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High cerebrospinal fluid antioxidants and interleukin 8 are protective of hypoxic brain damage in newborns

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Abstract

The objective was to explain the discrepancy in the development of hypoxic ischemic brain injury (HIE) in some asphyxiated newborns rather than others. Forty newborns were classified according to their cerebrospinal neuron-specific-enolase (CSF-NSE) levels on their 5th-day of life; group 1 with low-NSE (n = 25). The remaining 15 newborns had high-NSE and were further divided into a group with no HIE (n = 10, group 2) and another with HIE (n = 5, group 3). CSF-NSE, total-hydroperoxide (TH), biological-antioxidant-potentials (BAPs), 12 cytokines and Erythropoietin (EPO) were measured. The TH/BAP gave the oxidative-stress-index (OSI). The BAPs of serial dilutions of three types of EPO were tested. CSF-NSE and TH and mean OSIs were higher in group 3. IL-8 and mean BAPs were higher in group 2 than in group 1. EPO was less detected in group 3. Serial EPO dilutions correlated with their BAPs. Compensatory antioxidants and IL-8 elevation could be protective of perinatal asphyxic brain injury. Antioxidative effect of EPO could be neuroprotective.

Keywords: *Brain injury, neuron-specific enolase, neonate, cytokines, erythropoietin*

Introduction

Perinatal asphyxia is still a matter of great concern because of its high rate of mortality and morbidity [1,2]. Yet, there is discrepancy in the development of hypoxic ischemic brain injury in asphyxiated newborns rather than others. Hypoxic-ischemic encephalopathy (HIE) after perinatal asphyxia is a condition in which cerebrospinal fluid (CSF) concentrations of brain-specific biochemical markers, cytokines and radicals may be elevated [3–8]. Of these brain-specific proteins, the neuron-specific enolase (NSE) has been found to be released in high concentrations into the CSF of asphyxiated newborns and correlates significantly with long-term prognosis and neurological impairment [3].

On the other hand, the pathogenesis of brain injury in newborns is not entirely clear, though ischemia-reperfusion and infection/inflammation appear to be important [6]. The proinflammatory cytokine Interleukin-8 has been previously reported to be markedly elevated in the CSF of asphyxiated newborns with brain injury [7]. Yet IL-8 action is not fully understood.

It has been proven recently that hydroxyl radicals are generated in the central nervous system during

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asphyxiation [8]. The total hydroperoxide (TH) represents a measure of overall oxidative injury because they are the intermediate oxidative product of lipids, peptide and amino acids [9]. On the other hand, the biological antioxidant potentials (BAPs) represent the total antioxidative activity [10].

A homeostatic balance exists between the formation of reactive oxygen species (ROS) and their removal by endogenous antioxidant-scavenging compounds [11]. Oxidative stress occurs when this balance is disrupted by any excessive production of ROS or by inadequate antioxidant defenses.

Newborns and particularly pre-term infants are at high risk for oxidative stress and are very susceptible to oxidative injury by ROS [12]. This is because newborns have less protection against oxidation [13].

Erythropoietin (EPO) belongs to the cytokine superfamily and has traditionally been viewed as a haematopoiesis-regulating hormone. Hypoxia provokes the increase of endogenous erythropoietin (EPO) in the CSF of newborns [14]. In experimental animals, locally or systemically administered recombinant human erythropoietin (rHuEpo) is neuroprotective against a variety of insults, including cerebral ischemia and experimental autoimmune encephalomyelitis [15].

In order to understand the inter-relationship between the different markers and mediators released in the CSF of asphyxiated newborns, we studied the levels of TH, BAPs, cytokines and EPO in the CSF of asphyxiated newborns classified by their CSF levels of NSE with and without brain injury and to evaluate whether the changes in CSF levels of oxidative stress mediators and cytokines in asphyxiated newborns could explain the discrepancy in the development of hypoxic ischemic brain injury in some asphyxiated newborns rather than others.

Furthermore, we studied the BAPs of three different biochemical types of EPO *in vitro*: recombinant human (rh)-EPO, Asialo EPO and Carbamide EPO.

Patients and methods

The procedures followed were in accordance with the ethical standards of the ethical committee of both Nagoya City University and Daini Red Cross hospital.

