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Clinical significance of dynamic measurements of seric TNF-α, HMGBl, and NSE levels and aEEG monitoring in neonatal asphyxia

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Abstract. – OBJECTIVE: This study investigates the clinical value of monitoring blood levels of tumor necrosis factor-α (TNF-α), high mobility group protein Bl (HMGBl), and neuron-specific enolase (NSE), and examining an amplitude-integrated electroencephalogram (aEEG) for the diagnosis and short-term prognosis of brain damage caused by neonatal asphyxia.

PATIENTS AND METHODS: Sixty full-term neonates born in Yidu Central Hospital from January to December 2015 were enrolled in the study. The neonates were classified into one of 3 groups: 23 neonates in the mild asphyxia group, 7 in a severe asphyxia group and 30 in a control group admitted to the NICU but without asphyxia. The neonates presenting asphyxia received standard neonatal resuscitation before they were transferred to the NICU. The dynamic changes of the umbilical artery/peripheral blood TNF-α, HMGBl, and NSE levels and aEEG results were monitored and compared among the groups.

RESULTS: The umbilical artery and serum TNF-α, HMGBl, and NSE levels at day 1 were significantly higher in the two asphyxia groups than in the control group; and the values were higher in the severe asphyxia group (p <0.05). Furthermore, the correlation coefficients between TNF-α and HMGB1, TNF-α and NSE, and HMGB1 and NSE at all the monitoring time points were positive: 0.5516, 26.943 and 15.87, respectively (p <0.001). Additionally, the neonates with abnormal aEEG results at 6 hours postpartum had higher serum TNF-α, HMGBl and NSE levels than those with normal aEEG results (p <0.05). The patients with persistently abnormal or progressively deteriorating aEEG results usually had a poor evolution.

CONCLUSIONS: The dynamic monitoring of TNF-α, HMGBl, and NSE levels combined with aEEG can provide useful evidence for the early diagnosis, the determination of severity and the short-term prognosis of brain damage caused by neonatal asphyxia.

Key Words:
Neonatal asphyxia, Inflammatory cytokines, Amplitude-integrated electroencephalogram, Tumor necrosis factor-α, High mobility group protein Bl, Neuron-specific enolase.

Introduction

Perinatal asphyxia is an important cause of neonatal mortality in the world[1,2]; it is also one of the main causes of cerebral palsy, learning disabilities, sensory ataxia and other developmental and behavioral disorders in children[3,4]. Each year, about 4 million newborns die of perinatal asphyxia worldwide[5]. Early diagnosis of brain damage is of great importance to treat and improve the clinical outcomes of these patients. In recent years, a number of animal studies have suggested that inflammation reactions are involved in the hypoxia-reperfusion process of the brain tissues of patients with neonatal asphyxia. Asphyxia, premature birth and other perinatal risk factors could promote the activation of the coagulation system in newborns, causing damage to endothelial cells and the release of various inflammatory mediators, resulting in tissue hypoxia-ischemia, and reperfusion injuries, eventually manifesting as a systemic inflammatory response syndrome[6-8].

Amplitude-integrated electroencephalogram (aEEG) can be used to assess brain function by detecting electrical activity on the cerebral cortex. It has been shown to provide great value in the treatment of neonate asphyxia and the prognosis of brain injury[1,2]. A long period of aEEG monitoring representing the patient's brain activity levels can be accurately used to assess function9-11. In this study, we explore the combi-

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nation of TNF-α, HMGB1 (high mobility group protein B) and NSE (neuron-specific enolase) levels with aEEG monitoring in the diagnosis and prognosis of neonate asphyxia and the resulting brain damage, in an effort to provide evidence to secure an early diagnosis of brain damage caused by neonatal asphyxia.

Patients and Methods

Patients

The asphyxia groups included 30 full-term neonates with asphyxia who were born in Yidu Central Hospital from January to December 2015. These patients were transferred to the NICU after standard neonatal resuscitation. After assessment of their hypoxia, they were classified as having either severe (7 cases) or mild hypoxia (23 cases). The same amount of neonates born during the same period of time, admitted to the NICU but not presenting asphyxia, were selected to be in the control group. Neonates with intrauterine infection, premature birth, birth trauma, electrolyte imbalance, genetic and other congenital and metabolic diseases were excluded. The Ethics Committee of Yidu Central Hospital approved this study, and the families of the patients signed informed consent forms.

