INTERNATIONAL JOURNAL OF MEDICAL AND APPLIED SCIENCES
E-ISSN:2320-3137

Research Article

CORRELATION OF OXIDATIVE STRESS BIOMARKER AND SERUM MARKER OF BRAIN INJURY IN HYPOXIC ISCHEMIC ENCEPHALOPATHY

Shah Seema*[1], Goel Anil Kumar[2*], Padhy Mamta[3], Bhoi Sumitra[4]

1. **Senior Resident , Biochemistry , Maulana Azad Medical College, New Delhi**
2. **Associate Professor , Paediatrics , SHKM,GMC, Nalhar**
3. **Padhy Mamta (Biochemistry ,Maulana Azad Medical college**
4. **Assistant Professor , Biochemistry , VSS MCH , BURLA**

Note: First author and Corresponding Author: Dr. Seema Shah and Dr. Anil Kumar Goel

Received on 25 Jan 2014, Published on 9 Feb 2014

ABSTRACT

Neonatal hypoxic ischemic encephalopathy has a highly complex pathogenesis. Despite advances it is one of the leading cause of neonatal mortality. The aim of this study was to assess levels of free radical biomarkers – MDA(malondialdehyde) and NO (nitric oxide) in different stages of HIE and to correlate it with neuron specific enolase(NSE)- a biomarker of neuronal injury. Blood brain barrier (BBB) permeability was also evaluated to indicate increased vascular permeability as an added mechanism of neuronal function compromise
A group of 58 term babies with HIE were selected as control and 20 healthy gestational age and sex matched babies were taken as control. Serum levels of MDA, NO, NSE were measured by standard biochemical tests and BBB permeability was evaluated as a ratio of CSF albumin and Serum albumin. Datas were analysed by Medcalc software. Serum biomarkers of oxidative stress – MDA and NO were found to be significantly high in cases and also across different stages of HIE indicating increased free radical damage in more severe HIE babies. MDA and NO were positively correlated with NSE – a biomarker of neuronal damage .Blood brain permeability was also significantly altered in cases postulating a role of free radicals in damaging the blood brain barrier. Our study shows that free radicals have a causative role in extent of neuronal damage in HIE and hence a possible benefit by antioxidants may be predicted

Keywords: blood brain barrier permeability, hypoxic ischemic encephalopathy, malondialdehyde, nitric oxide , neuron specific enolase, oxidative stress

INTRODUCTION

Birth asphyxia and the resultant HIE is a major cause of neonatal morbidity and mortality and also of subsequent brain damage in the form of seizures, learning disability, mental retardation or as cerebral palsy if profound brain damage has occurred. HIE prevalence ranges from 0.1% to 0.5 % of total live births and is a cause of around 23% of neonatal death worldwide (1).

The pathogenesis of hypoxic ischemic brain damage is highly complex and starts with compromised placental blood flow leading to anaerobic glycolysis and exhaustion of ATP reserve with subsequent accumulation of lactic acid , failure of ion pumps and rise in intracellular calcium , cellular edema and release of neurotransmitters (2,3). All these events results in the formation of various free radicals and subsequent lipid peroxidation , DNA

damage , inactivation of enzymes and degradation of protein structure(4) .The cell membrane having the highest concentration of PUFA is extremely vulnerable to free radical induced lipid peroxidation and this can be assessed by measuring serum MDA as a marker of free radical production (5). Nitric Oxide too play a important role in free radical mediated injury during cerebral reperfusion .Perinatal asphyxia also results in disrupted blood brain barrier(BBB), resulting in increased vascular permeability, brain edema and further brain damage (6). Neuron specific enolase(NSE) an isoenzyme of glycolytic enzyme enolase is a brain specific protein specifically found in neurons and is released in CSF and blood after brain injury . Serum NSE was researched as a marker of extent of brain damage and subsequent adverse neurological outcome (7,8).

This study was undertaken to assess the role of free radicals in Perinatal hypoxic ischemic brain injury and its correlation with serum NSE (neuron specific enolase) as a marker of the extent of brain injury. Free radical injury was assessed by measuring the serum levels of MDA (malondialdehyde) and NO (nitric oxide) as an indirect estimation of oxidative stress during Perinatal hypoxic ischemic insult. The levels of MDA , NO and NSE were further correlated with permeability of BBB , which was measured as cerebrospinalfluid (CSF) albumin/ plasma albumin ratio.