Patients and classification

Among the infants admitted to the Neonatal Intensive Care Unit of Nagoya City University Hospital and the Nagoya Daini Red Cross Hospital were 58 newborns enrolled in the study. They all had abnormal neurological signs as described by Sarnat and Sarnat [2], such as increased irritability and jittery abnormal tones, abnormal primitive reflexes, altered consciousness or convulsions. After obtaining a parental informed consent, the CSF samples were withdrawn within the 5th and 6th post-delivery days for electrolytes, glucose, culture and Gram stain as part of screening for neonatal sepsis. All babies with congenital malformations, inborn error of metabolism, sepsis, diabetic mothers, blood group incompatibility, increased leukocytes and/or erythrocytes in CSF and positive bacterial cultures of CSF or blood were excluded. Among the 58 newborns, 40 newborns (25 male and 15 female) had Apgar scores lower than 5 at 1 and/or 5 min and the remaining 18 newborns were excluded from the study. Another parental informed consent was obtained to enrol the infant to the study. The clinical status of the enrolled cases was assessed daily and the infants were diagnosed for the development of neonatal hypoxic ischemic encephalopathy (HIE) according to Sarnat and Sarnat [2] and magnetic resonance imaging (MRI) examinations were performed within the first 3 months of life and/or 1 year of life and the final imaging diagnosis of intracranial lesions was made by a radiologist. Follow-up period of all enrolled newborns was more than 18 month after birth. Neurological examinations were performed on infants of more than 1 year and psychomotor development was assessed by calculation of the Developmental Quotient (DQ). The criteria for 'normal development infant' at a given age was the absence of neurological abnormalities and a DQ higher than 80.

The study protocol was approved by the ethics committee of both hospitals. The 40 newborns were classified according to their CSF-NSE level of 10 ng/ml (8.4 $\pm$ 1.6 ng/ml), which is the mean $\pm$3 SEM of what Sobajima and Togari [16] previously reported in normal newborns. The low-level group, which had CSF-NSE levels from 3.7–9.1 ng/ml (mean 6.7 ng/ml), included 25 newborns; all had developed normally and were considered as group 1 (Tables I and II). A high CSF-NSE group, which had CSF-NSE levels

Table I. The clinical data of the newborns in each group.

Items	Group 1 (n = 25)	Group 2 (n = 10)	Group 3 (n = 5)	p-value
Gestational age	39.59 $\pm$ 0.27 w	39.03 $\pm$ 0.67 w	38.83 $\pm$ 0.98 w	NS
Mode of delivery VD/CS	16/9	5/5	1/4	NS
Body weight	2949.2 $\pm$ 81 g	2967.1 $\pm$ 200.3 g	2825 $\pm$ 145.5 g	NS
Apgar at 1 m	3 (0–5)	3 (1–5)	2 (1–5)	NS
Apgar at 5 m	6 (3–9)	7 (5–10)	4 (1–7)	NS

w: weeks; NS: not significant; VD/CS: vaginal delivery/cesarean section; g: grams; m: minute. Apgar scores are expressed as median; range in parentheses.

Table II. Diagnosis and complications of the newborns in groups 1 and 2.

Groups	Asphyxia only	Asphyxia with other complications			
		TTN	MAS	Apnea	PPHN
Group 1	16	2	5	1	1
Group 2	3	0	5	1	1

TTN: Transient tachypnea of the newborn. MAS: Meconium aspiration syndrome. PPHN: Persistent pulmonary hypertension of the newborn.

from 10.2–44.3 ng/ml (mean 20 ng/ml), included 15 newborns; among them 10 had developed normally with no evidence of brain injury and were considered as group 2 (Tables I and II). The remaining five newborns had brain injury and were considered as group 3 (Tables I and III).

The clinical data are presented in Tables I–III. The Apgar scores of the enrolled newborns at 1 and 5 minutes are presented in Figure 1A and B.

Table III. Diagnosis and clinical profile of the newborns in group 3.

	Diagnosis	Complication	Profile
Case 1	HIE (severe)		Quadriplegia. MR, epilepsy. Diffused abnormal SI T2 & Bilateral atrophy of the basal ganaglia
Case 2	HIE (moderate)	TTN	Quadriplegia. Epilepsy. Bilateral white matter atrophy
Case 3	HIE (severe)	MAS	Quadriplegia. MR, epilepsy. Diffused abnormal SI T2 and brain atrophy.
Case 4	HIE (moderate)	Pulmonary haemorrhage	No hand movements Bilateral atrophy of the basal ganaglia
Case 5	HIE (severe)		Quadriplegia MR Extensive atrophy of the basal ganglia, and decrease in white matter.