Diagnosis and Classification Criteria for Neonatal Asphyxia

The diagnosis and classification criteria were based on the "Recommended standards of neonatal asphyxia diagnosis and classification (2013)"2 and included any of the following findings: (1) Severe respiratory depression at birth. (2) Absence of independent breathing 1 to 5 minutes after birth. (3) 1.5-minute Apgar score lower than 7 points or high initial Apgar score but lower than 7 points Apgar score at 5-minutes. (4) Umbilical arterial blood gas pH < 7.15. Additionally, patients without hypoxic-ischemic organ damage were considered to have mild asphyxia; and those suffering from hypoxic-ischemic organ damage were placed in the severe asphyxia group. The criteria used to determine organ damage can be found in the references[3,4].

Collection of Umbilical Artery/Peripheral Arterial Blood and Determination of TNF-α, HMGB1, and NSE

After birth and before the establishment of independent breathing, a 20-30 cm fragment of the umbilical cord at the fetal end was isolated with two hemostats. 1-2 ml of the umbilical cord blood were drawn with a dry syringe. After centrifugation at 3000 rpm for 10 min, the serum was separated and stored at -80°C. In addition, 0.8-1 ml of arterial blood were collected from the patients at day 0 (within 30 minutes after birth), 1, 2 and 3 days after birth. Sera were prepared with the same centrifugation method as mentioned above. Enzyme-linked immunosorbent assay (ELISA) was used to detect the levels of TNF-α, HMGB1 and NSE from the samples. All kits were provided by R & D Co. (Marburg, Germany). Specialized technical personnel conducted sample collection, processing and detection in strict accordance with the kit instructions.

Evaluation of Brain Function

Within 6 hours after birth, the neonates' brain electrical activities were monitored by using an aEEG machine (Nicolet, Thermo Scientific, Waltham, MA, USA). Sustained normal voltage indicated normal brain activity, while non-continuous normal voltage, burst suppression, continuous low voltage, or flat waves were considered abnormal1. Patients with abnormal aEEG results were checked again 2-3 days after birth.

Short-term Follow-up

The neonates in the asphyxia group were re-examined 1 month after birth. The short-term prognosis of their neurological functions was evaluated using the neonatal behavioral neurological assessment (NBNA).

Statistical Analysis

Statistical analyses were conducted using the SPSS13.0 statistical software (IBM, Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation. The t-test was used for comparisons between two groups; while comparisons among more groups were done by ANOVA. Count data were compared using the chi-square test. $p < 0.05$ was taken to indicate a significant difference.

Results

General Information

30 patients were included in the asphyxia group, including 18 males and 12 females. Their gestational ages ranged from 37 to 42 weeks, with an average of 39.5 ± 1.6 weeks. Birth weights

Table I. Comparisons of serum TNF-α levels at different time points (X ± s, ng/L).

Group	No.	Umbilical cord blood	Age				F	*p*
			0 days	1 day	3 days	7 days		
Control	30	141.3±53.2	159.3±43.8	155.3±43.8	144.3±32.8	139.4±43.2	0.187	0.398
Mild asphyxia	23	989.3±159.3	1015.2±145.8	1298.3±132.3	1128.3±132.8	972.3±153.2	1.289	0.021
Severe asphyxia	7	1872.3±378.4	2035.3±382.4	1827.3±323.3	1678.3±354.8	1433.3±382.3	1.487	0.008
F		8.398	6.382	6.483	6.482	6.387		
p		0.000	0.000	0.000	0.000	0.000		

Table II. Comparisons of serum HMGB1 levels at different time points (X ± s, ng/L).

Group	No.	Umbilical cord blood	Age				F	*p*
			0 days	1 day	3 days	7 days		
Control	30	80.6±34.3	102.8±48.3	112.9±49.3	101.2±48.3	102.4±34.2	0.987	0.332
Mild asphyxia	23	394.5±122.8	439.2±153.2	450.4±119.3	446.3±163.2	439.2±151.2	2.127	0.001
Severe asphyxia	7	843.3±219.3	974.3±247.2	1178.2±219.4	1272.3±219.3	1069.3±213.2	3.143	<0.001
F		5.689	5.987	6.494	6.441	6.493		
p		0.000	0.000	0.000	0.000	0.000		

Table III. Comparisons of serum HMGB1 levels at different time points (X ± s, ng/L).

