RESEARCH ARTICLE

Endothelial dysfunction and developmental outcomes of very low birth weight newborns with hypoxic encephalopathy

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Abstract

Objective: To investigate the levels of endothelial constricting and dilating mediators in preterm infants with hypoxic-ischaemic encephalopathy and prospectively evaluate the association between levels measured during the perinatal period and the diagnosis of neurodevelopmental disorders at 3 years of age.

Methods: This regional observational cohort study was conducted at the Azerbaijan Medical University, Baku, Azerbaijan, from November 2011 to January 2013, and comprised very-low-birth-weight infants admitted to the intensive care unit during the perinatal period. Blood concentrations of nitric oxide, endothelin-1 and endothelial nitric oxide synthase were measured on days 1-3 and 5-7 of the neonatal period. Concentrations of neuron-specific enolase and antibodies against N-methyl-D-aspartate glutamate receptors were measured in peripheral blood samples for detection of brain damage in the early neonatal period of life. The infants were divided in 3 different groups: those diagnosed with moderate-to-severe neurodevelopmental disorders or cerebral palsy were included in the first group; those with mild neurologic changes were in the second group; and children without evidence of neurological impairment were in the third group. The fourth group comprised controls. SPSS 20 was used for data analysis.

Results: Of the 62 participants, there were 8(12.9%) in the first group, 20(32.3%) in second, 14(22.6%) in third and 20(32.3%) in the control group. The activity of endothelial nitric oxide synthase was reduced and nitric oxide concentrations were increased in the first group compared to those in the third group ($p<0.05$). Deep endothelial nitric oxide synthase depression and insufficient endothelin-1 synthesis were associated with diagnosis in the first group ($p<0.05$). No differences in concentrations of neuron-specific enolase and NR2 antibodies were identified among infants with and without a subsequent diagnosis of neurodevelopmental disorders ($p>0.05$).

Conclusion: The association between depressed endothelial nitric oxide synthase activation and insufficient endothelin-1 synthesis in the early days of life of very-low-birth-weight infants might be one of the causes of more serious and irreversible injury of brain tissue.

Keywords: Perinatal hypoxia, Hypoxic-ischaemic encephalopathy, Endothelial dysfunction, Neurodevelopmental disorders, Cerebral palsy. (JPMA 67: 1857; 2017)

Introduction

Perinatal hypoxic-ischaemic encephalopathy (HIE) is a major cause of neonatal death and long-term disability. Approximately 15-25% of newborns with HIE die in the postnatal period, with those who survive being at risk of severe and permanent impairments in physical, neurological and cognitive function, including cerebral palsy, seizure disorder, blindness, mental retardation, learning and cognitive disabilities.[1-3] Severe hypoxia/ischaemia can lead to permanent brain damage, and also affects other tissues of the body. Specifically, disorders of peripheral organs are often caused by haemodynamic disturbances due to the centralisation of the blood flow and consequent poor circulation within internal organs.[4,5] Hypoxia and energy deficiency generate the synthesis of free radicals, which are highly reactive with the polyunsaturated fatty acids of the brain. Moreover, following hypoxic-ischaemic injury, concentrations of brain-specific proteins rise in blood and neuronal tissue. Previous studies have suggested that elevated serum concentrations of antibodies against N-methyl-D-aspartate (NMDA) subtype of the glutamate receptors (NR2 antibodies) and neuron-specific enolase (NSE) are capable of identifying newborns with a risk of HIE after birth asphyxia.[6,7] A series of investigations confirmed prolonged elevation in blood levels of these markers to be reflective of neuronal cell injury, with the magnitude of increase being positively related to the severity of process.[7]

Energy failure in hypoxic injury also activates neuronal nitric oxide synthase (nNOS) and increases nitric oxide

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(NO) production, whereas primary source of NO in the vasculature is endothelial nitric oxide synthase (eNOS) under normal conditions. Cellular energy failure, acidosis, glutamate release, and nitric oxide neurotoxicity lead to cytotoxic oedema and neuronal death.[7,8] The resultant hypoxic inflammation is regulated via biological active mediators synthesised by the endothelium. Whereas endothelin-1 (ET-1) being a potent endothelium-derived vasoconstriction peptide, NO, and the source of its synthesis, play a special role in the pathophysiology of vasodilatation.[7] The imbalance in ET-1 and NO concentrations as a result of endothelial dysfunction may be one of the main causes of cerebral haemodynamic disturbance in preterm infants. Previous in vitro research has demonstrated an association between impairment in vascular growth and endothelial function in infants with intrauterine growth retardation and with profound and prolonged brain damage.[9] Despite the widespread confirmation of a significant role for NO in physiological regulation and pathological changes of vascular functions, the impact of endothelial vasoregulator factors in the formation of neuronal damage and neurodevelopmental disorders (NDD) has not been fully investigated. The current study was planned to evaluate the changes in blood levels of vasoregulatory markers in infants with HIE, and the association of these changes with the pathophysiology leading to early onset of NDD.