HIE: Hypoxic-ischemic encephalopathy. MR: Mental retardation. SI: Signal intensity TTN: Transient tachypnea of the newborn. MAS: Meconium aspiration syndrome.

Methods

A small portion, ~60 μL, of the CSF samples which were taken as explained previously were used after obtaining parental informed consent to be enrolled in this study.

TH production was measured by using a d-ROMs kit (Diacron srl, Proma, Italy) in the free radical analytic system (FRAS), as previously described [9]. Briefly, in the presence of iron (which is released from the proteins by an acidic buffer), free radicals are able to generate alkoxyl and peroxyl radicals, according to Fenton's reaction. Such radicals, in turn, are able to oxidize an alkyl-substituted aromatic amine (A-NH2, dissolved in a chromogenic mixture), which transforms them into a pink-coloured derivative. Finally, this coloured derivative was photometrically quantified. The intensity of the developed colour is directly proportional to the concentration of TH.

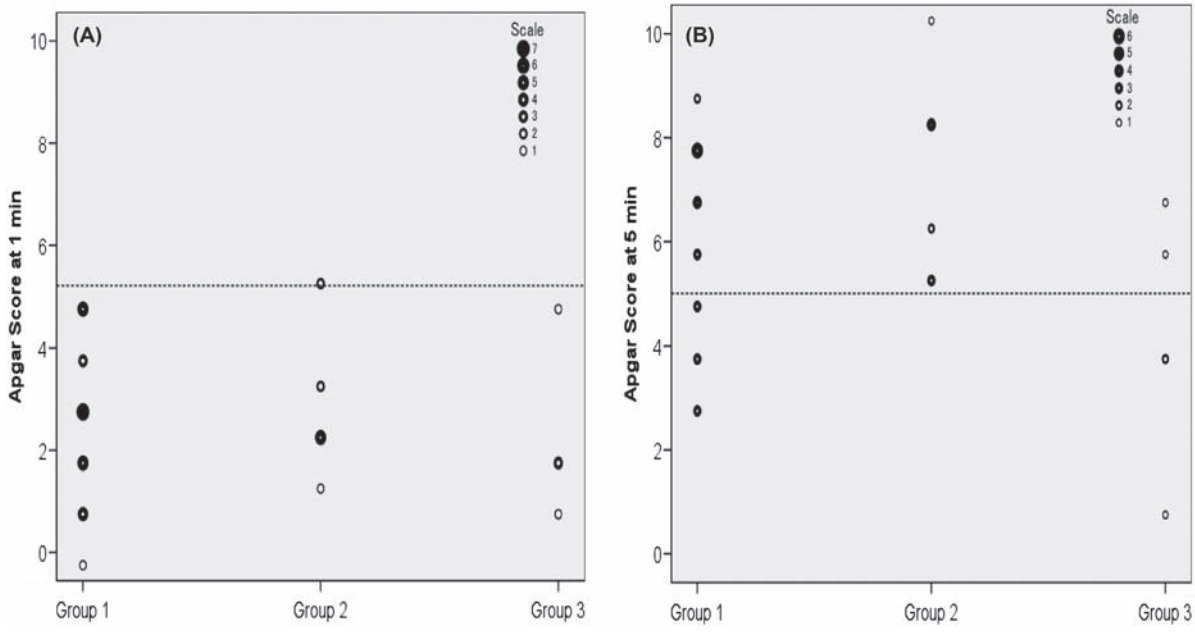

Figure 1. The Apgar score at (A) 1 min and (B) 5 min in 40 newborns, group 1 low CSF-NSE newborns (n = 25), group 2 newborns with high CSF-NSE but without brain injury (n = 10) and group 3 newborns with high CSF-NSE and brain injury (n = 5).

Results were expressed in conventional arbitrary units, called Carr units, equal to a concentration of 0.08 mg/dl of hydrogen peroxide.