Group	No.	Umbilical cord blood	Age				F	*p*
			0 days	1 day	3 days	7 days		
Control	30	12.4±4.3	12.8±8.3	12.9±9.3	11.2±8.3	10.4±3.2	0.487	0.132
Mild asphyxia	23	40.4±12.8	49.2±13.2	50.4±19.3	46.3±13.2	39.2±11.2	3.289	0.002
Severe asphyxia	7	68.3±19.3	70.3±17.2	78.2±19.4	72.3±19.3	69.3±13.2	2.893	0.004
F		2.389	4.387	5.424	6.231	7.493		
p		0.000	0.000	0.000	0.000	0.000		

ranged from 2,726 to 4,239 g with an average of 3,629 ± 856 g. Twenty-three neonates were assigned to the mild asphyxia group, and the other 7 to the severe asphyxia group. The control group was also comprised of 30 patients, including 16 males and 14 females. Their gestational ages ranged from 37 to 41 weeks, with an average of 37.2 ± 1.3 weeks. Birth weights ranged from 2,700 to 4,200 g, with an average of 3,562 ± 591 g. No significant differences were found between the two groups of patients when gender, gestational age and birth weights ($p > 0.05$).

Relationship Between Blood Marker Levels and Asphyxia Severity

The mild and severe asphyxia groups had significantly higher serum TNF-α, HMGB1, and NSE levels than the control group. The levels were highest in the severe asphyxia subgroup ($p < 0.05$). The correlation coefficients between TNF-α and HMGB1, TNF-α and NSE, HMGB1 and NSE were 0.5516, 26.943 and 15.87, respectively ($p < 0.001$) (Tables I-III and Figure 1A-C).

Relationship Between Blood Marker Levels and Brain Function

A total of 12 patients showed abnormal aEEG results within 6 hours after birth. Their blood TNF-α, HMGB1, and NSE levels were significantly higher than those of the patients with normal aEEG results at 1-day-post partum ($p < 0.05$) (Table IV).

Prognosis

Only three patients in the control group had abnormal aEEG results within 6 hours after birth. And their aEEG results became normal when they were checked at 3-days-post partum. In contrast,