MATERIAL AND METHODS :

The study was conducted in the Department of Biochemistry in association with Department of Paediatrics of V.S.S. Medical College and Hospital, Burla over a period of 3 years from May 2006 to Oct. 2009 on 58 full term infants with history of birth asphyxia who subsequently developed HIE .Birth asphyxia was defined as requirement for positive pressure ventilation including bag and mask ventilation or intubations for more than 1 minute during postnatal resuscitation and an Apgar score of 6 or less at 5minutes. Progression of birth asphyxia to HIE was defined as presence of minimum of 2 abnormal neurological findings out of the following as alteration of muscle tone either hypotonia / hypertonia, abnormal neonatal reflexes including the Moro's, rooting and sucking, failure to arouse the infant even after vigorous stimulation, presence of convulsion. All neonates with HIE included in this study belong to stage [I, II, III] of Sarnat and Sarnat classification (9). Twenty full term healthy, gestatinal age and sex matched healthy neonates without any birth asphyxia who did not required anypositive pressure ventilation during resuscitation and who cried immediately after birth were enrolled in the study as control. Exclusion criterias for selection of cases were history of maternal drug addiction or analgesia which might cause neonatal depression , severe infection congenital or acquired , respiratory distress syndrome, Congenital anomaly or tumors and any type of birth injury of the neonate.

This research work was approved by the institutional ethical committee, VSS Medical College Burla and informed consent was obtained from the parents of the study group.

Peripheral venous sample from all the infants was collected in sterilized tubes at 24 hour of life and stored at -20 degree C until analyzed. Malondialdehyde levels were measured by the thiobarbituric acid assay (10). Serum NO was measured indirectly by measuring amount of nitrates and nitrites formed by it in the body by modified Griess reaction (11).Serum albumin and CSF albumin were measured by BCG method and pyrogallol red method respectively using commercial kits from Crest Diagnostics. Serum NSE was measured by ELISA .

Statistical calculations were done using Medcalc software . Students t test and one way of analysis of variance (F) to find out the significant difference between the groups. Student –

Newman-Keuls (SNK) test applied to test the significance of difference among the means of different groups . Correlation coefficient between different variables were also calculated .

RESULTS:

We studied 78 neonates in total. The patients of HIE were divided in three stages as per Sarnat and Sarnat staging and accordingly for statistical calculation five different groups consisting of group A which included 20 healthy control , group B consists of neonates progressing to stage I (n=19), group C consists of neonates progressing to stage II (n=30), group D consists of neonates progressing to stage III (n=9) and group E consists of all HIE patients (n=58).

Sex distribution , birth weight and gestational age were similar among the neonates with HIE I, II, III or in control group(table 1). Apgar score at 1 minute and at 5 minute are shown in table 1 . Apgar score was significantly different between case and control and also between group A and group D at $p<0.05$.

Table 1 : Anthropometric profile of neonates across different groups

	HIE I	HIE II	HIE III	HIE TOTAL	CONTROL
No. of patients	n=19	n=30	n=9	n=58	n=20
Male / female baby	11/8	18/12	5/4	34/24	12/8
Gestational age(weeks)	39.0±1.0	38.7±2.0	39.0±1.4	38.8±1.68	37.8±1.72
Birth weight (kgs)	2.66±1.66	2.65±1.6	2.37±0.09	2.61±0.18	3.0±0.22
Apgar at 1 minute	4.3±1.3	4.3±0.96	3.2±1.2	4.17±1.11	8.0±0 .85
Apgar at 5 minute	5.8±0.37	5.7±0.46	4.0±0.86	5.48±0.82	9.0±0.79

The mean MDA and NO were found to be significantly higher in cases than among controls with $p<0.001$. Serum MDA and NO were found to be increasing with higher staging of HIE and values in HIE III was significantly higher as compared to HIE I and HIE II. A significant correlation was observed between serum malondialdehyde and NO level($r= 0.96$, $p<0.05$).