BAPs were measured using a commercial assay kit (Diacron srl, Proma, Italy) in the FRAS, as previously described [10]. The BAPs test is based on the ability of a coloured solution, containing a source of ferric (Fe^{3+}) ions adequately bound to a special chromogenic substrate, to decolour when Fe^{3+} ions are reduced to ferrous ions (Fe^{2+}), which occurs through the addition of a reducing/antioxidant system.

The ratio of TH to BAP gave the oxidative stress index (OSI), because the shift of oxidative/antioxidative balance toward the oxidative side is considered to be oxidative stress [17].

CSF-NSE was measured by using the enzyme immunoassay procedure previously described [16]. Erythropoietin was measured by using a Human Erythropoietin (EPO) immunoassay kit (Stem Cell Technologies, Vancouver, B.C., Canada). Twelve cytokines, Tumor necrosis factor (TNF), Interleukin (IL-1a), IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, Granulocyte Macrophage colony-stimulating factor (GM-CSF) and Interferon (IFN)-γ, were measured by using the Proteoplex human cytokine array kit (Novagem, Merck, Darmstadt, Germany).

Measuring the BAPs of three different biochemical types of erythropoietin. We used concentrated rh-EPO, Asialo EPO and Carbamide EPO (20 000 IU/ml) to produce 13 serial dilutions of each drug and then we measured their BAPs by using the commercial assay kit (Diacron srl, Proma, Italy), as explained previously.

Statistical analysis. The distribution of data were tested using the Shapiro-Wilk test. The means of the three groups (inter-groups) were compared using the analysis of variance (ANOVA), followed by the Bonferroni multiple comparison procedure. If the data were not normally distributed, the Kruskal-Wallis test was used. When significance was detected the Mann-Whitney test was used. The coefficient of relation was studied by using the Pearson two tailed correlation coefficient, and if the data were non-parametric the Spearman two tailed test was used. Data were reported as mean ± SEM, unless mentioned otherwise. Probability values <0.05 were considered significant. All data analyses were performed with commercially available statistical analysis software package SPSS 14 (Statistical Package for Social Sciences, Chicago, IL).