Patients and Methods

This regional observational cohort study was conducted at the Azerbaijan Medical University, Baku, Azerbaijan, from November 2011 to January 2013, and comprised very-low-birth-weight (VLBW) infants admitted to the intensive care unit (ICU) during the perinatal period. The study was approved by the national ethics committee, and informed parental consent was obtained in all cases.

The inclusion criteria were clinical diagnosis of severe/moderate HIE and birth weight $\leq$1,500 grams. Patients who survived to age three years were included. We assessed the degree of concordance between measured vasoregulatory markers and the diagnosis of a NDD at 6 months, and at 1, 2 and 3 years. Neurodevelopmental outcomes were evaluated by a multidisciplinary team using neurodevelopmental assessment tools and/or via medical interviews and physical examination. The team of health professionals consisted of psychologist, psychiatrist, neurologist and paediatrician with at least three years of experience. Mild motor impairments, transient or mild psychomotor delay, mild mental delay, hyperactivity and mild language delay was classified as mild NDD; moderate-to-severe forms of psychomotor, mental, language delay and cerebral palsy were included in severe/moderate NDD. Cerebral palsy was defined as an impairment of movement and posture attributed to an abnormality of the brain at 1-year follow-up.

The study was not blinded, and according to further neurodevelopmental status VLBW infants were divided into 3 groups: infants diagnosed with moderate-to-severe NDD or cerebral palsy later in life were included in the 1st group; infants with mild neurologic changes at an early age were included in the 2nd group; and children without evidence of neurological impairment in the post-neonatal period were classified into the 3rd group. VLBW infants who fulfilled the following criteria were included in the control group: no maternal illness; after 5 minutes, an Appearance, Pulse, Grimace, Activity, Respiration (Apgar) score of $\geq$7; capillary or arterial blood cord pH of $\geq$7;an uneventful course during the first 10 days of life; absence of intrauterine infection or sepsis, absence of manifestations of neurological impairment during the neonatal and post-neonatal period; no drugs administered during first weeks of extrauterine life. Gestational age was based on the Ballard et al. scale.[10] Growth delay was determined by estimated anthropometric parameters of a foetus and newborn infant according to percentile growth charts.[11] We collected neonate data prospectively, and obstetric data from the hospital records. The study exclusion criteria comprised clinical or laboratory evidence of toxoplasmosis, other [syphilis, varicella-zoster, parvovirus B19], rubella, cytomegalovirus, and herpes (TORCH) infections, sepsis, or congenital malformation.

The diagnosis of perinatal asphyxia was determined based on the guidelines of American Academy of Paediatrics.[12] The grades of HIE were identified based on the behavioural assessment of a newborn on day 1 of life.[13]

The results of the physical examination were reviewed for level of consciousness, spontaneous activity, posture tone, primitive reflexes, and autonomic nervous system signs. Infants were categorised as having mild, moderate, or severe HIE with the modified Sarnat staging for HIE, and infants with clinical diagnosis of severe/moderate HIE were included in study.

Intraventricular haemorrhage (IVH) was diagnosed based on Papille's classification.[5]

Peripheral blood samples were collected on postnatal days 1-3 and 5-7. Specimens were obtained in an

ethylenediaminetetraacetic acid (EDTA) tubes for study purposes and fractioned by centrifuging for 15-20 minutes. The specimens were stored at -70°C. Haemolysed specimens were excluded from this study.

Commercially available kit was used for NO measurements (Thermo Scientific, Pierce Biotechnology, Rockford, Illinois, United States [US]). NO levels in plasma samples were determined by Griess reaction based on the converting of nitrate to nitrite through the nitrate reductase. Before direct assay, it was exposed ultrafiltration of all specimens with the filters of 10,000 molecular weight cut-off membrane. NO levels were expressed in mmol/l.

The levels of ET-1 (Cayman Chemical Company, Ann Arbor, Michigan, US), eNOS (antibodies-online, Aachen, Germany), NR2 antibodies (CIS Biotech, Inc., Decatur, Georgia, US), and NSE (Life Science, Wuhan, China) were determined in peripheral blood with aforementioned commercially available enzyme-linked immunosorbent assay (ELISA) kits. ET-1 levels were expressed in pg/ml, eNOS in IU/ml, NR2 antibodies in ng/ml, and NSE in ng/ml.