Table 2 : Biochemical tests finding across different groups

	HIE I n=19	HIE II n=30	HIE III n=9	HIE TOTAL n=58	CONTROL n=20
S.MDA (mmol/L)	3.03±0.33	4.04±1.18	6.75±0.54[2]	4.13±1.51[1]	2.25±0.32
S.NO ((mmol/L)	54.26±5.48	61.1±11.65	95.22±7.34[2]	64.15±16.61[1]	36.05±5.79
S. Albumin (gm/dl)	3.98±0.34	3.77±0.52	3.8±0.42	3.84±0.42	3.78±0.42
CSF albumin (mg/dl)	178.4±70.52	253.3±56.77	336.88±65.70[2]	241.77±81.56[1]	46.15±9.08
BBB permeability (x 10^{-3})	45.51±19.76	68.42±18.28	88.69±16.35[2]	64.06±23.48[1]	12.47±3.47
S. NSE (µg/L)	16.0±8.96	40.25±17.06	52.01±24.36[2]	34.14±20.88[1]	7.64±4.45

[1] :$p<0.001$ when compared to control

[2] : $p<0.05$ when compared to HIE I and HIE II

Serum albumin levels (gm/dl) were comparable in neonates of HIE and control group whereas CSF albumin (mg/dl) and BBB permeability were significantly higher in HIE group ($p<0.001$, table 2) . The increase in CSF albumin and BBB permeability were found to be increasing with worsening stage of HIE and significant intragroup difference were observed in the SNK test (gr B vs C $p< 0.01$, gr C vs D $p< 0.01$, gr B vs D $p< 0.01$). Serum NSE as a marker of neuronal damage was found to be significantly high in cases as compared to control($p< 0.001$).Significant correlation of NSE was observed with BBB permeability and also with MDA and NO (r=0.49 for BBB permeablility , r=0.58 for MDA and r=0.58 for NO) with p value < 0.0001(fig 1, 2, 3).

One way analysis of variance was also found to be significant with following values F= 52 for MDA , 60 for NO and 19.2 for NSE and a p value of <0.001 in SNK test.

Figure 1 : Correlation of NSE with BBB permeability

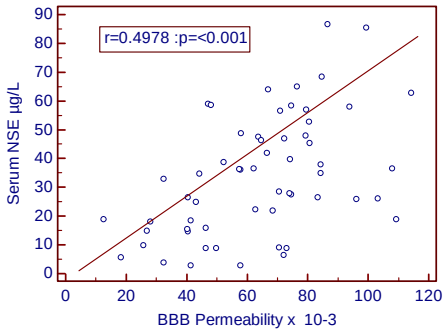


Figure 2: Correlation of NSE with MDA

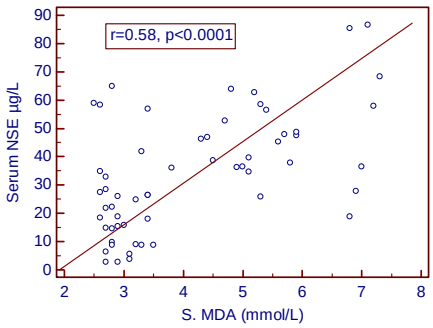
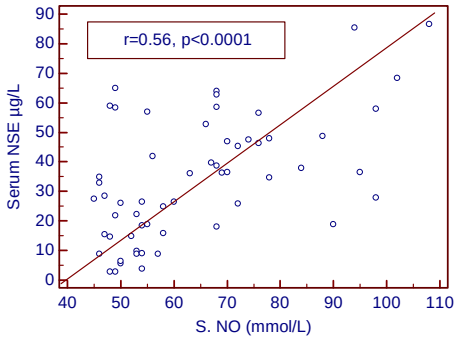


Figure 3: Correlation of NSE with NO



DISCUSSION ;

Birth asphyxia is a major cause of neonatal mortality and also chronic neurological sequelae in the survivors (12). The incidence of HIE is reported as 3-5 per 1000 full term infants and upto 60% in preterm neonates (13).this increased vulnerability in immature brain can be explained , in part by the density of NMDA receptors , Nnos positive cells, poor antioxidant defensive mechanisms, and a high concentration of free iron and lipids (14).