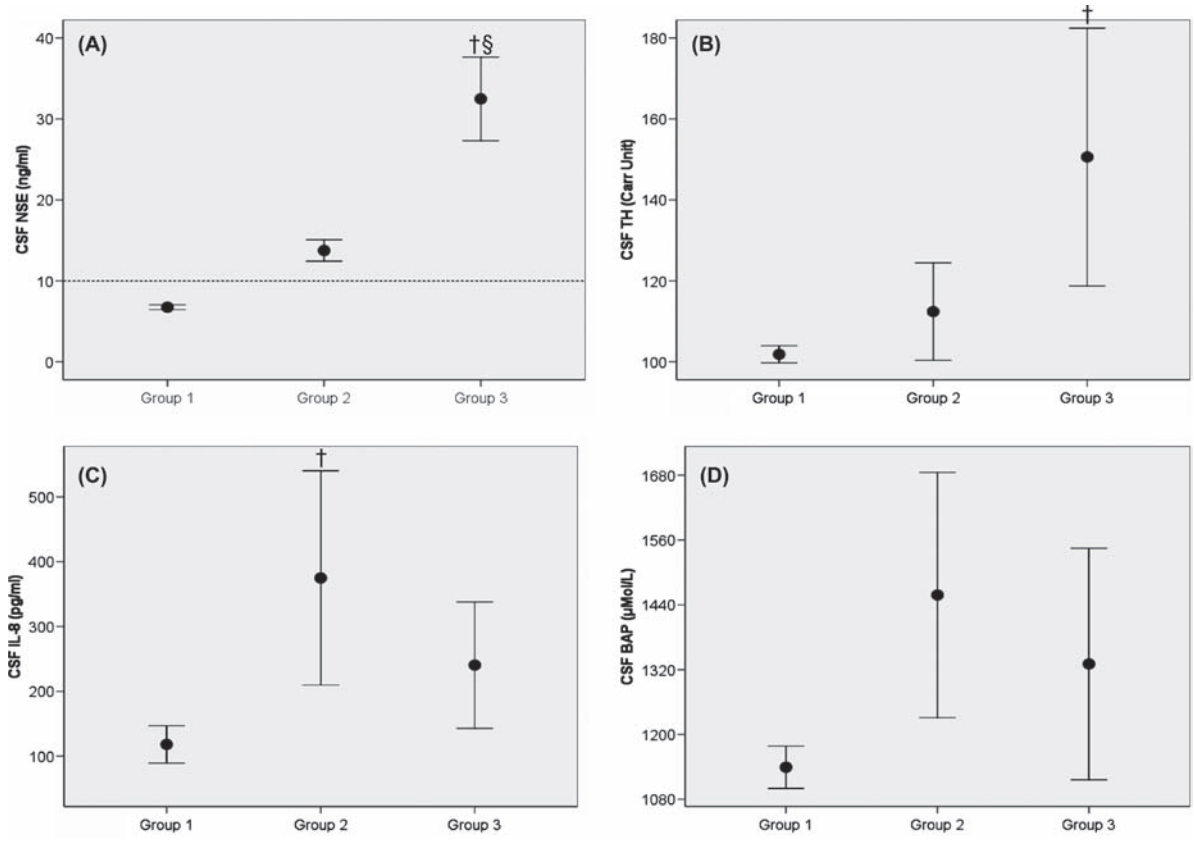

Figure 2. Cerebrospinal fluid (CSF) measurements. (A) Neuron-specific enolase (NSE), (B) Total hydroperoxide (TH), (C) Biological antioxidant potentials (BAPs) and (D) Interleukin (IL)-8. In 40 newborns, group 1 low CSF-NSE newborns ($n = 25$), group 2 newborns with high CSF-NSE but without brain injury ($n = 10$) and group 3 newborns with high CSF-NSE and brain injury ($n = 5$). † and § $p < 0.05$ compared to group 1 and group 2, respectively, CSF-NStE level of 10 ng/ml was used to classify the enrolled newborns as low CSF-NSE and high CSF-NSE (dotted line in figure 2-A).

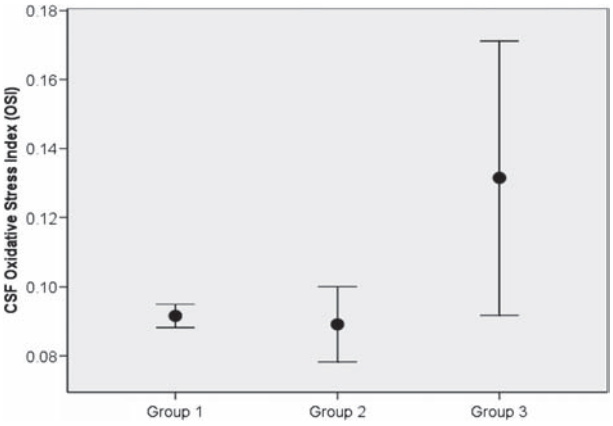

Figure 3. The oxidative stress index (OSI) is the ratio of total hydroperoxide (TH) to biological antioxidant potentials (BAPs) in cerebrospinal fluid (CSF). In 40 newborns, group 1 low CSF-NSE newborns (n = 25), group 2 newborns with high CSF-NSE but without brain injury (n = 10) and group 3 newborns with high CSF-NSE and brain injury (n = 5).

Results

Outcome

All cases in groups 1 and 2 developed normally with no evidence of brain injury during the follow-up period of more than 18 month. Cases in group 3 had variable degrees of brain damage and HIE classified according to Sarnat and Sarnat [2], as mentioned in Table III.

The CSF-NSE levels in group 3 were higher than in group 2 (32.48 ± 5.16 ng/ml vs 13.7 ± 1.3 ng/ml, $p < 0.001$). The CSF-NSE levels in groups 2 and 3 were higher than in group 1 (6.7 ± 0.3 ng/ml, $p < 0.005$ and 0.001, respectively) (Figure 2A).

The CSF-TH levels were higher in group 3 than in both groups 1 and 2 (150.6 ± 31.8 Carr units vs 101.8 ± 2.1 Carr units and 112.4 ± 12 Carr units), respectively, but this difference was significant only compared to group 1, $p < 0.01$. The CSF-TH levels in groups 1 and 2 showed no significant differences (Figure 2B).

The CSF-BAPs and IL-8 levels were higher in group 2 than in group 1 (1458.3 ± 227 μMol/L vs 1139.4 ± 39.3 μMol/L and 374.9 ± 165.3 pg/ml vs 118.2 ± 29 pg/ml, respectively), but this difference was significant only in the case of IL-8, $p < 0.05$ (Figures 2C and D).

