

# The Role of Oxidative Stress in Perinatal Hypoxic-Ischemic Brain Injury

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## SUMMARY

**Introduction** The pathogenesis of perinatal hypoxic-ischemic brain damage is highly complex.

**Objective** The aim of this study was to assess the role of oxidative stress in hypoxic-ischemic brain injury and subsequent abnormal neurological outcome in infants with perinatal hypoxic-ischemic encephalopathy (HIE). We estimated perinatal oxidative brain damage measuring activity of glutathione peroxidase (GPX) in cerebrospinal fluid (CSF) as an indirect biomarker of free radical production during cerebral hypoxia-ischemia in correlation with the level of intracellular enzyme neuron specific enolase (NSE) in CSF as a biomarker of extent of brain injury.

**Methods** Ninety neonates (>32 GA) with perinatal HIE were enrolled prospectively. HIE was categorized into three stages according Sarnat and Sarnat clinical scoring system and changes seen on amplitude integrated EEG. CSF for GPX analysis and NSE analysis was taken in the first 72 hours of life. Neurodevelopment outcome was assessed at 12 months of corrected gestational age.

**Results** GPX activity in CSF was in good relation with clinical stage of HIE ( $p < 0.0001$ ) and GA ( $p < 0.0001$ ) and significantly corresponded with subsequent neurodevelopment outcome ( $p < 0.001$ ). GPX activity in CSF showed a strong correlation with NSE levels in CSF ( $p < 0.001$ ) as the biomarker of extent of brain injury.

**Conclusion** Our results suggest that oxidative stress might be important contributing factor in perinatal hypoxic-ischemic brain damage, particularly in preterm neonates.

**Keywords:** hypoxic-ischemic encephalopathy; glutathione peroxidase; neuron specific enolase; neonates; neurodevelopment outcome

## INTRODUCTION

Perinatal hypoxic-ischemic encephalopathy (HIE) is a common cause of neonatal morbidity and mortality and neurological disabilities among survivors [1]. Each year 1.2 million neonates die and about one million infants have permanent neurological disability caused by perinatal HIE [2]. The pathogenesis of perinatal hypoxic-ischemic brain damage is highly complex, and involves impaired blood-brain barrier permeability, energy failure, loss of cell ion homeostasis, acidosis, increased intracellular calcium, excitotoxicity, free radical mediated toxicity, growth factor deficiency or upregulation and activation inflammatory cascade in immature brain [3]. Free radicals are highly reactive molecules generated predominantly during cellular respiration and normal metabolism. Imbalance between cellular production of free radicals and the ability of cells to defend against them is referred to as oxidative stress. Glutathione peroxidase (GPX) is a principal antioxidant enzyme and protects the cells against intracellular radicals and peroxides coming from the respiratory chain or other metabolic pathways. A number of studies documented that HIE were associated with increased production of free radicals in animal models [4]. The direct measurement of free radicals in biological samples is difficult because they are extremely reactive and have a short half-

life. Therefore, particularly in human studies, indirect approaches have been used to demonstrate free radical production during cerebral hypoxia-ischemia, measuring the products of free radical reaction with other molecules, such as lipids, proteins, and DNA, and the level or activity of antioxidant molecules. Neuron specific enolase (NSE) was originally described by Moore and McGregor [5] in 1965. NSE is intracytoplasmic glycolytic enzyme in neurons. Enolase has five isoenzymes. Those containing the gamma subunit, predominantly found in neurons of the central and peripheral nervous system are called NSE [6]. The major distinctive feature of NSE compared with other enolases is its high degree of stability.

## OBJECTIVE

In this study, we assessed the role of oxidative stress in perinatal hypoxic-ischemic brain injury. We estimated perinatal oxidative brain damage measuring activity of GPX in cerebrospinal fluid (CSF) as the indirect biomarker of free radical production during cerebral hypoxia-ischemia in correlation with level of intracellular enzyme NSE in CSF as a good biomarker of the extent of brain injury. Understanding the mechanisms of perinatal hypoxic-ischemic brain injury is essential to the design of effective neuroprotective interventions.

