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Cord Blood Brain Derived Neurotrophic Factor: Diagnostic and Prognostic Marker in Fullterm Newborns with Perinatal Asphyxia

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Abstract: This prospective case control study was designed to evaluate cord blood brain derived neurotrophic factor level in full term newborns with perinatal asphyxia as a marker of central nervous system insult and predictor of severity of hypoxic ischemic encephalopathy, with follow up of its level during the reperfusion phase. The study included twenty fullterm neonates with perinatal asphyxia (cases) and twenty controls. Cord blood samples were obtained at birth and peripheral blood samples at 72 h postnatal from cases only. Plasma brain derived neurotrophic factor level was measured using enzyme linked immunosorbent assay. The clinical severity of encephalopathy was graded based on Sarnat and Sarnat staging. Cord Plasma brain derived neurotrophic factor level was significantly increased among cases compared to controls. Among cases, brain derived neurotrophic factor level at delivery and after 72 h significantly correlated with the severity of encephalopathy according to Sarnat staging being higher as severity increases. Brain derived neurotrophic factor level significantly increased after 72 h of life compared to its level at delivery among cases. Brain derived neurotrophic factor levels at delivery and at 72 h postnatal were predictors of severe Sarnat stage and poor outcome. We concluded that brain derived neurotrophic factor level as a marker of central nervous system insult is increased in full term newborns with perinatal asphyxia. It can serve as an indicator for the severity of encephalopathy and adverse outcomes.

Key words: Brain derived neurotrophic factor, perinatal asphyxia, cord blood, hypoxic ischemic encephalopathy

INTRODUCTION

Hypoxic Ischemic Encephalopathy (HIE) is an important cause of mortality and morbidity in full-term newborns. Neurological handicaps develop in 25-28% of these infants (Hankins and Speer, 2003). Despite advances in supportive care, no treatments for HIE are available at present (Pimentel-Coelho and Mendez-Otero, 2009). To date, despite accurate perinatal and intra-operative monitoring, the post-insult period is crucial, since clinical symptoms and monitoring parameters may be of no avail and therapeutic window for pharmacological intervention (6-12 h) may be limited, at a time when brain damage is already occurring (Gazzolo *et al*., 2009). Neurotrophins are growth factors that regulate cell growth, differentiation and apoptosis in the nervous system (Assimakopoulou *et al*., 2007). They form a large family of dimeric polypeptides that include Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, NT-6 and NT-7 (Kolbeck *et al*., 1994). Neurotrophin expression is known to be up-regulated during injury and stress to the central nervous system (Hicks *et al*., 1999), resulting in significant changes in the levels and states of the neurotrophins (Stanzani *et al*., 2001) presumably affecting extent of injury and subsequent repair (Miyata *et al*., 2001). Neurotrophins (BDNF, NGF), their proteolytic processing and their receptors (TrkB and $p75^{NTR}$) would affect central nervous system cell behaviors including cell survival, proliferation, migration and differentiation, which in turn would have dramatic effects on the genes involved with synaptic maturation (Kim *et al*., 2004). Hypotension, cerebral ischemia and reperfusion are the main events involved in vascular auto-regulation leading to cell death and tissue damage. Reperfusion could be critical since organ damage, particularly of the brain, may be amplified during this period (Gazzolo *et al*., 2009).

The present study aimed at assessment of cord blood BDNF level in full term newborns with perinatal asphyxia, following up its level during reperfusion phase and studying its possible relation to the development and severity of HIE.

MATERIALS AND METHODS

This prospective case-control study was conducted at Gynecology and Obstetrics department and NICU at Ahmed Maher Teaching Hospital over a period of

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4 months from October 2007 till January 2008. The study was approved by the Ethical Committee of the Pediatric Department at Ain Shams University. An informed verbal consent was obtained from the parents before enrollment of patients.

Neonates included in this study were full terms ≥37 completed weeks of gestation, appropriate for gestational age. Newborns with congenital malformations, chromosomal abnormalities, suspected inborn error of metabolism, congenital heart disease, blood group incompatibility, sepsis, diabetic or preeclamptic mothers and those with multiple gestations were excluded from the study.