The means of both the CSF-BAPs and IL-8 were higher in group 2 than in group 3 (1330.6 ± 214. 3 μMol/L and 240.5 ± 97.4 pg/ml, respectively), but the differences did not reach significance.

The OSIs were higher in group 3 than in both groups 1 and 2 (0.13 ± .04 vs 0.09 ± 0.03 Carr units and 0.089 ± 0.03 Carr units, respectively), but the differences did not reach significance.

The OSI levels in groups 1 and 2 showed no significant differences (Figure 3). In the CSF of all cases enrolled in the study, NSE levels correlated with the levels of the TH and the BAPs levels correlated with the levels of IL-8 (r^2 = 0.4 and 0.6, $p < 0.01$ and 0.001, respectively).

The CSF-EPO levels were detected in 15 out of the 25 in group 1 (60%), seven out of 10 in group 2 (70%) and one out of five in group 3 (20%); there were no differences in CSF-EPO levels between groups 1 and 2 (Figure 4).

The serial dilutions of rh-EPO, Asialo EPO and Carbamide EPO significantly correlated with their biological antioxidative potentials (r^2 = 0.66, 0.58 and 0.71, p = 0.01, 0.03 and 0.006, respectively) (Figure 5).

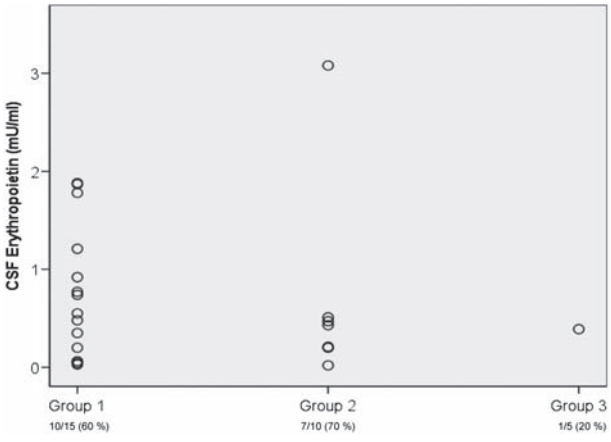

Figure 4. Cerebrospinal fluid erythropoietin (CSF-EPO) box plot detected in 23 newborns from 40 newborns: Group 1 low CSF-NSE newborns (n = 15 from 25), group 2 newborns with high CSF-NSE but without brain injury (n = 7 from 10) and group 3 newborns with high CSF-NSE and with brain injury (n = 1 from 5).

Discussion

Our findings indicate that oxidative stress as a result of an over-production of free radicals with failure of the compensatory endogenous antioxidative scavengers to counteract this over-production resulted in the loss of the oxidative homeostatic balance, which could be an important contributing factor to the hypoxic-ischemic brain injury of newborns.

This was clear where group 2 had higher mean BAPs than group 1, which rendered a lower level of CSF-TH and similar to levels in group 1. In spite of the higher NSE in group 2, which indicates some degree of neuronal hypoxia, both groups developed without detectable brain damage. Meanwhile, group 3 had higher CSF-NSE and TH, but less mean BAPs than group 2 did. Thus, the ability of the free radicals scavenger system of the newborns to maintain the levels of free radicals could be protective from hypoxic-ischemic injury. Moreover, the loss of this ability, as in group 3, resulted in an elevation of harmful free