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## METHODS

Our prospective study was performed from January 2007 to January 2009 and was approved by the Ethical Committee for the Medical Research of the School of Medicine, University of Belgrade. We studied 90 neonates (gestational age >32 weeks) with perinatal asphyxia who subsequently developed HIE admitted to the neonatal intensive care unit at the Institute of Gynaecology and Obstetrics, Clinical Centre of Serbia, Belgrade. Our Institute serves as a referral centre for high-risk pregnancies, with delivery numbers of 7000–7500 per year. Written consent was obtained from all parents. Perinatal HIE was diagnosed if foetal distress (meconium staining of liquor or abnormal foetal heart rate), immediate neonatal depression (Apgar score <6 at 5 minute and/or necessary intubations in delivery room), metabolic acidosis (pH<7.20, BE≥10 mmol/l and lactate >3 mmol/L on arterial cord blood within 60 minutes of birth) and early neonatal encephalopathy (within the first 24 hours of life) were present. All of the neonates were resuscitated according to the guidelines of the Newborn Resuscitation Program of the American Academy of Pediatrics and American Heart Association. [7].

Complete obstetrical history and physical examinations were obtained on admission. Perinatal HIE was categorized into three stages according to Sarnat and Sarnat clinical scoring system and changes seen on amplitude integrated EEG. Amplitude-integrated electroencephalography (aEEG) was recorded during the first three days of life using cerebral function monitor-CFM Olympic 6000 (Olympic Biomedical, USA). Mild HIE (HIE stage I) was defined as an altered level of consciousness that included irritability with periods of spontaneous eye opening and jitteriness, slightly abnormal muscle tone, exaggerated Moro and absence of autonomic dysfunction and with normal continuous aEEG patterns (upper margin of the trace >10  $\mu$ V and lower margin >5  $\mu$ V) and without seizures. Moderate HIE (HIE stage II) was defined as somnolence with hypotonia, weak primitive reflexes, constricted pupils, bradycardia or periodic breathing and moderately abnormal aEEG patterns (upper margin of the trace >10  $\mu$ V and the lower margin <5  $\mu$ V) and with early seizures. Severe HIE (HIE stage III) was defined as stupor or coma with decerebrate posture, absent spontaneous activity, flaccid hypotonia, absent reflexes, seizures, nonreactive pupils and brainstem dysfunction with abnormal cranial nerve function and severely abnormal aEEG patterns (upper margin of the trace <10  $\mu$ V and the lower margin <5  $\mu$ V). A severely abnormal aEEG patterns may be accompanied by burst suppression or seizure activity.

Head sonograms were performed on all neonates before enrolment. Lumbar puncture was indicated for neonates as a part of the diagnostic work-up for suspected perinatal HIE to rule out other intracranial disorders (e.g., congenital or acquired neonatal infections, neonatal meningitis, inborn errors of metabolism) that may mimic the clinical features of perinatal HIE. Lumbar puncture for GPX analyses and NSE analyses were done in the first 72 hours of life. All samples were immediately frozen and stored at -80°C

until analysed. Hemorrhagic traumatic CSF samples were discarded. GPX activity in CSF was measured indirectly by a coupled reaction with glutathione reductase (GR) as determined by a modified method originally performed by Paglia and Valentine. The GPX activity was measured spectrophotometrically by GR recycling method, using an available commercial assay kit, according to the manufacturer's instructions (Ransel test kit, Randox Laboratories Ltd, UK). Concentrations of NSE in CSF were measured using commercial NSE kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Neurodevelopment outcome was assessed at 12 months of corrected gestational age by the neonatologist and the paediatric neurologist using the Denver Developmental Screening Test (DDST). Neurodevelopment outcome was classified as normal outcome, mild motor development delayed (slight abnormality in muscular tone or mild delayed of motor development) and severe adverse outcome (cerebral palsy, epilepsy or if died in follow up period).

We excluded infants with congenital malformations, inherited metabolic disorders, congenital or acquired neonatal infections and maternal drug addiction.

All statistical analyses were performed using SPSS for Windows (Chicago, IL, USA). For all tests values of  $p < 0.05$  were considered statistically significant. Data were expressed as mean value with standard deviation or as percentages when appropriate. Group comparisons were performed with the Pearson  $\chi^2$ -test or Fisher exact test or Kruskal-Wallis test and Wilcoxon rank sum test. Correlations were calculated by the Spearman rank method. Receiver operating curve (ROC) was used to determine a cut-off point for the prediction of abnormal neurodevelopment outcomes. Sensitivity, specificity and accuracy were then calculated.