They were classified into 2 groups:

- Group1 (cases) included 20 neonates suffering from perinatal asphyxia as evidenced by the presence of at least two of the following conditions: Apgar score≤3 at 1 min or ≤6 at 5 min, umbilical cord arterial pH ≤7.2 with base deficit≥10 mmol L^{-1}, or the presence of postnatal clinical complications attributed to perinatal asphyxia, such as neurological manifestations, multiorgan failure, hypotension requiring inotropic support, severe apnea and oliguria (Glistrap *et al*., 1989)
- Group 2 (control) included 20 apparently healthy newborns without perinatal asphyxia

The following was done to all neonates included in the study:

Clinical data: Full history taking with special emphasis on antenatal maternal history and diseases, labor, mode of delivery and delivery circumstances. Gestational age was calculated based on the date of last menstrual period and confirmed by examination using the modified Ballard score within the 1st 24 h of life (Ballard *et al*., 1991).

Birth weight, sex and Apgar score at 1 and 5 min (Apgar, 1953) were recorded. Complete physical examination was done with special emphasis on neurologic examination for the presence of any neurological abnormalities. Patients were graded into three subcategories according to Sarnat and Sarnat staging (Sarnat and Sarnat, 1976). Follow up of all cases during their stay in the neonatal intensive care unit was done with daily clinical assessment.

Radiologic findings: Brain imaging was done, either ultrasound or CT brain as required to detect congenital anomalies, hemorrhage and brain hypoxia.

Sample collection: Blood samples were withdrawn from cord blood of all patients just after delivery and the samples were divided into 3 parts: part on EDTA tubes for CBC and nucleated RBCs and another part then centrifuged, the separated plasma stored at -20°C for assessment of BDNF. Another part was withdrawn on plain tubes for CRP. Cord blood arterial blood gas assessment was also done.

Seventy two hours postnatal a peripheral blood sample was withdrawn from the study groups, centrifuged and the separated plasma stored at -20°C for assessment of BDNF.

Analytical tests

- Complete blood picture (CBC) assessment: CBC was assayed by Hitachi 917 autoanalyzer and Roche reagents
- Nucleated RBCs: Counted in blood film stained with leishmann stain
- CRP: Measured by latex coagglutination test
- Cord blood gases: Cord blood gases were assayed by Bayer 348 rapid lab using Bayer reagents
- Plasma level of BDNF in cord blood with follow up in the patient group after 48-72 h

Plasma levels of BDNF in the samples were detected by ELISA technique using Quantikine human BDNF immunoassay kit supplied by R and D systems, 614 MCKinley Place NE, Minneapolis, MN 55413, United States of America (R and D systems, 2006). This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for BDNF had been pre-coated onto a microplate. Standards and samples are pipette into the wells and any BDNF present was bound by the immobilized antibody. An enzyme-linked monoclonal antibody specific for BDNF was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of BDNF bound in the initial step. The color development was stopped and the intensity of the color was measured.

Statistical analysis: Data were analyzed using SPSS software Package (Chicago, IL, USA). Quantitative variables were described as Mean±SD, median and (interquartile range); categorical variables were expressed as numbers and percentages. Mann-Whitney U test and Kruskal Wallis tests were used to compare non parametric data. Chi-Square and Fisher exact tests were used to compare categorical variables. Correlation study was done between all studied parameters using spearman correlation coefficient (r). The diagnostic performance of

BDNF level was done using Receiver Operator Characteristics (ROC) curve analysis from which the best cut-off value was chosen. Results were considered significant when $p<0.05$, highly significant when $p<0.01$.

