenichel (modified by Ellis and Costello).

Enzyme-linked immunosorbent assay (ELISA) by using the enzyme assay kit were used for estimation of Caspase 9 and Cytochrome c. The average absorbance values for each set of duplicate standards and samples was calculated and standard curve was made by plotting the mean absorbance for each standard concentration on the ordinate against the Human Cytochrome C concentration on the abscissa made.

Statistical analysis was done using the SPSS version 16.0 (NY, USA). Data were expressed as mean ± standard deviation for continuous variables and percentage for categorical variables and were analyzed using Student's t-test or Mann–Whitney U-test, Fisher's exact test respectively. Comparison among multiple groups was done using ANOVA and *post hoc* Bonferroni test. Pearson correlation coefficient was calculated for different variables. *P* value of <0.05 was considered statistically significant.

RESULTS

Among cases, 8 (16%) neonates progressed up to HIE stage I, 6 (12%) up to stage HIE II and 36 (72%) up to stage HIE III. During hospital stay, 16 (32%) neonates with HIE expired.

In our study population both cases and controls had a male preponderance. The incidence of perinatal asphyxia specially HIE stage III was significantly higher in unbooked mothers ($p<0.05$), extramural deliveries ($p<0.05$), and those born through SVD compared to emergency CS ($p<0.05$). There was no statistical difference in gravidity, parity, gestation, birth weight and delivery through MSAF between cases and controls (Table 1.) In sample 1, cases has significantly higher levels of Cytochrome C ($p<0.001$) and Caspase 9 ($p<0.001$) compared to controls (Table 2). Similarly in sample 2, the values of Cytochrome c and Caspase 9 were statistically significantly higher in stage III compared to stage I ($p<0.001$), II ($p<0.05$) and controls ($p<0.01$) (Table 3). There was no statistically significant difference among males and females biochemical markers.

Table 2: CSF Caspase 9 and Cytochrome c levels in study population (mean ± SD).

Parameter	Cases	Controls	p-value
Cytochrome c (ng⁄mL)			
Sample 1	1.46 ± 0.71 (n=42)	1.02 ± 0.27 (n=17)	0.001
Sample 2	1.31 ± 0.54 (n=19)	-	-
Caspase 9 (ng⁄mL)			
Sample 1	0.29 ± 0.27 (n=42)	0.13 ± 0.16 (n=17)	0.009
Sample 2	2.36 ± 2.91 (n=19)	-	-

Table 1: Baseline characteristics of the study population.

Parameters	Case (n=50)			Control (n=20)	Comparison between cases and controls (p-value)
	Stage I (n=8)	Stage II (n=6)	Stage III (n=36)		
Maternal Booked Unbooked	3 (37.5%) 5 (62.5%)	6 (100%) 0 (0%)	10 (27.77%) 26 (72.22%)	12 (60%) 8 (40%)	0.012
Gravida Median	1	1	1	1	0.645 (NS)
Parity Median	1	1	1	1	0.452 (NS)
Birth weight (g)	2800 ± 460	2883 ± 365	2788 ± 342	2830 ± 435	0.139 (NS)
GA (wk)	37.5 ± 1.1	37.8 ± 1.2	37.8 ± 1.3	38.2 ± 1.1	0.514 (NS)
Delivery Intramural Extramural	4 (50%) 4 (50%)	1 (16.67%) 5 (83.33%)	2 (5.56%) 34 (94.44%)	8 (40%) 12 (60%)	0.024
Mode of delivery SVD Cesarean Section	6 (75%) 2 (25%)	5 (83.33%) 1 (16.67%)	29 (80.56%) 7 (19.44%)	11 (55%) 9 (45%)	0.042
Sex Male Female	6 (75%) 2 (25%)	5 (83.33%) 1 (16.67%)	24 (66.67%) 12 (33.33%)	13 (65%) 7 (35%)	0.778 (NS)
Fetal distress Present Absent	3 (38%) 5 (63%)	1 (16.67%) 5 (83.33%)	24 (66.67%) 12 (33.33%)	0 (0%) 20 (100%)	0.0001
MSAF Present Absent	4 (50%) 4 (50%)	3 (50%) 3 (50%)	16 (44.4%) 20 (55.56%)	5 (25%) 15 (75%)	0.176 (NS)
Age of first sampling (days) Median (range)	3 (1-13) (n = 42)			1 (1-6) (n = 17)	0.012
Age of second sampling (days) Median (range)	9 (8-17) (n = 19)			-	-
Outcome Improved Expired	8(100%) -	5 (83.33%) 1 (16.67%)	21 (58.33%) 15 (41.67%)	20 (100%) 0 (0%)	0.001

Table 3: Comparison of CSF Caspase and Cytochrome c (mean ± SD) in HIE stages and controls.