## RESULTS

We studied 90 neonates (gestational age >32 weeks) with perinatal asphyxia who subsequently developed HIE. Fifty-seven neonates had mild HIE, 24 moderate HIE, and 9 severe HIE. Birth weight (BW) and gestational age (GA) were similar among the neonates with mild, moderate or severe HIE, while male and female proportions varied between different HIE groups ( $p < 0.05$ ) (Table 1). Distributions values of Apgar scores (AS) at 1 minute ( $W=166$ ;  $p < 0.05$ ) ( $W=166$ ;  $p < 0.05$ ) and AS at 5 minutes ( $W=181$ ;  $p < 0.01$ ) and values of arterial blood cord pH ( $W=184$ ;  $p < 0.001$ ), base deficit (BE) ( $W=167$ ;  $p < 0.05$ ) and lactate ( $W=51$ ;  $p < 0.05$ ) are shown in Table 1. Arterial cord blood pH had best predictive capacities for the severity of HIE and subsequent abnormal neurological outcome ( $W=916$ ;  $p < 0.001$ ) (Table 1). We did not detect a significant difference among the values of arterial blood pH, BE and lactate or AS at 1 minute and AS at 5 minutes in preterm and term neonates.

Concentrations of NSE correlated significantly with the clinical severity of HIE ( $\chi^2=48.364$ ;  $p < 0.0001$ ) and

**Table 1.** Clinical and biochemical characteristic at birth in neonates with different stage of hypoxic-ischemic encephalopathy (HIE) and those with subsequent neurological sequels

Parameter	HIE	HIE stage I	HIE stage II	HIE stage III	Neurological sequels
Gestational age (weeks)	36.6±2.6	36.4±2.5	37.4±2.6	36.2±3.1	35.8±2.7
Birth weight (g)	2711±810	2699±775	2889±796	2311±1000	2450±93
Male/Female (%)	62/38	54/46	67/33*	100/0*	67/33*
Apgar score (1st minute)	3.4±1.4	4.1±1.1	2.6±1.3	1.3±0.7*	2.5±1.6*
Apgar score (5th minute)	5.5±1.7	5.6±0.5	4.3±1.2	2.7±1.0*	3.8±1.8**
pH	7.09±0.11	7.15±0.05	7.03±0.11**	6.88±0.09***	6.97±0.14***
Base deficit (mmol/L)	-13.1±4.2	-11.0±1.7	-15.5±4.5*	-19.7±3.9*	-16.5±5.1*
Lactate (mmol/L)	7.4±4.4	5.4±2.9	9.6±4.6*	13.9±3.9*	11.0±5.1*
Numbers of neonates	90	57	24	9	15

Values are expressed as mean ± standard deviation (SD), percentages (%) or total numbers (Kruskal–Wallis chi-squared test and Wilcoxon rank sum test with continuity correction).

\* p<0.05; \*\* p<0.01; \*\*\* p<0.001

**Table 2.** Concentrations of biochemical markers in cerebrospinal fluid in neonates with different stage of hypoxic-ischemic encephalopathy (HIE) and those with subsequent neurological sequels

Parameter	HIE	HIE stage I	HIE stage II	HIE stage III	Neurological sequels
NSE (µg/L)	24.3±17.7	15.4±8.2****	33.9±16.2**	54.8±19.5***	49.5±18.7****
GPX (U/L)	118.4±34.9	106.6±29.5****	128.6±33.1**	166.1±27.4****	153.7±28.7****

Values are expressed as mean ± SD (Kruskal–Wallis chi-squared test and Wilcoxon rank sum test with continuity correction).