RESULTS

The present study included 20 neonates with perinatal asphyxia as cases and 20 healthy neonates as the control group. Both cases and controls were statistically matched as regards gestational age, birth weight, sex and mode of delivery. Cases had statistically significant lower apgar score compared to controls (Table 1). Postnatal complications attributed to hypoxia included oliguria in 18 (90%) of cases, respiratory affection in 17 (85%), hypotension requiring inotropes in 15 (75%) and convulsions in 13 (65%) of cases. Fourteen neonates (70%) with perinatal hypoxia survived whereas 6 (30%) died. According to Sarnat staging, five (25%) cases had mild, 10 (50%) cases had moderate and five (25%) cases had severe grade of encephalopathy. Cord blood BDNF level was statistically significantly higher among cases with perinatal hypoxia compared to the control group. Neonates with perinatal asphyxia had significantly higher total leucocytic count and nucleated red blood cells while significantly lower PH, PO2 and HCO3 compared to controls (Table 2). Severe Sarnat stage of encephalopathy was significantly associated with poor outcome among cases (Table 3).

Among cases, BDNF level at delivery and after 72 h was significantly correlated with the severity of encephalopathy according to Sarnat staging being higher as severity increases. In addition, cases with severe Sarnat stage had significantly higher nucleated RBCs and lower PH values in cord blood (Table 4). Increased echogenicity was the most common cranial ultrasound finding among the studied cases followed by periventricular leukomalacia and ventricular attenuation (Table 5). There was non significant difference between male and female among cases and controls as regards BDNF level at delivery($p>0.05$). Statistically non significant correlation was demonstrated between GA, pH,

Table 1: Clinical characteristics of the studied neonates

	Cases	Controls	Test used			
Clinical characteristics	----------(n = 20)----------		χ^2-value	Z-value	Fisher exact	p-value
Sex						
Male	12 (60)	12 (60)	0			1
Female	8 (40)	8 (40)				
Wt (kg)	3.265±0.3675	3.209±0.4128		182		0.625
GA (week)	38.35±1.089	38.55±1.128		165.5		0.334
Maternal age (year)	28.05±4.249	29.91±3.59		175.5		0.505
APGAR 1	2.65±0.671	7.55±0.688		0.0000000		0.00000002**
APGAR 5	5.05±0.826	8.91±0.302		0.00000000		0.00000002**
APGAR 10	5.9±0.852	8.91±0.302		0.00000000		0.00000017**
Mode of delivery						
VD	11 (55)	13 (65)	0.417			0.748
CS	9 (45)	7 (35)				
Parity						
Multi.	13 (65)	17 (85)	2.133			0.273
Primi.	7 (35)	3 (15)				
PROM						
Yes	4 (20)	2 (10)			0.784	0.661
No	16 (80)	18 (90)				

Values are expressed as Mean±SD. Values in brackets are percentage. *$p<0.05$: Significant, **$p<0.01$: Highly significant, $p\geq0.05$: Non significant, SD: Standard deviation, wt: Weight, GA: Gestational age, VD: Vaginal delivery, CS: Cesarean section, PROM: Premature rupture of membrane

Table 2: Laboratory variables of the studied neonates

Laboratory variables	Cases	Controls	Mann-Whitney	p-value
TLC ($\times10^3$ cmm^{-1})	16.89±6.3016	9.964±3.1819	35.000	0.0000802**
NRBCs	19.5±6.581	2.18±0.751	0.00000000	0.0000508**
PH	7.055±0.10473	7.3664±0.04843	0.00000000	0.0000006**
PCO_2 (mmHg)	40.255±9.3882	35.735±5.3865	146.000	0.144
PO_2 (mmHg)	53.79±22.604	75.75±6.397	44.500	0.00002529**
HCO_3 (mmol L^{-1})	11.015±2.8322	20.9±4.0596	4.500	0.0000012**
Cord blood BDNF (pg mL^{-1})	1477.5±733.67	162.27±66.271	0.00000000	0.0000006**

Values are expressed as Mean±SD. *$p<0.05$: Significant, **$p<0.01$: Highly significant, $p\geq0.05$: Non significant, TLC: Total leucocytic count, NRBCs: Nucleated red blood cells, HCO_3: Bicarbonate, BDNF: Brain derived neurotrophic factor