Alongwith hypoxic ischemic neuronal damage which occurs in around 30% of cases of birth asphyxia , dysfunction of pulmonary, cardiac or renal system may complicate the outcome adversely(15).With increasing severity of HIE , the risk of neurological sequels and death

also increases. Impaired cerebral circulation causes generalized brain edema and neuronal death in vulnerable areas of brain and prolonged anoxia might lead to frank infarctions in cortical and subcortical region(16).

The pathogenesis of hypoxic ischemic brain damage is highly complex and starts with compromised placental blood flow leading to anaerobic glycolysis and exhaustion of ATP reserve with subsequent accumulation of lactic acid , failure of ion pumps and rise in intracellular calcium , cellular edema and release of neurotransmitters (2,3). All these events results in the formation of various free radicals. The brain contains lower concentrations of Super Oxide Dismutase (SOD), catalase and glutathione peroxidase activity as compared to kidney or liver(17). The neonatal brain is very vulnerable to oxidative damage because of its high concentration of lipids , very high oxygen requirement , decreased levels of antioxidant enzymes and increased availability of free iron (18,19).Oxidative injury is further increased during reperfusion phase via production of highly reactive nitric oxide (NO) molecule(20). Free radicals injury leading to lipid peroxidation , DNA and protein damage , is further added by increased production of superoxide and hydrogen peroxides during reperfusion which though less reactive combines with NO to form highly toxic peroxynitrite compound. This peroxynitrite is very diffusible and easily crosse the BBB and damages the brain tissues(21). Free radicals can also induce inflammation and further production of more oxidative radicals during reperfusion phase via enzymes such as nitric oxide synthase(NOS), cyclooxygenase, lipoxygenase and xanthine oxidase (22). The increased free radicals level causes destruction of tight junction of endothelial layer of BBB and increases its permeability(23). Disrupted BBB and resultant increased vascular permeability leads to brain edema and secondary brain damage(24).

In the present study both cases and control group are matched with respect to gestational age and birth weight (table 1).Apgar score was significantly different between cases and control suggesting increased stress and profound anoxia in HIE group. We found significantly higher concentration of serum Malondialdehyde and serum NO levels in HIE group as compared to control group indicating increased oxidative stress in HIE cases. Further higher serum MDA serum NO were associated with worsening stage of HIE . similar findings have been reported by other authors(25,26). We also found a significant linear correlation between serum MDA and NO , which can be explained on the basis of increased lipid peroxidation (increased MDA levels) due to a progressive increase in generation of NO during reperfusion stage.

The role of NO in hypoxic ischemic injury is very complex and contradictory in various studies as it has been proposed to mediate both protective and pathologic response(27,28,29). In acute hypoxia , excess of NO may be harmful as it causes vasodilatation and subsequent reperfusion injury , but in chronic hypoxia ,improved cerebral blood flow by NO dependent vasodilatation may infact improves the neurological outcome .NO also acts as a superoxide radical scavenger and may also inhibits platelet aggregation and Neutrophil adhesion , thus limiting the inflammation(30).In the brain there are 2 isoforms of NOS present constitutively – nNOS present in neurons and eNOS present in vascular endothelium. A third isoform inducible NOS (iNOS) is synthesized after induction by endotoxins and proinflammatory cytokines (31).In hypoxic ischemic brain , NO derived from nNOS is cytotoxic and contributes to HIE because of its reaction with superoxide radicals and generation of peroxynitrite ; however NO generated from the eNOS causes vasodilatation , improves cerebral circulation and is beneficial (32).

This contradictory action of NO in HIE is dependent on the amount of NO produced , the time course of hypoxia when it is produced , the isoform or cellular location of NOS whether

eNOS or nNOS and also om the availability of superoxide radicals(32). It has also been proposed that following cerebral ischemia persistent activation of glutamate induced increased calcium concentration in ischemic neurons leads to persistent activation of nNOS , resulting in continuous NO production (33).