NSE – neuron specific enolase; GPX – glutathione peroxidase

\*\* p<0.01; \*\*\* p<0.001; \*\*\*\* p<0.0001

corresponded well with subsequent neurological outcome ( $W=65.5$ ;  $p<0.0001$ ) (Table 2). We did not detect a significant difference in concentrations of NSE in CSF between preterm and term neonatal groups (Table 3). Negative linear correlation between the values of arterial cord blood pH and concentrations of NSE in CSF ( $r=-0.672$ ;  $p<0.001$ ) are shown in Graph 1. Elevated levels of NSE in CSF (cut off value for poor neurodevelopmental outcomes was found 25.5 µg/L) were observed in all neonates with subsequent adverse neurological outcome (AUC of NSE in CSF: 0.942 (0.895-0.998);  $p<0.001$ ) (Graph 2).

GPX activity in CSF was significantly higher in the neonates with moderate and severe HIE than in those with mild HIE ( $\chi^2=23.301$ ;  $p<0.0001$ ) and corresponded well with subsequent neurological outcome ( $W=179$ ;  $p<0.0001$ ) (Table 2). GPX activity in CSF was higher in preterm neonates compared with term neonates ( $W=1649$ ;  $p<0.0001$ ) (Table 3). Negative linear correlation between values of arterial cord blood pH and GPX activity in CSF ( $r=-0.384$ ;

**Table 3.** Comparison values of biochemical markers in cerebrospinal fluid between preterm and term neonates

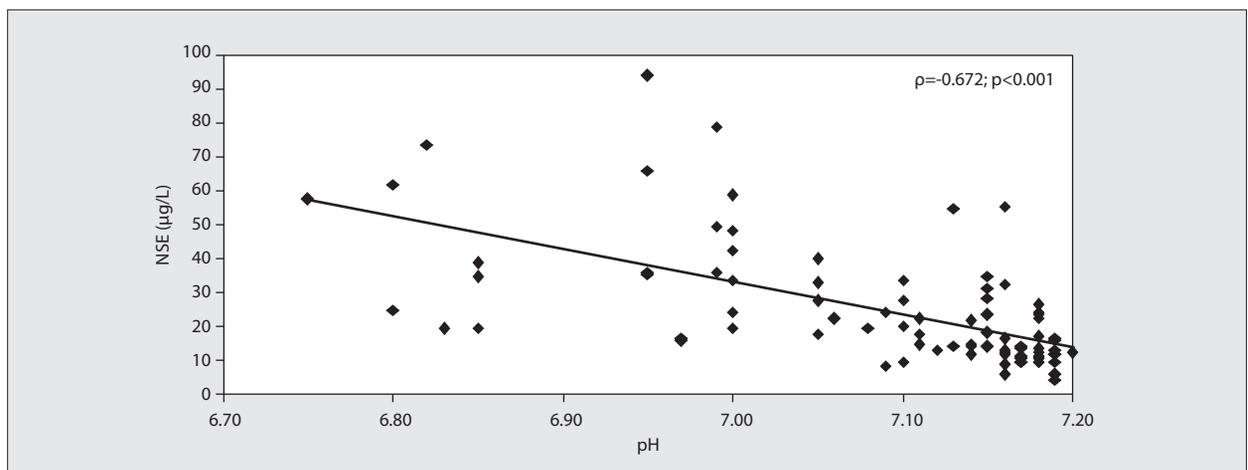
Neonates	Number	NSE (µg/L)	GPX (U/L)
Preterm	45	26.7±20.6	137.2±33.5****
Term	45	21.9±14.1	99.6±25.9

Values are expressed as mean ± SD (Fisher exact test).