Few studies have evaluated neurodevelopmental disorders in VLBW infants with signs of HIE. Depending on the definition used, the reported incidence across studies varies from 1.3/1000 live births to 5-9/1000 live births.[1,3] The sample size was calculated using the formula:

$n= Z2p(1-p)/e^2$ where 'e' is the margin of error. During the study period, 42 VLBW infants with severe/moderate HIE were seen at follow-up. This sample size provided 95% confidence interval (CI) with 5% margin of error.

Data collection and statistical analysis was conducted using SPSS 20. We performed analyses in duplicate. Intra- and inter-assay coefficients of variation were <5% and <15%, respectively. Data was tested for normal distributions and found to be nonparametric. First we examined whether the values for each parameter fit a Gaussian distribution, using the Kolmogorov-Smirnov test, and the distribution for all the examined data found to be nonparametric. Differences in concentrations of endothelial activity markers and neurospecific proteins were using Kruskal-Wallis test. Qualitative variables regarding maternal and neonatal characteristics were compared using Chi-square test. P<0.05 was considered significant.

Results

Of the 62 participants, there were 8(12.9%) in the first

Table-1: Maternal and neonatal characteristics of study groups.

	1st group (n=8)	2nd group (n=20)	3rd group (n=14)	[a]p value
Age	25.2 (19-31)	26.4 (19-34)	24.6 (20-37)	
Gravidity	2.3 (1-7)	2.1 (1-5)	2.4 (1-5)	
Premature rupture of membranes	2 (25)	3 (15)	3 (21.4)	
Preeclampsia	2 (25)	2 (10)	1 (7.1)	
Anaemia	4 (50)	6 (30)	5 (35.7)	
Caesarean section	2 (25)	4 (20)	4 (28.6)	
Gestational age, weeks	31.4 (28-34)	32.5 (28-35)	31.9 (28-33)	
Sex (M/F)	4/4	11/9	6/8	
Birth weight, g	1345.6 (980-1480)	1310.4 (1030-1500)	1398.7 (1100-1450)	
Small for gestational age	2 (25)	5 (25)	4 (28.57)	
Apgar 1 min	4.2 (3-6)	5.1 (2-6)	5.5 (4-7)	
Apgar 5 min	5.3 (4-6)	5.6 (4-7)	6.2 (4-7)	
pH	7.15 (6.85-7.29)	7.19 (6.81-7.31)	7.21 (6.98-7.32)	
RDS	4 (50)	8 (40)	6 (42.86)	
Seizures	6 (75)	5 (25)	3 (21.43)	*p<0,05
IVH, grade I	1 (12.5)	4 (20)	3 (21.43)	
IVH, grade II-III	4 (50)	6 (30)	6 (42.86)	
PVL	1 (12.5)	1 (5)	-	

Data are shown as mean (range) or n (%); ap<0,05 is considered statistically significant..
M/F: Male/female
pH: Potential of hydrogen
Apgar: Appearance, Pulse, Grimace, Activity, Respiration
RDS: Respiratory distress syndrome
IVH: Intraventricular haemorrhage
PVL: Periventricular leukomalacia.

Table-2: Blood concentrations of vasoregulatory markers in the early neonatal period by study groups.

Variables	Groups	N	Mean±SD	Minimum	Maximum	[a]p value
eNOS, IU/ml, day 1-3	1st group	8	4,65±1,67	2,50	7,60	[1-c]p<0,01
	2nd group	20	7,71±3,55	3,20	15,00	[2-c]p<0,01
	3rd group	14	6,71±3,83	2,80	15,00	[3-c]p<0,01
	control group	20	2,09±0,63	,70	3,20	
eNOS, IU/ml, day 7-10	1st group	8	1,37±0,43	,70	1,90	[1-2]p<0,05
						[1-3]p<0,05
	2nd group	20	2,64±1,01	,60	4,10	[2-3]p<0,05
	3rd group	14	7,57±3,27	3,90	14,00	[2-c]p<0,05
	control group	20	1,85±0,74	,50	3,20	[3-c]p<0,01
NO, mmol/l, day 1-3	1st group	8	47,50±17,51	23,00	72,00	[1-c]p<0,05
	2nd group	20	45,60±12,39	23,00	72,00	[2-c]p<0,01
	3rd group	14	42,85±13,40	19,00	68,00	[3-c]p<0,01
	control group	20	25,00±7,72	16,00	41,00	
NO, mmol/l, day 7-10	1st group	8	69,37±13,74	45,00	87,00	[1-3]p<0,05
						[2-3]p<0,05
	2nd group	20	65,00±31,04	17,00	108,00	[1-c]p<0,01
	3rd group	14	43,57±14,71	19,00	68,00	[2-c]p<0,05
	control group	20	26,50±7,97	16,00	41,00	[3-c]p<0,01
Endothelin-1, pg/ml, day 1-3	1st group	8	5,71±2,60	3,20	7,20	[2-3]p<0,05
	2nd group	20	4,43±1,55	1,30	6,70	[1-c]p<0,01
	3rd group	14	5,70±1,73	3,20	7,90	[2-c]p<0,01
	control group	20	2,71±0,56	1,60	3,90	[3-c]p<0,01
Endothelin-1, pg/ml, day 7-10	1st group	8	3,92±1,08	2,60	5,60	[1-2]p<0,05
						[2-3]p<0,05
	2nd group	20	5,91±1,61	3,20	8,60	[1-c]p<0,01
	3rd group	14	4,02±0,93	2,60	5,60	[2-c]p<0,01
	control group	20	1,82±0,69	,90	3,90	[3-c]p<0,01