	Cytochrome c (ng/mL)		Caspase 9 (ng/mL)	
	Sample 1	Sample 2	Sample 1	Sample 2
Controls	1.02 ± 0.27 (n=17)		0.13 ± 0.16 (n=17)	
Stage I	0.82 ± 0.43 (n=8)	1.01 ± 0.14 (n=4)	0.11 ± 0.07 (n=8)	0.13 ± 0.10 (n=4)
Stage II	0.99 ± 0.18 (n=6)	0.94 ± 0.38 (n=6)	0.12 ± 0.08 (n=6)	0.13 ± 0.11 (n=6)
Stage III	1.74 ± 0.68 (n=28)	1.68 ± 0.50 (n=9)	0.38 ± 0.30 (n=28)	4.84 ± 2.44 (n=9)

ANOVA (p value)	11.027 (<0.001)	8.192 (<0.001)	5.977 (<0.01)	33.147 (<0.001)
Stage I vs. Stage II	1.000 (NS)	1.000 (NS)	1.000 (NS)	1.000 (NS)
Stage I vs. Stage III	<0.001	0.023	0.032	<0.001
Stage I vs. Controls	1.000 (NS)		1.000 (NS)	
Stage II vs. Stage III	0.014	0.002	0.103 (NS)	<0.001
Stage II vs. Controls	1.000 (NS)		1.000 (NS)	
Stage III vs. Controls	<0.001		0.004	

DISCUSSIONS

In the present study we observed a significant increase in the levels of cytochrome c and caspase 9 in CSF of term newborns with perinatal asphyxia compared to controls. The markers were measured twice in cases, first at median age of 3 days and again at 9 days and once in controls at median age of 1 day. Both cytochrome c and caspase 9 levels were significantly higher in cases compared to controls on day 3 of age. On day 7 of age, the caspase 9 levels showed a further increase, whereas cytochrome c levels decreased from the sample 1 value. When biomarkers levels were analysed as per the severity of hypoxic ischemic encephalopathy (HIE), though there was no difference in cytochrome c and caspase 9 levels among stage 1 and stage II of HIE and controls, stage III showed a significant rise in the levels of biomarkers.

Mechanism of neuronal injury

The mechanisms that lead to irreversible brain injury and apoptosis in HIE are complex and still partly unknown. Apoptosis is the more prevalent type of delayed cell death in the perinatal brain and both caspase-dependent and caspase-independent mechanisms of apoptosis have been proven. Mitochondria are predominantly injured in both 1st and 2nd phase.[14] In later mechanism shift from aerobic to anaerobic metabolism and decrease in the ratio of phosphocreatine/inorganic phosphate, lactate formation with an unchanged intracellular pH will occur that contributes to additional brain injury. Oxidative stress is associated with inactivation of a number of enzymes,[15] including mitochondrial respiratory enzymes, low capacity of the antioxidant mechanism, high oxidative phosphorylation, high free iron producing hydroxyl radicals, high fatty acid content, high metabolism and low metabolic reserves, high oxygen consumption, and immaturity at birth.[16] Circulatory and endogenous inflammatory cells/mediators also contribute to ongoing brain injury.[17] This deficit in ATP production leads to loss of resting membrane potential,[18] disturbances in ionic homeostasis, membrane depolarisation, and an increase in extracellular glutamate concentration.[19]. This mechanism will results in over-activation of the ionotropic NMDA (N-methyl-D-aspartic acid).[20] AMPA/KA(Alpha-aminoacid-3-hydroxy-5-methyl-4-isoxazolepropionic acid/Kainic acid) receptors as well as the G-protein-linked glutamate receptors (mGluR),[21] inducing a massive influx of Ca2+ and Na+ into cells. Other factors i.e. excitatory amino acids, nitric oxide, inflammation, trophic factor withdrawal, and an increased pro- versus anti-apoptotic Bcl-2 protein ratio also have been found to trigger pro-apoptotic multidomain (Bax)-dependent mitochondrial outer membrane permeabilization (MOMP), Cytochrome c efflux activates caspase 9 and 3, leading to DNA fragmentation.[22] Asphyxia mechanism finally results in over-production of reactive oxygen species[23] that result in cell death.

Different biochemical markers i.e. S100B,[24] NSE,[25] IL-6, urinary uric acid, cardiac troponin-I, creatinine kinase,[26] Glial Fibrillary Acidic Protein (GFAP),[27] Cytochrome C, Caspase 9 have been studied as indicators of diagnostic and prognostic indicators in Hypoxic ischemic encephalopathy. In this study we evaluated the role of cytochrome c and caspase 9 in perinatal asphyxia.