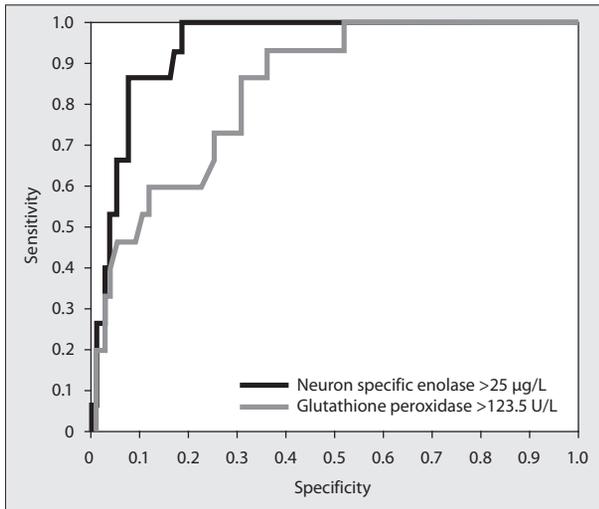
\*\*\*\* p<0.0001

$p<0.001$ ) are shown in Graph 3. Elevated GPX activity in CSF (cut off value for poor neurodevelopmental outcomes was 123.5 U/L) was observed in all neonates with subsequent adverse neurological outcome (AUC of GPX in CSF: 0.841 (0.756-0.935);  $p<0.001$ ) (Graph 2). GPX activity in CSF showed a strong correlation with NSE levels in CSF ( $r=0.543$ ;  $p<0.001$ ) (Graph 4).

Neurodevelopment outcome at 12 months of corrected gestational age corresponded well with severity of HIE ( $p<0.01$ ) and gestation age ( $p<0.05$ ) (Table 4) All infants with severe HIE developed severe adverse outcome



**Graph 1.** Negative linear correlation between arterial cord blood pH and neuron specific enolase (NSE) in cerebrospinal fluid ( $p<0.001$ )