BBB separates and regulates the microenvironment of cerebral tissues from the peripheral circulation. Disruption of BBB in hypoxic conditions is contributed by oxidative stress , increased production of VEGF , excess of NO,and also by inflammatory cytokines . Disrupted BBB causes increased BBB permeability and subsequent brain edema and secondary brain damage. In our study we assessed the permeability of BBB by measuring the CSF albumin /serum albumin ratio , a method adopted by various previous studies(34,35).BBB permeability was found to be significantly high in HIE group and also across different stages of HIE , suggesting profound disruption of BBB in severe HIE with widespread neuronal damage. Our findings are in agreement with the findings of other researchers(36,37) . Several biochemical factors have been studied as possible marker of neuronal damage following hypoxic ischemic insult including various cytokines like IL-1b and IL-6 , S-100 B protein , NSE(neuron specific enolase), nitrotyrosine , adrenomedullin , activin A , VEGF, isoprostanes , MMPs and also non protein bound iron(38).After irreversible cellular injury brain cell death occurs with release of intracellular enzymes such as NSE, CK-BB, LDH and also AST(39). A high level of NSE in serum and CSF is a good biomarker of brain damage (40).Garcia et al reported a significant association between increased CSF NSE level and severity of encephalopathy(41).similarly Thorberg et al found significantly high levels of CSF NSE in HIE newborn (median value of 25.4 µg/l) compared to control group (median value of 10 µg/l, $p< 0.001$) (42). It was further reported that serum values were roughly double of those of CSF values and a serum NSE cutoff of 40 µg/l was suggested for moderate or severe HIE(43).

Our study shows a significantly higher values of NSE in HIE group than in control group with significant difference between different stages of HIE. We further found a positive correlation between serum NSE and BBB permeability and also between serum NSE and serum MDA and NO (figure 1,2,3). this can be explained in terms of increased free radical mediated lipid peroxidation during cerebral anoxia and reperfusion stage (as evidenced by increased serum MDA and NO levels) leading to disruption of BBB integrity (BBB permeability increases). The increased BBB permeability in turn leads to more pronounced neuronal death and consequent increased NSE levels in both serum and CSF.

There are a few limitations to our study. First , we did not measure F2 –isoprostanes ,derived from non enzymatic oxidation od arachidonic acid , which is a better indicator of lipid peroxidation than MDA(44).secondly , serum NO is measured only indirectly by measuring the amount of nitrates and nitrites . Thirdly , we did not measure MDA, NO, NSE in CSF , which could have helped us to correlate between plasma and CSF levels .

CONCLUSION:

In our study increased serum MDA and serum NO levels in HIE patients , suggests oxidative stress as a causation of hypoxic ischemic encephalopathy. Further increased BBB permeability and increased NSE levels in different stages of HIE , indicates extent of neuronal damage and severity of HIE. Increased free radical mediated injury in HIE proposes a possible preventive or therapeutic role of antioxidants in reducing the neuronal damage and subsequent outcome, which can be confirmed by large multicentric studies in different experimental and clinical setups.

Contributions: Seema Shah collected the data and prepared the manuscript. Anil Kumar Goel conceptualized and designed the study and reviewed the manuscript. Mamta Padhy helped with statistical calculation. Sumitra Bhoi helped in carrying out the tests

Disclaimer: None

REFERENCES

1. Bryce J, Boschi-Pinto C, Shibuya K, Black RE; WHO Child Health Epidemiology Reference Group. Who estimates of the causes of death in children. Lancet. 2005;365:1147-52.

2. Alonso-Spilsbury M, Mota-Rojas D, Villanueva-Garcia D, Martinez-Burnes J, Orozco H, Ramirez-Necoechea R, Mayagoitia AL, Trujillo ME. Perinatal pathophysiology in pig and human: A review. Anim Reprod Sci. 2005;90:1-30.

3. Shalak L, Perlman JM. Hypoxic-ischemic brain injury in the term infant – current concepts. Early Hum Dev. 2004; 80: 125-141

4. Saugstad OD. Oxygen toxicity in the neonatal period. *Acta Paediatr Scand.* 1990;79(10):881–892

5. Dormandy TL. In praise of peroxidation. *Lancet.* 1988;2(8620):1126–1128

6. Anagnostakis D, Messaritakis J, Damianos D, Mandyla H. Blood brain barrier permeability in "healthy" infected and stressed neonates. *J Pediatr.* 1992;121(2):291–294

7. Celtik C, Acunas B, Oner N, Pala O. Neuron specific enolase as a marker of the severity and outcome of hypoxic ischemic encephalopathy. Brain Dev. 2004;26:398-402.