Note: [a]p<0,05 is considered statistically significant between main groups (1-2, 1-3, 2-3), and between main and control groups (1-c, 2-c, 3-c).
eNOS: Endothelial nitric oxide synthase
NO: Nitric oxide
SD: Standard deviation.

group, 20(32.3%) in second, 14(22.6%) in third and 20(32.3%) in the control group. Maternal and neonatal data showed no difference in characteristics identified between the groups, including factors that may have contributed to utero-placental insufficiency (anaemia, preeclampsia) and the mode of delivery. The groups were similar with regard to baseline neonatal characteristics, including gestational age, gender, Apgar scores, and small for gestational age data. Children with severe NDD had a high incidence of perinatal seizures compared to children in the 2nd and 3rd group ($p<0.05$). The incidence of respiratory distress syndrome (RDS), periventricular leukomalacia (PVL), and IVH grades were similar in study groups (Table-1).

Changes in concentrations of molecules acting in regulation of vascular tone were different among the study groups during the early neonatal period. Specifically, activity of eNOS was reduced on days 5-7 of infants of the 1st and 2nd groups, compared to the 3rd group infants ($p<0.05$), while NO concentrations were increased in the 1st and 2nd groups compared to the 3rd group. No difference in ET-1 concentrations between the 1st and 3rd groups was identified. However, ET-1 levels on days 5-7 for the 2nd group were considerably higher compared to the 1st and 3rd groups, the difference being significant ($p<0.01$) between groups (Table-2).

In spite of higher levels of neuron-specific injury markers in the study groups, compared to the control group, we did not identify a significant difference between-group in the mean total neonatal serum concentrations of neurospecific proteins, regardless of the type and severity of NDD (Table-3).

Table-3: Blood concentrations of neuron-specific markers in the early neonatal period by study groups.

Variables	Groups	N	Mean±SD	Minimum	Maximum	[a]p value
NR2 antibodies, ng/ml, day 1-3	1st group	8	4,37±3,27	,60	8,60	1-c p<0,05
	2nd group	20	4,60±1,86	1,50	7,80	2-c p<0,01
	3rd group	14	4,20±1,00	2,90	5,90	3-c p<0,01
	control group	20	1,14±0,71	,20	2,70	
NR2 antibodies, ng/ml, day 1-3	1st group	8	4,58±1,14	3,70	6,80	1-c p<0,01
	2nd group	20	3,86±2,72	,10	8,80	2-c p<0,01
	3rd group	14	4,07±2,83	,90	8,60	3-c p<0,01
	control group	20	1,05±1,79	,10	7,80	
NSE, ng/ml, day 1-3	1st group	8	63,12±14,42	39,00	87,00	1-c p<0,01
	2nd group	20	54,35±10,90	36,00	78,00	2-c p<0,01
	3rd group	14	57,42±12,01	42,00	86,00	3-c p<0,01
	control group	20	26,30±8,66	11,00	45,00	
NSE, ng/ml, day 7-10	1st group	8	46,12±27,72	19,00	89,00	1-c p<0,05
	2nd group	20	44,30±27,58	11,00	89,00	3-c p<0,01
	3rd group	14	42,28±18,76	11,00	69,00	
	control group	20	25,20±8,56	13,00	45,00	

[a]Note: p<0,05 is considered statistically significant between main groups (1-2, 1-3, 2-3), and between main and control groups (1-c, 2-c, 3-c).
SD: Standard deviation.
NSE: Neuron-specific enolase.
NR2: N-methyl-D-aspartate subtype of the glutamate receptors.