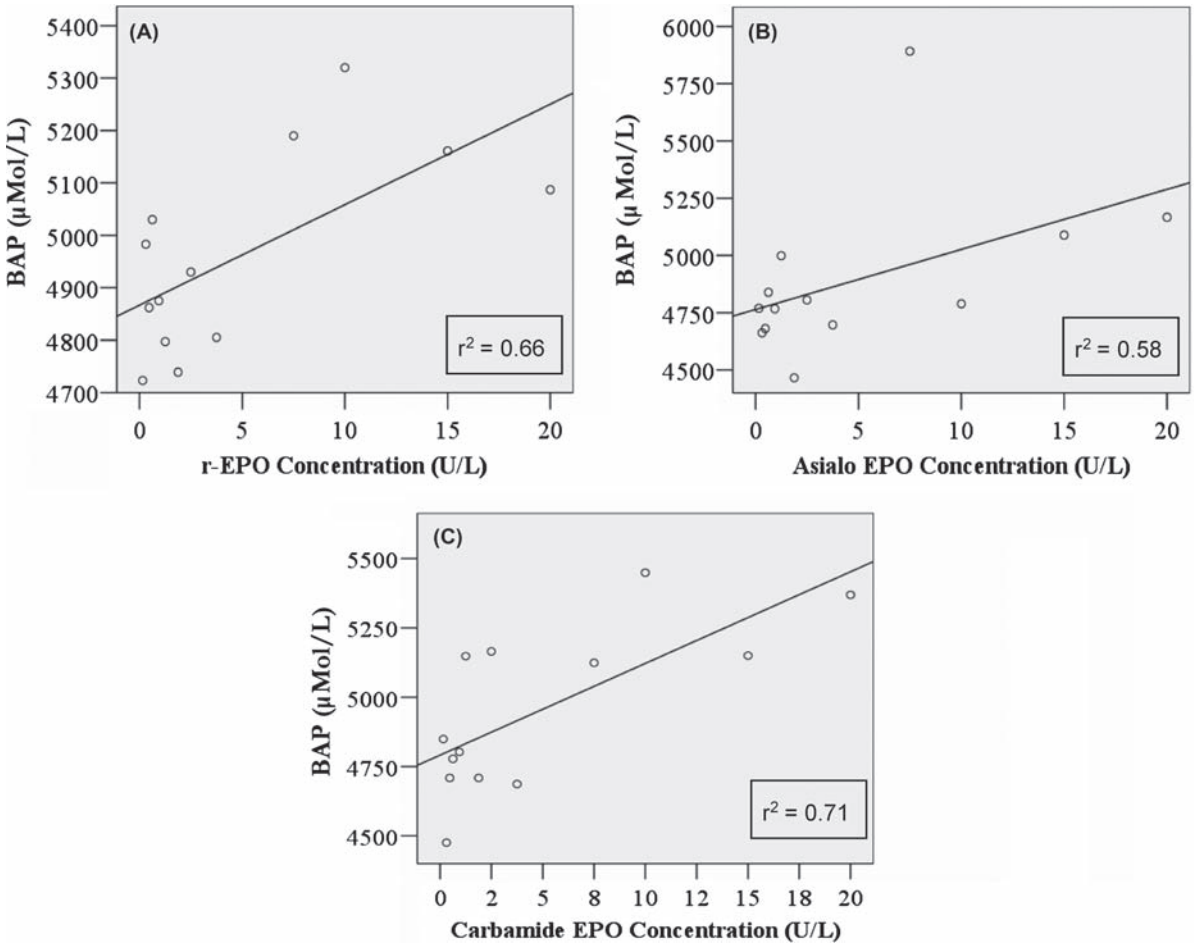

Figure 5. Thirteen serial dilutions of (A) rh-Erythropoietin (EPO), (B) Asialo EPO and (C) Carbamide EPO correlated with their biological antioxidant potentials (BAPs). The coefficient of relation was detected by using Pearson's two-tailed correlation coefficient for rh-EPO and Spearman's two-tailed correlation coefficient for Asialo EPO Carbamide EPO.

radicals [12] which provoked a higher mean OSI with more brain injury and higher NSE levels. For sure, this ability would depend on the degree of the stressful insult. Further, Celtik et al. [4] showed that NSE is initially high in samples taken from 4–48 h after birth in all newborns with significantly higher levels of NSE in the newborns who developed HIE and these levels decreased later in their 2nd measurements at 5–7 days after birth, but yet there were significantly higher levels of NSE in the samples drown from newborns who developed HIE.

Free radical mediated injury has been hypothesized to be the final common pathway to oligodendroglial cell death in newborns as a result of brain injury [8].