8. Verdu Perez A, Falero MP, Arroyos A, Estevez F, Felix V, Lopez Y, Pantoja A, Ureta A. Blood neuronal specific enolase in newborns with perinatal asphyxia. Rev Neurol. 2001;32:714-7.

9. Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress: A clinical and electroencephalographic study. Arch Neurol 1976;33:696–705

10. Sinnhuber RO, Yu TC. Characterization of red pigment formed in the Z-thiobarbituric acid determination of oxidative rancidity. *Food Res.* 1958;23(6):626–633.

11. Evans T, Carpenter A, Kinderman H, Cohen J. Evidence of increased nitric oxide production in patients with sepsis syndrome.*Circ Shock.* 1993;41(2):77–81

12. Vannucci RC. Mechanisms of perinatal hypoxic-ischemic brain damage. Semin Perinatol. 2003; 17(5):330-7.

13. Vannucci SJ, Hagberg H. Hypoxia-ischemia in the immature brain. J Exp Biol. 2004; 207(Pt 18):3149-54.

14. Brankica Vasiljević, Svjetlana Maglajlić-Djukić, Miroslava Gojnić, Sanja Stanković .The Role of Oxidative Stress in Perinatal Hypoxic-Ischemic Brain Injury. Srp Arh Celok Lek. 2012;140(1-2):35-41

15. Finer NN, Robertson CM, Richards RT, et al. Hypoxicischemic encephalopathy in term neonates: perinatal factors and outcome. J Pediatr 1981;98: 112–17

16. Auer RN, Benveniste H. Hypoxia and related conditions. In: Graham DI, Lantos PL, eds. Greenfield's Neuropathology.London, UK: Edward Arnold 1997:263–314

17. Shouman BO, Mesbah A, Aly H. Iron metabolism and lipid peroxidation products in infants with hypoxic ischemic encephalopathy. J Perinatol. 2008; 28(7):487-91

18. Saugstad OD. Oxidative stress in the newborn: 30 year perspective. Biol Neonate. 2005; 88:228-36.

19. Schulz JB, Lindenau J, Seyfried J, Dichgans J. Glutathione, oxidative stress and neurodegeneration. Eur J Biochem. 2000; 267(16):4904-11.

20. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med.* 1985;312(3):159–163

21. Beckman JS. The double-edged role of nitric oxide in brain function and superoxide-mediated injury. *J Dev Physiol.* 1991; 15(1):53–59

22. Lewen A, Matz P, Chan PH. Free radical pathway in CNS injury. JNeurotrauma. 2000; 17:871-90.

23. Witt KA, Mark KS, Hom S, Davis TP. Effects of hypoxia-reoxygenation on rat blood-brain barrier permeability and tight junctional protein expression. Am J Physiol Heart Circ Physiol. 2003; 285(6):H2820-31.

24. Persidsky Y, Ramirez SH, Haorah J, Kanmogne GD. Blood-brain barrier: structural components and function under physiologic and pathologic conditions. J Neuroimmune Pharmacol. 2006;1(3):223-36.

25. Chu GL, Xin Y, Cheng J, Bi SY. Expression of neuronal nitric oxide synthase in brain tissue of hypoxic-ischemic neonatal rat

and the cerebral protective effect of neuronal nitric oxide synthase inhibitor 7-nitroindazole. *Zhonghua Yi Xue Za Zhi.* 2004;84(2):156–158

26. Schmidt H, Grune T, Mu¨ ller R, Siems WG, Wauer RR. Increased levels of lipid peroxidation products malondialdehyde and 4-hydroxynonenal after perinatal hypoxia. *Pediatr Res.* 1996;40(1):15–20

27. Wainwright MS, Grundhoefer D, Sharma S, Black SM. A nitric oxide donor reduces brain injury and enhances recovery of cerebral blood flow after hypoxia-ischemia in the newborn rat. *Neurosci Lett.* 2007;415(2):124–129