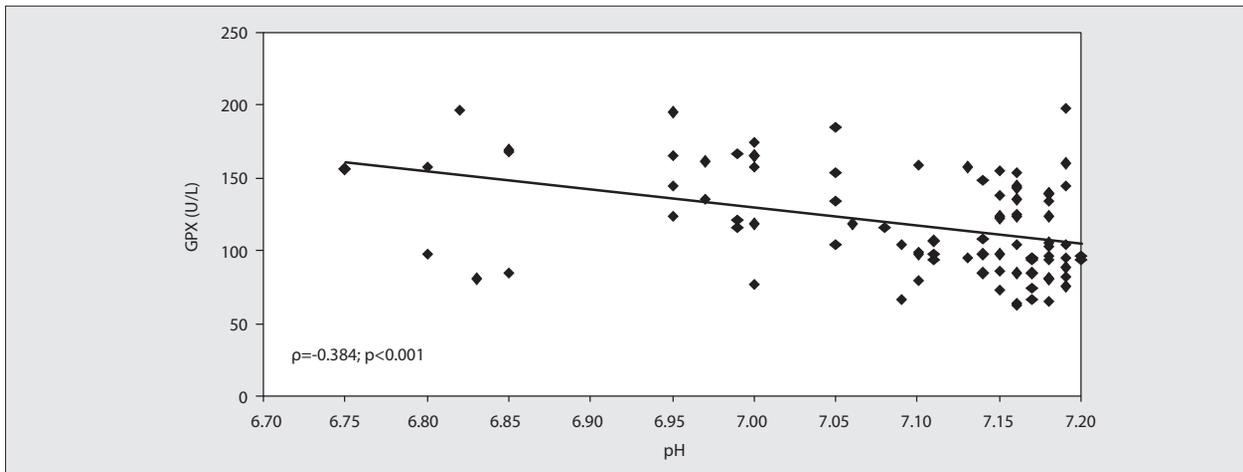


**Graph 2.** Comparison of receiver operating characteristics (ROC curve) estimated laboratory tests

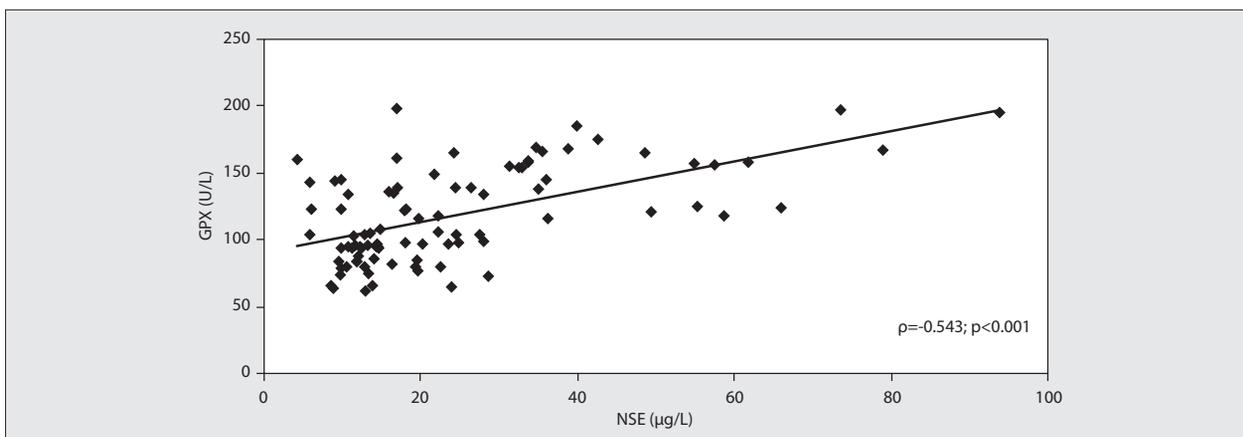
(cerebral palsy, epilepsy or died in follow up period). Two preterm neonates with severe HIE died, one within early neonatal period because of multiorgan failure, the other died later because of respiratory dysfunction. Incidences of neurological sequels were significantly higher in preterm infants ( $p < 0.05$ ). NSE exhibited superior prediction of abnormal outcomes at 12 months of age (sensitivity was found 100% and specificity 81%) when compared to GPX activity in CSF (sensitivity was 87% and specificity 69%) (Graph 5).

## DISCUSSION

Perinatal hypoxic-ischemic brain damage is a major cause of acute mortality and chronic neurological morbidity in infants and children [8]. The incidence of perinatal HIE



**Graph 3.** Negative linear correlation between values of arterial cord blood pH and concentrations of glutathione peroxidase (GPX) in cerebrospinal fluid ( $p < 0.001$ )

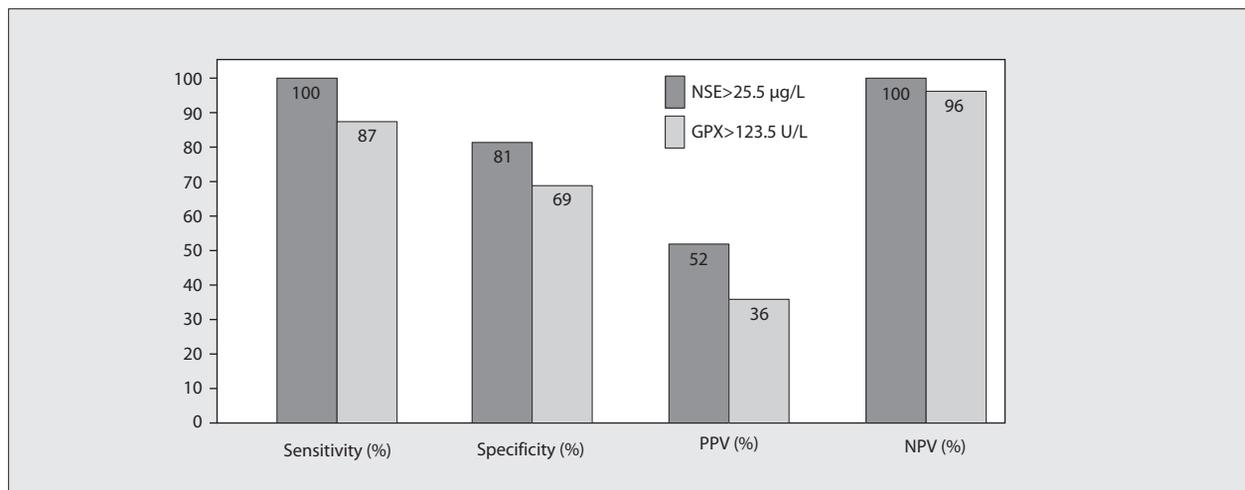


**Graph 4.** Linear correlation between concentrations neuron specific enolase (NSE) and glutathione peroxidase (GPX) in cerebrospinal fluid ( $p < 0.001$ )

**Table 4.** Neurodevelopment outcome in infants with different stage of hypoxic-ischemic encephalopathy (HIE) and different gestation age at birth (Fisher exact test)