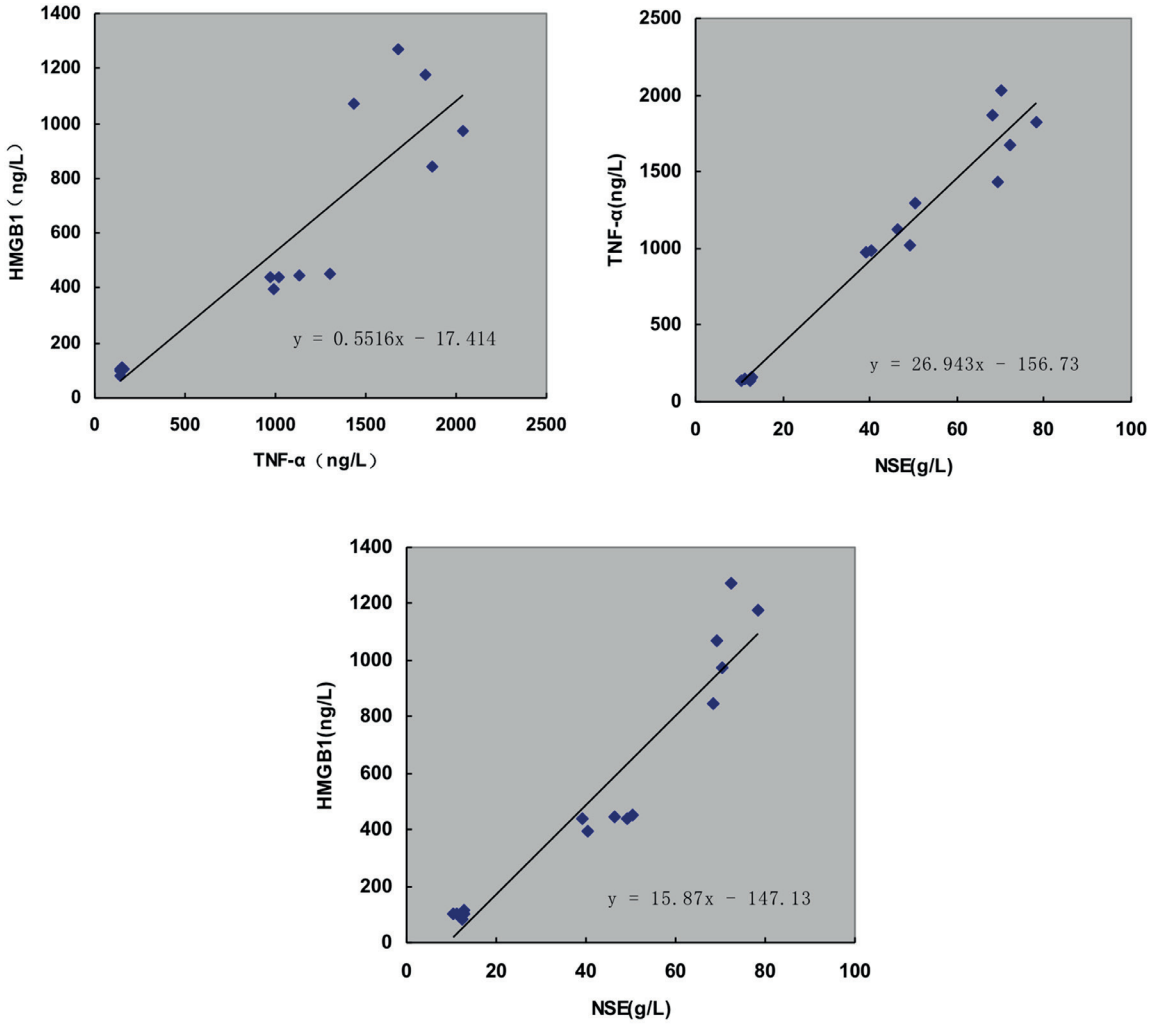

Figure 1. Coefficient analysis of the tested blood markers. ***A,*** The coefficient between TNF-α and HMGB1was 0.5516 (p<0.001); ***B,*** The coefficient between NSE and TNF-α was 26.943 (p<0.001); ***C,*** The coefficient between NSE and HMGB1 was 15.87 (p<0.001).

Table IV. Comparisons of TNF-α, HMGB1 and NSE levels at 1-day-old between patients with normal and abnormal aEEG results within 6 hours after birth.

Group	No.	TNF-α (ng/L)	HMGB1 (ng/L)	NSE (g/L)
Normal aEEG	48	213.3±67.3	313.2±98.3	24.3±9.3
Abnormal aEEG	12	1344.3±332.3	674.3±213.4	60.2±19.3
t		4.389	3.298	2.987
p		0.000	0.000	0.000

there were 9 other cases with abnormal aEEG results within 6 hours after birth. Among them, 3 were in the mild asphyxia group; they showed a normal aEEG at the 3-day post-partum check, and good prognosis at the 1 month-old follow-up. The other 6 cases were in the severe asphyxia group. These patients still had varying degrees of abnormality at the 3-day-old aEEG check. One of them showed progressively deteriorating results and passed away. The 2 patients with abnormal aEEG results at 7-days post-partum had lower than 35 NBNA points at the 1-month-old follow-up and presented varying degrees of neurobehavioral abnormalities.

Discussion

Hypoxic-ischemic encephalopathy (HIE) is a serious consequence of neonatal asphyxia with high morbidity and mortality rates. Early diagnosis of perinatal asphyxia has always been a hot topic in perinatal medicine. Previous animal experiments found that maternal infection and perinatal hypoxia affect brain development and movement ability in rats, by causing a synergistic deleterious effect by inflammatory responses and oxidative stress in the brain cortex[5]. In consequence, brain damage after asphyxia is thought to be related to excessive local inflammatory responses caused by ischemia and reperfusion of brain tissues. Among the different inflammatory cytokines playing a role, TNF-α was confirmed to be involved in the early onset of HIE caused by asphyxia, and functions in its development[6,7]. Also, the NF-κB signaling pathway stimulates the secretion of IL-6 and TNF-α by a variety of ways[7]. Animal experiments have shown that the TNF-α content in brain tissues increases at 3-6 hours after the establishment of a cerebral ischemia model, and 24 hours after asphyxia, the level of IL-6 increased, while that of TNF-α either increased or decreased[8]. Our research showed that the asphyxia group had significantly increased TNF-α in the umbilical arterial and the peripheral arterial blood compared with the control group. The arterial blood TNF-α level peaked at day 1 after birth and then declined rapidly. Additionally, the levels of inflammatory cytokines were positively correlated to the severity of asphyxia. These results suggest that the high umbilical blood TNF-α level is closely related to the presence of asphyxia. It is possibly a reliable indicator of neonatal asphyxia.