28. van den Tweel ER, Nijboer C, Kavelaars A, Heijnen CJ, Groenendaal F, van Bel F. Expression of nitric oxide synthase isoforms and nitrotyrosine formation after hypoxia-ischemia inthe neonatal rat brain. *J Neuroimmunol.* 2005;167(1–2):64–71

29. Zhao P, Zuo Z. Prenatal hypoxia-induced adaptation and neuroprotection that is inducible nitric oxide synthase-dependent. *Neurobiol Dis.* 2005;20(3):871–880

30. Rodrigo J, Ferna´ndez AP, Serrano J, Peinado MA, Martı´nez A. The role of free radicals in cerebral hypoxia and ischemia. *Free Radic Biol Med.* 2005;39(1):26–50

31. Serrano J, Encinas JM, Ferna´ndez AP, Rodrigo J, Martı´nez A. Effects of acute hypobaric hypoxia on the nitric oxide system of the rat cerebral cortex: protective role of nitric oxide inhibitors. *Neuroscience.* 2006;142(3):799–808

32. Wei G, Dawson VL, Zweier JL. Role of neuronal and endothelial nitric oxide synthase in nitric oxide generation in the brain following cerebral ischemia. *Biochim Biophys Acta.* 1999; 1455(1):23–34

33. Choi DW. Glutamate receptors and the induction of excitotoxic neuronal death. *Prog Brain Res.* 1994;100:47–51

34. Seyfert S, Faulstich A. Is the blood-CSF barrier altered in disease? *Acta Neurol Scand.* 2003;108(4):252–256

35. Andersson LM, Hagberg L, Fuchs D, Svennerholm B, Gissle´n M. Increased blood-brain barrier permeability in neuro-asymptomatic HIV-1-infected individuals: correlation with cerebrospinal fluid HIV-1 RNA and neopterin levels. *J Neurovirol.* 2001;7(6):542–547

36. Anagnostakis D, Messaritakis J, Damianos D, Mandyla H. Blood brain barrier permeability in “healthy” infected and stressed neonates. *J Pediatr.* 1992;121(2):291–294

37. van der Flier M, Stockhammer G, Vonk GJ, et al. Vascular endothelial growth factor in bacterial meningitis: detection in cerebrospinal fluid and localization in postmortem brain. *J Infect Dis.* 2001;183(1):149–153

38. Marianna Varsami, Theodoros Xanthos, Filippia Aroni, Ioanna Argyri, Pavlos Lelovas, Ismene Dontas, Apostolos Papalois, Vassilios Fanos, Nicoletta Iacovidou : Journal of Pediatric and Neonatal Individualized Medicine 2013;2(2):e020203

39. Reddy S, Dutta S, Narang A. Evaluation of lactate dehydrogenase, creatinine kinase and hepatic enzymes for the retrospective diagnosis of perinatal asphyxia among sick neonates. Indian Pediatr. 2008;45:144-7.

40. Nygaard O, Langbakk B, Romner B. Neuron-specific enolase concentrations in serum and cerebrospinal fluid in patients with no previous history of neurological disorder. Scand J Clin Lab Invest. 1998; 58:183-6.

41. Garcia-Alix A, Cabanas F, Pellicer A, Hernanz A, Stiris TA, Quero J. Neuron specific enolase and myelin basic protein; relationship of cerebrospinal fluid concentrations to the neurologic condition of asphyxiated full-term infants. Pediatrics. 1994;93:234-40.

42. Thornberg E, Thiringer K, Hagberg H, Kjellmer I. Neuron specific enolase in asphyxiated newborns: association with encephalopathy and cerebral function monitor trace. Arch Dis Child Fetal Neonatal Ed. 1995;72:39-42.

43. Celtik C, Acunas B, Oner N, Pala O. Neuron specific enolase as a marker of the severity and outcome of hypoxic ischemic encephalopathy. Brain Dev. 2004;26:398-402.

44. Milne GL, Sanchez SC, Musiek ES, Morrow JD. Quantification of F2-isoprostanes as a biomarker of oxidative stress. *Nat Protoc.* 2007;2(1):221–226