Table 3: Clinical characteristics and outcomes of cases in relation to their Sarnat grading for the severity of encephalopathy

	Sarnat staging					
	Mild and moderate (n = 15)		Severe (n = 5)			
Clinical characteristics and outcome	Frequency	%	Frequency	%	Test value	p-value
Mode of delivery						
VD	7	46.7	4	80	1.684	0.319
CS	8	53.3	1	20		
Chest comp. During resusc.						
Yes	2	13.3	5	100	12.381	0.001**
NO	13	86.7	0	0		
Postnatal convulsions						
Yes	10	66.7	3	60	0.073	1.00
NO	5	33.3	2	40		
IVH						
Yes	2	13.3	3	60	4.356	0.073
NO	13	86.7	2	40		
Outcome						
Survivors	13	86.7	1	20	7.937	0.014*
Non survivors	2	13.3	4	80		

Fisher exact test used, *p<0.05: Significant, **p<0.01: Highly significant, p≥0.05: Non significant, VD: Vaginal delivery, CS: Cesarean section, IVH: Intraventricular heamorrhage

Table 4: Comparative study between mild, moderate and severe HI cases according to Sarnat as regards some laboratory data

	Mild			Moderate			Severe				
Variable	Mean±SD	M	Range	Mean±SD	M	Range	Mean±SD	M	Range	Test	p-value
BDNF at delivery (pg mL^{-1})	900±190.394	900	650-1150	1305±275.328	1350	900-1750	2400±891.628	2300	1200-3500	11.432	0.003**
BDNF after 72 h (pg mL^{-1})	1520±454.973	1400	1200-2300	1960±343.835	2000	1500-2500	2850±961.769	2750	1500-4000	8.192	0.017*
N RBCS	12.8±1.924	12	11-16	19.6±5.797	18	15-34	26±4.183	25	20-30	12.274	0.002**
PH	7.14±0.0295	7.15	7.1-7.18	7.02±0.12365	7.01	6.78-7.17	7.03±0.06385	7.04	6.93-7.09	5.799	0.055*
PCO_2 (mmHg)	40.28±2.6976	40.5	35.8-42.8	45.08±8.7616	41.45	34-63.6	30.58±7.9654	33	18.3-37.3	9.004	0.011*
PO_2 (mmHg)	53.32±7.923	50.4	45-66	55.7±29.548	49.55	18-127	50.44±19.398	60	27-72	0.134	0.935

M: Median, SD: Standard deviation, Kruskal-Wallis test used, *p<0.05: Significant, **p<0.01: Highly significant, p≥0.05: Non significant. BDNF: Brain derived neurotrophic factor; NRBCs: Nucleated red blood cells

Table 5: Cranial ultrasound findings among cases

Ultrasound findings	Frequency	Percent
Increased echogenicity		
Yes	12	60
No	8	40
PVL		
Yes	8	40
No	12	60
Ventricular attenuation		
Yes	8	40
No	12	60
IVH		
Grade III		
Yes	4	20
No	16	80
Grade IV		
Yes	1	5
No	19	95

PVL: Periventricular leukomalacia, IVH: Intraventricular heamorrhage

PO_2, PCO_2 and BDNF level both at delivery and after 72 h (p>0.05), whereas significant positive correlation was found between nucleated RBCs and BDNF level both at birth (r = 0.62, p = 0.003) (Fig. 1) and at 72 h posnatal (r = 0.49, p = 0.02) (Fig. 2). BDNF level at delivery among the control group showed non significant correlation with GA (r = 0.046, p = 0.846). Among cases, significant negative correlation was found between BDNF level and Apgar score at 1 min (r = -0.798, p = 0.000) while non significant correlation was detected with 5 min Apgar score (r = -0.380, p = 0.09).