Human immature white matter appears to have a reduced antioxidant defense system. Houdou et al. [18] demonstrated a delay in catalase expression in white matter glial cells until ~31 weeks of gestation and thereafter only a slow development in its expression. Therefore, the capacity to detoxify hydrogen peroxide may be limited early in neural cells development. Excessive free radical production may occur through a wide variety of mechanisms, many of which are commonly implicated in neonatal brain injury. These mechanisms include ischemia-reperfusion [19] and inflammation and infection [20]. Friel et al. [21] reported that all healthy newborn infants are experiencing some degree of oxidative stress that resolves only with age. This could be due to dramatic changes occurring during the foetal-neonatal transition period in the pO_2 in lung and blood cells, with a more gradual change in liver and brain [22].

IL-8 was concluded to have a neuroprotective effect where it enhanced the survival of hippcoampal neurons in cultures [23]. The higher levels of IL-8 in the CSF of group 2 cases, which were protected from hypoxic-ischemic brain injury, along with the correlation between the CSF-IL-8 and BAPs, supports that both IL-8 and BAPs production could be triggered for protection from further neuronal injury and failure of this process could be an important contributing factor in brain injury.

The specific elevation of IL-8 among the 12 cytokines measured, along with the elevation of antioxidants, reveals a certain degree of specificity, which leads us to the probability that these changes could

result because of an activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response pathway. Oxidation by ROS causes the dissociation of Keap1 of the Nrf2-Keap1 complex, allowing the free Nrf2 to migrate from the cellular cytoplasm to the nucleus [24]. Nrf2 in the nucleus shares in two processes. The first, Nrf2 is part of the transcription factor complex that activates the transcription of a series of genes coding for phase 2 enzymes, which upregulates the Glutathione synthesis, the main thiol antioxidant [25]. The second process, Nrf2/antioxidant response pathway, induces the expression of IL-8 by a mechanism that mostly depends on mRNA stabilization [26]. IL-8, in turn, blocks neutrophil emigration to the site of inflammation [27] and suppresses their adhesion to activated endothelial cells [28]. Furthermore, IL-8 showed many protective effects, such as a tissue-protective effect during ischemia reperfusion injury [29], wound healing [30] and decreased scar forming [28], promoting tissue remodelling by endothelial proliferation and angiogenesis [31]. Thus, the Nrf2/antioxidant pathway could be of crucial importance in the protection of the degree of stress that all newborns are exposed to during the perinatal period [21]. However, further confirmative experiments focusing on the role of Nrf2/antioxidant pathway would need cellular studies that were not available in our recent settings. Before this report, there was no evidence of the relation between the CSF-NSE levels as a biochemical indicator of brain injury and the CSF-TH levels as a mediator of oxidative stress, or between the CSF-BAPs levels as an indicator of the endogenous antioxidative scavenger system and the CSF-IL-8 levels as a possible neuroprotective mediator.

Juul et al. [14] had concluded that endogenous EPO increased in the CSF of asphyxiated newborns.

Our results did not show such high levels of CSF-EPO as those seen in the previous study; this can be due to the differences in the timing of sample withdrawal between the two studies. Our study was mainly restricted to the 5th and 6th day of life, but in the previous study CSF samples were obtained within 2 days after the asphyxia insult occurred. Furthermore, the absence of CSF-EPO in the brain-injured group could be due to its utilization and/or the EPO's short half-life. The mechanism by which EPO was neuroprotective has been suggested in several studies, such as reducing nitric oxide overproduction [32] band by showing an anti-apoptotic effect [33].

Moreover, we add the antioxidant effect of three different biochemical types of EPO to the different mechanisms by which EPO could be a protective mediator.

Previously, we have shown that pre-treatment with low doses of rh-EPO (50–100 U/kg) ameliorates brain damage in a rat model of neonatal hypoxic ischemic brain injury [34]. This pre-clinical study along with our recent results in the present study would support our ongoing clinical trial in Japan for the use of low doses of rh-EPO in high risk newborns of hypoxic ischemic brain injury.

Conclusion

The increase of free radicals with the failure of the homeostatic balance to lower its levels in the brain of hypoxic newborns could play an important role in the development of perinatal hypoxic-ischemic brain injury and the ability to increase endogenous BAPs and IL-8 could be important neuroprotective factors. The EPO's neuroprotective effect along with its antioxidant effect could make it an important preventive and/or therapeutic drug in perinatal hypoxic-ischemic brain injury.

Limitations

1) The inclusion criteria limited the number of enrolled newborns.
2) The clinical follow-up of the newborns failed in several cases due to moving to distant areas and resulted in their exclusion from the study.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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