Cytochrome c and caspase 9 levels in cerebrospinal fluid in perinatal asphyxia

After neonatal insult, markers of apoptosis (cleaved caspase-3) and necrosis (calpain-dependent fibrin breakdown product) can be expressed by the same damaged neurons, suggesting that the “continuum” could be explained by a failure of some dying cells to complete apoptosis, due to a lack of energy and mitochondrial dysfunction. Cytochrome c is primarily known as an electron-carrying mitochondrial protein that transport electrons from cytochrome c reductase (Complex III) to cytochrome oxidase (Complex IV). Hypoxia is known to lead to mitochondrial release of cytochrome c to the cytosol, there by initiating apoptotic cascade or intrinsic pathway.[28] In this cascade, cytochrome c binds to the scaffold protein Apaf-1 in a reaction that requires deoxy-ATP (dATP), after which procaspase-9 attaches, forming a complex known as the apoptosome,[29] and activates an initiator caspase 9. During hypoxia Apoptosis occurs after bound procaspase-9 is cleaved to become activated caspase-9resulting in cytoskelet al disruption, cell shrinkage, and membrane blebbing[30] and DNA fragmentation. Another determinant of apoptosis is loss of neuronal connections, which can continue days to weeks after injury, because groups of cells seem to commit to die.[31]

Caspases (cysteinyl aspartate-specific proteinases) are a family of 14 proteases that are activated by regulated

proteolysis of proenzymes. Caspase 9 can itselfalso induce permeability transition (PT)-independent cytochrome c release and serve to amplify further release of cytochrome c.[32]

The extrinsic apoptotic pathway is initiated by the ligation of death receptors with their cognate ligands, leading to the recruitment of adaptor molecules such as FAS-associated death domain protein and then caspase 8. This results in the dimerization and activation of caspase 8, which can then directly cleave and activate caspase 3 and caspase 7, leading to apoptosis.[33] Similarly in severe traumatic brain injurycaspase-9 and cytochrome c were present in the CSF of patients and higher level were associated with poor neurologic outcome.[34] Effect of neuroprotection in rodents and its association has been seen in few studies. The neuroprotective effects of Hsp70 overexpression, specifically by upregulating FLIP and sequestering Apaf-1, leading to reduced cleavage of caspase-8 and caspase-9 was observed.[35]With use of pentapeptide-based group II caspase inhibitor, TRP601/ORPHA133563 in rodents, mitochondrial release of cytochrome c, and apoptosis in vivo was inhibited and pharmacological inhibition of caspases for neuroprotection was proved.[36]

Very few studies have measured cytochrome c and caspase 9 levels in CSF of human newborns with perinatal asphyxia, though few studies are available in animals. Pothana, et al (1998) studied the mechanism of apoptosis in cultured kidney cells in rats and found that the levels of cytochrome c and caspase 9 were increased during hypoxia.[37] Feng et al, (2003) studied the effect of hypoxia in seven-day-old rats after ligating carotid artery and subjecting them to 2.5 h of 8% oxygen. Rats were given intraperitoneal injection of 10 mg/kg of TPCK (Tosyl-L-phenylalanyl-chloromethyl ketone) a proteinase inhibitor 3 h after hypoxia. Caspase 9 and caspase 3 activity in blood were measured enzymatically 24 h after injury. The authors found that the extent of injury and levels of these biomarkers were significantly higher in the rats not treated with TPCK.[38]

In previous studies in rats after induction of hypoxia, the features of apoptosis and necrosis were seen in majority of striatal cells. There was formation of a functional apoptosome, and activation of caspases 9 and 3, occurring simultaneously with loss of structurally intact mitochondria and loss of mitochondrial cytochrome c oxidase activity levels. Apoptotic cells and the cytochrome c mRNA expression in the cortical and hippocampal areas increased 6 hour after hypoxic injury, peaked at 1 day and then decreased gradually.[39]

Strength of the study was that we did not find any study which compared cytochrome c and caspase 9 in CSF of neonates with perinatal asphyxia. We measured cytochrome c and caspase 9 at an interval of 7 days to document any ongoing neuronal injury in perinatal asphyxia. On day 7 of age, the values of cytochrome c and caspase 9 were significantly higher in stage III in comparison to stage I ($p<0.03$) and stage II ($p<0.01$). Values of caspase 9 were also higher in stage III in comparison to stage I ($p<0.01$) and stage II ($p <0.01$). Our findings suggest that neuronal injury continues in perinatal asphyxia beyond first week of age. More studies are needed to confirm these findings. These observations have important therapeutic implications. Currently neuroprotective interventions are limited to first 6 hours of age. Ongoing neuronal injury during and beyond first week of age suggests that window of opportunity for therapeutic neuroprotection may be longer. Lack of study was that sample size was small and it is a costly investigation.

CONCLUSIONS

In the present study we compared the CSF levels of cytochrome c and caspase 9 between 50 term newborns with perinatal asphyxia and 20 controls. The markers were measured twice, first at median age of 3 days (sample 1) and again at 9 days (sample 2) in cases and at median age of 1 day (sample 1) in controls. A significant increase in the levels of cytochrome c and caspase 9 was documented in CSF neonates with perinatal asphyxia compared to controls. The increase in cytochrome c and caspase 9 levels was most marked in stage III HIE. Further trials may be required in future to include these investigation in management of perinatal asphyxia.

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Abbreviations: HIE- Hypoxic ischemic encephalopathy, CSF- Cerebrospinal fluid

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