HMGB1 is an important inflammatory cytokine involved in late inflammatory responses in humans. Rat studies with an endotoxin-induced death model found that in the process of dying, HMGB1 was closely associated with the development of cerebral ischemia-reperfusion injury[9]. HMGB1 is an extracellular protein inducing cytokine secretion, and a DNA-binding nuclear protein activating inflammatory cytokines. HMGB1 is widely present in all eukaryotic lymphoid tissues, in the cytoplasm and nucleus of cells of brain, liver, lung, heart and other important organs. Its sequence is highly conserved. HMGB1 can be regarded as an inflammatory cytokine with pro-inflammatory activities. Under normal circumstances, endotoxin and inflammatory cytokines induce the release of HMGB1 to mediate inflammatory responses. Also, when cells undergo necrosis, or get damaged, HMGB1 gets released into the extracellular space. It can then stimulate monocyte-macrophage cells and promote cell adhesion; thus, a cascade of more cytokines and pro-inflammatory mediators ensues[10]. Studies[11,12] have shown that HMGB1 gets transferred to the plasma during the early stages of cerebral ischemia-reperfusion in humans, the concentration of the protein in cerebrospinal fluid increases, and anti-HMGB1 antibodies can specifically antagonize the formation of cerebral infarction caused by cerebral ischemia. We found that the seric HMGB1 level was closely related to the occurrence of neonatal asphyxia, and was positively correlated with the degree of asphyxia.

On the other hand, even though TNF-α peaked when the patients were 1-day-old and then gradually decreased, and a similar changing trend was found for HMGB1, we found that the peak duration of TNF-α was shorter than that of HMGB1, which might be related to HMGB1's biological effects in late inflammatory responses.

NSE is a key 78kD enzyme in the process of glycolysis, and its biochemical structure consists of two isomers of 7 subunits. NSE is mainly found in neurons of the central nervous system and cytoplasm of neuroendocrine cells. It is highly specific to nervous tissues, and can be used as a marker for mature neurons *in vivo* and *in vitro*[13]. Under normal circumstances, the NSE level is very low in body fluids. However, damage to the nervous system produces a large amount of NSE released into the cerebrospinal fluid and blood. Thus, the NSE content can be a reliable marker for neuronal axonal injury, targeted regeneration, and reinnervation[14] and also for hypoxia-ischemia, trauma or stress. The more severe the brain damage, the higher the levels of NSE released[15]. NSE has a high degree of sensitivity and specificity for the early diagnosis of central nervous system damage[16,17]. We observed serum NSE levels significantly higher in neonates with asphyxia than the in those of the control group. Its peak level was observed 1 day after birth. The higher the NSE concentration and the longer the duration of the abnormally high level, the more severe the degree of asphyxia and the more likely the patients would have a poor aEEG result indicating brain injury. Furthermore, NSE showed a positive correlation with serum TNF-α and HMGB1.

aEEG has commonly been used in the diagnosis of epilepsy and other brain diseases. It is a simple, straight forward technology that allows for continuous monitoring of patients. It is always more and more widely used in the diagnosis and evaluation of neonatal brain injury[18]. A meta-analysis[19] showed that children with normal aEEG results usually have a good prognosis, and abnormal aEEG results at 3-hours-post partum could predict poor prognosis with a sensitivity of 85% and a specificity of 77%, while aEEG results at 6-hours-post partum could do the prediction with a sensitivity of 91% and a specificity of 86%. The same meta-analysis in full-term HIE patients found the sensitivity and specificity of the aEEG for predicting neonatal death or disability, were 91% and 88%, respectively. Choi et al[20] found that the absence or abnormality of a sleep-wake cycle, and the recovery time reported in the aEEG results could reflect the severity of perinatal asphyxia. This work showed that the results of the aEEG tended to be more severe with cases of severe asphyxia. However, 6 hours after birth, most patients in our research showed a discontinuous normal voltage, and only the one patient who passed away displayed a burst suppression, continuous low voltage, and a flat wave. Finally, only the one patient that did not survive the study had a missing sleep-wake cycle at 12 hours after birth.

Conclusions

We suggest that a combination of dynamic measurements of TNF-α, HMGBl, and NSE levels and aEEG monitoring can be a reliable approach for the early diagnosis, evaluation, and prognosis of neonates with asphyxia and brain damage.

Conflict of interest

The authors declare no conflicts of interest.

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