Neurodevelopment outcome	HIE	HIE stage I	HIE stage II	HIE stage III	Preterm neonates	Term neonates
Normal (%)	73.9	91.3**	54.2**	0**	62.7	84.4*
Mild motor developmental delay (%)	12.5	7**	29.2**	0**	16.3*	8.9
Cerebral palsy (%)	10.2	1.7**	12.5	71.4**	14*	6.7
Epilepsy (%)	3.5	0	4.1	28.6**	13.3*	0

\*  $p < 0.05$ ; \*\*  $p < 0.01$



**Graph 5.** Comparison of predictive value estimated laboratory tests

PPV – positive predictive value; NPV – negative predictive value; NSE – neuron specific enolase; GPX – glutathione peroxidase

varies at different gestational ages. Statistics suggest an incidence of HIE 3-5 per 1000 full-term births and an incidence approaching 60% in premature newborns [9]. In our study, the incidence of perinatal HIE was similar. The age-dependent regional vulnerability to hypoxic-ischemic insults seen in the immature brain can be explained, at least in part, by the density of NMDA receptors and nNOS-positive cells. The immature brain is especially sensitive to oxidative damage in comparison to the mature brain, because it has poor antioxidant capabilities and a high concentration of free iron and lipids. In our study males had a higher incidence of long term developmental disabilities than females. A recent analysis of a European database of 4500 children with cerebral palsy found that the incidence of CP was 30% higher in males. Sex differences in the immature brain appear to be strongly influenced by intrinsic differences between male and female cells and that this is influenced by the sex chromosomes and sex hormones [10]. The incidence of long-term complications depends on the severity of HIE. Understanding of the mechanisms of perinatal hypoxic-ischemic brain injury is essential to the design of effective neuroprotective interventions [11, 12]. Treatment is currently limited to supportive intensive care, but efforts have been made to develop more effective therapies. In adults, neuronal necrosis and apoptosis after global ischemia are slow, and last for several hours to several days. Studies in animals suggest a quicker cellular destruction and energy substrates in the neonatal brain continue to run down for 12 to 48 h after perinatal hypoxia [13]. Therefore, a neuroprotective intervention might be effective from 6 to 8 hours after perinatal asphyxia. As neuroprotective interventions may be harmful, it is important to find early and reliable indicators of brain damage or poor long-term prognosis to initiate neuroprotective treatment [14].

The information obtained by examination of CSF is often of crucial importance in the diagnosis of many neurological diseases (HIE, ischemic stroke and different types of meningitis). Several studies measured different biochemical factors in serum and CSF after the hypoxic-ischemic events [15, 16]. After irreversible cellular injury,

brain cells die by necrotic lysis or apoptosis, which releases intracellular enzyme such as NSE. A high level NSE in CSF is considered as the result of neural damage and it could be a good biomarker of brain damage or dysfunction of CNS [17, 18]. The optimal time for measuring released intracellular enzyme in CSF remains uncertain. In studies of adults, NSE increased 18 hours to four days after cerebral infarction and during the first days following anoxaemia [19]. As delayed neuronal death peaks between the 2<sup>nd</sup> and 3<sup>rd</sup> day of life, the optimal time is most likely to be at around 72 hours. Our results provide a strong association between concentrations of NSE in CSF and severity of stage of HIE, extent of brain damage and subsequent neurological outcome. CSF contains both powerful enzymatic and non-enzymatic antioxidants, and CSF analysis provides some important clues about the physiological or pathological changes in the central nervous system. There is little information in the literature on the evaluation of oxidative stress markers in CSF. Our present results showed that the activity of GPX in CSF was markedly higher in the preterm neonates and neonates with severe HIE. Increased GPX activity in CSF occurred in response to hypoxic-ischemia and might suggest the overproduction of reactive oxygen species and brain oxidative stress. In agreement with our results, a gradual increase of GPX activity in CSF have indicated advancing stage of HIE and the extent of brain damage. Free radicals, which are produced continuously during oxidative metabolism, are generated at high rates within the brain. Oxidative stresses due to overproduction of free radicals effectively overwhelms the reduced anti-oxidative mechanisms of the newborn and have been suggested as possible factors contributing to the pathogenesis of perinatal hypoxic-ischemic brain injury [20]. The brain contains lower superoxide dismutase, catalase and GPX activity compared with kidney or liver [21]. The neonatal brain is particularly vulnerable to oxidative damage because of its high concentration of lipids, high rate of oxygen consumption, decreased levels of antioxidant enzymes (i.e. GPX and catalase) and increased availability of free iron [22, 23]. Oxidative injury takes place mostly