Discussion

Cerebral ischaemia induces an inflammatory response in the parenchyma and systemic circulation.[6] Vasoregulatory mechanisms play an essential role in the formation of brain injury and the processes of tissue reperfusion in critically ill children. Endothelial dysfunction results in an imbalance between vasoconstriction and vasodilatation, causing tissue reperfusion, cytotoxic oedema, and brain injury.[5,7] Results of several investigations regarding the role of NO as biochemical marker to assess brain injury in newborns are controversial.[14,15] Consequently, interpretation of the role of NO in the genesis of brain injury is difficult to clearly discern; is the concentration of this molecule increased as a defensive mechanism, or does an increase in NO point to a more profound impairment? In one of our previous studies, we identified a high NO/eNOS ratio, resulting from a high NO concentration and low eNOS activity, in growth restricted infants with severe hypoxic-ischaemic injury, indicative of a probable key role of this molecule in the genesis of intrauterine hypoxic injury.[16] In the present study, we identified a significant association between peripheral blood vasoregulatory markers in the perinatal period and the diagnosis of an NDD at an early age. With high NO levels, eNOS activity was insufficient in the early postnatal days in infants with an NDD diagnosed later in life, compared to levels in neonates without current or subsequent neurological complications (Table-2). Therefore, depressed eNOS activity and increased non-endothelial NOS synthesis might play an important role in the formation of further developmental impairments.

Interestingly, a direct connection was identified between the severity of an NDD at an early age and perinatal regulatory markers of vascular tonus. As shown in Table-2, a deep eNOS depression in combination with insufficient ET-1 synthesis during neonatal period was associated with a more profound neurodevelopmental delay and cerebral palsy diagnosed at an early age. In contrast, when eNOS was depressed but in the presence of maintained sufficient vasoconstriction (due to increased ET-1 concentrations), a more moderate degree of neurological impairment was diagnosed at an early age, such as mild motor and cognitive delays or minimal brain dysfunction. Therefore, the association of insufficient eNOS activation with the lack of compensatory mechanisms, such as peripheral vasospasm and centralisation of circulation in vital organs during the early stage of the pathological process, might be one of the causes of more serious and irreversible injury to brain tissue.

A limitation of this study was the small number of children with cerebral palsy. It is obvious that only eight children diagnosed with severe NDD calls for caution

when drawing any conclusions. A strength is that the children were followed up prospectively without dropout, and the findings of our study were consistent with several experimental investigations that have evaluated the role of different NOS synthases in the pathogenesis of neuronal injury. It was shown that chronic hypoxia decreases eNOS and increases nNOS in brain tissue.[17] In addition, elevated synthesis of glutamate and extracellular amino-terminal fragment (ATF), and processes of long-term and severe hypoxia cause changes in enzyme activity related to 2, 4-Dinitrotoluene (DNT) structure, resulting in activation of neuronal and inducible forms of nitric oxide synthases.[18,19] Previous studies showed that NO generation by eNOS induces vasodilatation which might depress the hypoxic damage, while, conversely, elevated nNOS production results in neuronal injury.[20,21] Based on the role of eNOS depression in the formation of brain damage, several investigators have suggested a protective effect of nNOS inhibitors.[22] Our study confirms the results of a previous study which reported that blood concentrations of neuron-specific proteins are not sufficiently specific predictors for future neurodevelopmental delays.[23] In fact, despite investigation of a wide range of possible biomarkers of neonatal brain injury, predicting the risk of NDD in postnatal growth is not yet possible. Our findings do suggest that neuronal injury in preterm infants can be evaluated by detecting the plasma levels of vasoregulation markers, and this might be accepted of significant predictive value. Endothelial dysfunction might serve a detector of the severity of HIE in newborn infants and a predictor of further neurological complications. Assessment of brain injury in perinatal period may provide a useful tool for preventive proceedings, with targeted treatment possibly preventing developmental disorders in these newborns. We propose that depressed eNOS synthesis in the background of increased NO concentrations may be involved in the pathogenesis of perinatal HIE and indicates the severity of neuronal injury. Changes in the activity of endothelial and non-endothelial sources of NO generation as a function of the severity of neuronal injury would be of great clinical significance for predicting the development of future neurosomatic problems in preterm infants who experienced perinatal HIE.

Conclusion

Major pathophysiologic processes that contribute to permanent brain injury in preterm infants with HIE included vascular insufficiency and impaired cerebrovascular flow. Endothelial function in the perinatal period played a fundamental role in the autoregulation of vascular tone and a significant part in the formation of hypoxic brain damage.

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Conflict of Interest: None.

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