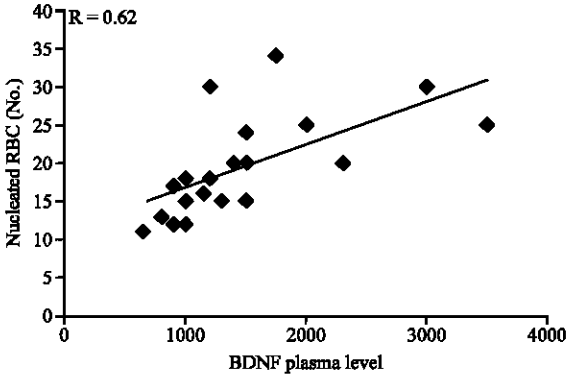

Fig. 1: Correlation between brain derived neurotrophic factor level at delivery and Nucleated red blood cells. p-value = 0.003 highly significant (r≥0.5 means strong correlation)

Significant association was detected between BDNF level both at delivery and at 72 h with chest compression during resuscitation (p = 0.004, 0.009, respectively), however statistically non significant association was detected between BDNF and mode of delivery, postnatal convulsions, IV Hge and outcome.

BDNF level significantly increased after 72 h of life compared to its level at delivery among cases

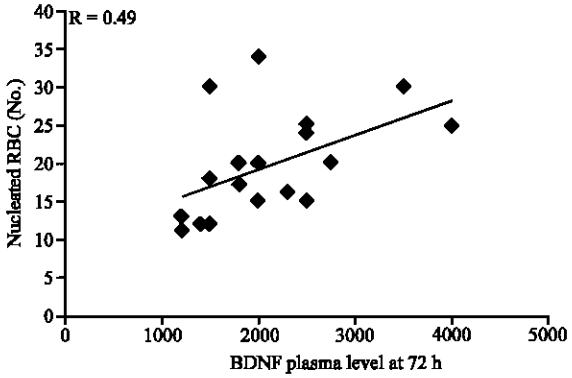

Fig. 2: Correlation between Brain derived neurotrophic factor level at 72 h and Nucleated red blood cells. p-value = 0.02 significant (r≥0.5 means strong correlation)

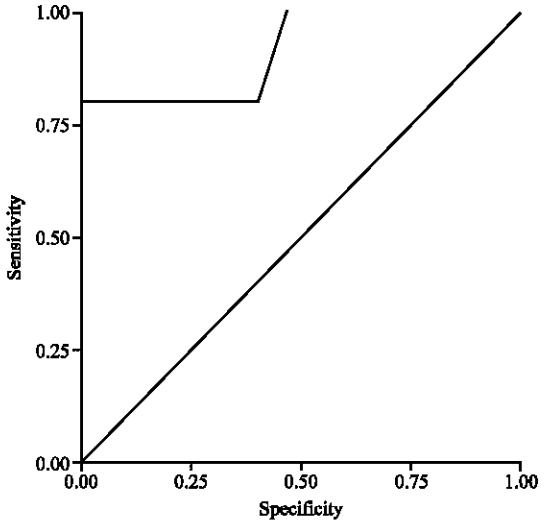

Fig. 3: ROC curve for prediction of severe Sarnat stage by level of Brain derived neurotrophic factor at delivery. Diagonal segments are produced by ties

(2072.5±735.111 and 1477.5±733.678 pg mL^{-1}, respectively, test value = -3.927 (p = 0.000086).

BDNF level at delivery was a predictor of poor outcome with area under the curve AUC (0.744), at value of 1875 pg mL^{-1} it was 50% sensitive and 92.9% specific. In Addition, BDNF level at 72 h after delivery was also a predictor of poor outcome with area under the curve AUC (0.702), at a cutoff value of 2400 pg mL^{-1} it was 66.7% sensitive and 85.7% specific.

Also, cord blood BDNF was a good predictor for the severity of encephalopathy with area under the curve AUC (0.913), at a cutoff value of 1875 pg mL^{-1} it was 80% sensitive and 100% specific (Fig. 3).

DISCUSSION

The present study demonstrated that BDNF level in cord blood was higher in asphyxiated newborns compared to control group. In addition, BDNF level increased significantly with increased disease severity.