during the reperfusion phase [24]. Free radicals can lead to lipid peroxidation as well as DNA and protein damage and can trigger apoptosis [25, 26]. Finally, free radicals can combine with nitric oxide to form peroxynitrite, a highly toxic oxidant. Free radicals also induce inflammation and further formation of more oxidative radicals during the reperfusion phase via non-protein bound and pro-oxidant enzymes, such as nitric oxide synthase, cyclooxygenase/lipoxygenase, and xanthine oxidase [27]. The increased free radicals level causes destruction of the endothelial monolayer tight junctions of blood-brain barrier (BBB) and increases its permeability [28]. Disruption of BBB results in increased vascular permeability, brain oedema, and secondary brain damage [29]. Data associated with GPX activity after acute hypoxic-ischemic brain injury is controversial. The GPX activity in patients with acute hypoxic-ischemic insult was reported to be decreased, increased or unchanged in both CSF and serum. In our study, we noted increased GPX activity in CSF in neonates with advanced stage of HIE and lower gestation age which is in accordance with published data, however, we observed a significant correlation between the severity of stage of HIE and GPX activity in CSF. Increased GPX activity of CSF in neonates with HIE might be an indicator of perina-

tal hypoxic-ischemic brain injury and improve our ability to optimize the timing and assessment of neuroprotective treatment at the same time.

## CONCLUSION

The results of our study may have clinical and research implications. We postulate that oxidative stress might be an important contributing factor in the pathogenesis of perinatal hypoxic-ischemic brain injury particularly in preterm neonates. Our results demonstrate that increased GPX activity in CSF is related to the extent of brain injury and subsequent neurological abnormalities in infants with perinatal HIE, which might be prevented by antioxidative treatment. Further experimental and clinical studies are needed to clarify this point.

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## Улога оксидативног стреса у настанку перинаталног хипоксично-исхемијског оштећења мозга

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### КРАТАК САДРЖАЈ

**Увод** Патогенетски механизам перинаталног хипоксично-исхемијског оштећења мозга је веома сложен.

**Циљ рада** Циљ ове студије био је да се процени значај оксидативног стреса у настанку перинаталног хипоксично-исхемијског оштећења мозга и каснијих неуролошких секвела код новорођенчади с хипоксично-исхемијском енцефалопатијом (ХИЕ). За процену перинаталног оксидативног оштећења мозга коришћена је индиректна метода којом се утврђује стварање слободних радикала током церебралне хипоксије, односно исхемије мерењем активности глутатион-пероксидазе (GPX) у ликвору у односу на ниво унутарћелијског ензима неурон-специфичне енолазе (NSE) у ликвору као добар биолошког показатеља оштећења мозга.

**Методе рада** Проспективном студијом обухваћено је 90 новорођенчади (старије од 32 недеље гестације) с перинаталном ХИЕ. Она је процењивана на основу „Сарнат и Сарнат“ клиничког скорa и промена регистрованих на енцефа-

лограму интегрисаних амплитуда, а потом категорисана на три степена. Активност GPX и концентрација NSE одређиване су у ликвору узетом у прва 72 часа по рођењу. Неуролошка процена испитиване деце вршена је у узрасту од 12 месеци, а коригована је према гестационој зрелости на рођењу. **Резултати** Активност GPX у ликвору је у корелацији с тежином ХИЕ и гестационом зрелошћу новорођенчета и у складу с каснијим неуролошким исходом ( $p < 0,001$ ). Активност GPX у ликвору је у јасној корелацији с нивоом NSE у ликвору ( $p < 0,001$ ), која је добар показатељ интензитета оштећења мозга.

**Закључак** Резултати истраживања показују да оксидативни стрес има важну улогу у настанку перинаталног хипоксично-исхемијског оштећења мозга, посебно код детета рођеног пре термина.

**Кључне речи:** хипоксично-исхемијска енцефалопатија; глутатион-пероксидаза; неурон-специфична енолаза; новорођенче; неуролошки развој

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