In accordance with the present study Korhonen *et al.* (1998) showed that BDNF can be detected in human CSF and that the levels increased following hypoxic ischemic brain injury. Nikolaou *et al.* (2006) documented that NT3 and NT4 decreased after hypoxia-ischemia in newborn infants, while NGF and BDNF increased.

This can be explained by the findings of Aloe *et al.* (1999), who reported that basal level of brain nerve growth factor can be influenced negatively or positively by local expression of cytokines namely TNF-α.

Karege *et al.* (2002) found that circulating BDNF levels correlated with cortical BDNF levels in newborn rats.

In contrary to the present study, Chouthai *et al.* (2003) demonstrated that infants with severe intraventricular hemorrhage had significantly lower cord blood BDNF levels (925+/- 513 pg mL^{-1}) compared with their normal counterparts (1650 +/- 674 pg mL^{-1}; p = 0.021). In addition, Scheepens *et al.* (2003) in their model of global birth asphyxia in the rat reported that asphyxia caused a delayed increase in BDNF content within the hippocampus but decreased BDNF levels within the cerebellum.

The results of Tsukahara *et al.* (1994), suggested that BDNF gene expression was enhanced by transient ischemia both in the hippocampus and in the cerebral cortex and that BDNF, at a sufficient dose, had a preventive effect on the delayed hippocampal neuronal death observed after transient forebrain ischemia in the rat brain. This goes with our study which demonstrated that BDNF level increased significantly after 72 h of birth that is the reperfusion phase of perinatal hypoxia.

The present study found non significant difference between males and females as regards BDNF level. On the contrary, Chouthai *et al.* (2003) found that BDNF levels were higher in newborn girls than in boys but did not reach statistical significance.

The results of the present study showed that increased echogenicity and periventricular leukomalacia then ventricular attenuation were the most common cranial ultrasonographic findings among HI neonates. This is in accordance with Zaharie *et al.* (2007) in his retrospective study on 38 newborns with the diagnosis of neonatal asphyxia, transfontanellar ultrasonography showed different grades of intraventricular hemorrhage, periventricular leukomalacia in 35% of cases.

The present study revealed that, asphyxiated neonates had higher cord blood TLC, nucleated RBCs and lower pH and PO_2 compared to normal neonates. In addition nucleated RBCs were significantly increased with increased disease severity. This was in accordance with Ikeno(1994), who showed that the percentage of neonates with higher G-CSF levels (> or = 100 pg mL^{-1}) was greater in neonates with perinatal complications than in normal neonates. Neonates with higher G-CSF levels had larger numbers of peripheral leukocytes and neutrophils.

The present study demonstrated that there was a highly significant correlation between nucleated red blood cells (NRBCs) and BDNF level. This is similar to the finding of Ghosh *et al.* (2003), who found that, a statistically significant negative correlation existed between NRBCs level and markers of acute intrapartum asphyxia (Apgar score and umbilical arterial pH). Positive correlation was demonstrated with evidence of chronic antepartum asphyxia, presence of pregnancy induced hypertension and intrauterine growth restriction. A high NRBC count in umbilical blood correlated with poor early neonatal outcome. This emphasizes the value of BDNF as a marker of perinatal asphyxia.

The present study demonstrated that cord blood BDNF at a cutoff value of 1875 pg mL^{-1} is 50% sensitive and 92.9% specific marker for prediction of poor outcome (mortality) after perinatal asphyxia. In addition, the cord blood BDNF at a cutoff value of 1875 pg mL^{-1} is 80% sensitive and 100% specific marker for prediction of severe Sarnat stage. To the best of our knowledge, this is the first report of cut off values for sensitivity and specificity of BDNF as a predictor of severity and outcome of perinatal asphyxia.

CONCLUSION

Cord blood BDNF was significantly higher among neonates with perinatal asphyxia and correlated significantly with its severity. Cord blood BDNF could be used as a predictor of the severity and outcome of perinatal asphyxia giving opportunities to early and new therapeutic interventions during the precious window gap that could avoid long term complications.

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