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Comparison of new Biomarkers in the Diagnosis of Perinatal Asphyxia

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Abstract

Objectives

Precise and early diagnosis of neonatal asphyxia may improve outcomes. Recent studies aim to identify diagnostic biomarkers in neonates at risk for brain damage. The current study was designed to evaluate the diagnostic value of new biomarkers for neonatal asphyxia.

Materials & Methods

This prospective study was conducted with an available sampling of infants upper 35 weeks of gestational age, including neonates with asphyxia (case group) and healthy controls, 2014-2022, in Ghaem Hospital, Mashhad, Iran. Data collection was performed utilizing a researcher-made questionnaire, including maternal and neonatal characteristics, as well as clinical and laboratory evaluation. Serum umbilical cord levels of interleukin-6 (IL6), interleukin-1-beta (IL-1β), pro-oxidant-antioxidant balance (PAB), and heat shock protein-70 (HSP70), as well as nucleated red blood cells count (NRBC), were determined. Data were analyzed by t-test, Chi-square, receiver operating characteristic (ROC), and regression models.

Results

The differences in variables IL6, IL1β, PAB, NRBC/100WBC, and HSP70 were statistically significant between the two groups (in all cases, $P<0.0001$). In the diagnosis of asphyxia, the most sensitive marker (89%) was IL1β more than 2.39 pg/ml and HSP 70 upper than 0.23 ng/ml, while IL6 was higher than 9pg/ml, determined as the most specific marker (85%). Furthermore, a combination of HSP + PAB and IL6 + lL1b + PAB + NRBC/100WBC possesses the prediction power of 93.2% and 87.3%, respectively, for diagnosing asphyxia.

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Conclusion

According to data analysis, the combination of new biochemical markers (NRBC count, IL6, IL1β, PAB, and HSP 70) could be a reliable marker for diagnosing infants with asphyxia.

Keywords: Neonatal asphyxia; Interleukin-6; Interleukin-1β; Pro-oxidant-antioxidant balance; nucleated red blood cells count; Heat shock protein.

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Introduction

Perinatal asphyxia is defined as a failure in oxygen supply to the infant around birth time. The annual burden of the disease is four million cases.1 Approximately 20-50% of asphyxiated infants with brain damage die, while 25-60% of survivors suffer from permanent neurological development problems such as cerebral palsy, epilepsy, intellectual disabilities, and learning disabilities.2,3 The perinatal period is critical for brain development.4 The diagnosis of perinatal asphyxia is based on medical history, obstetrics, perinatal injury, and radiological and laboratory assessments. Early detection of infants at risk for ischemic hypoxia is necessary to facilitate therapeutic strategies.5 Debate exists over the definition and diagnosis of asphyxia.6 Despite progress in understanding the mechanisms and effects of asphyxia, early and rapid detection of HIE-induced brain damage is still one of the most challenging steps in newborns care.7 Definition and identification of diagnostic biomarkers for neonates at risk of brain damage are the main goals of the studies.4

Currently, diagnosis is established using the Apgar score, arterial blood gases, nucleated red blood cells (NRBC)6, monitoring of abnormal fetus signs during parturition, involving multiple systems within 72 hours after delivery, early imaging with evidence of acute cerebral damage, and encephalopathy. Brain-damage-specific biochemical markers are used for evaluating regional brain injury after perinatal asphyxia. In recent decades, diagnostic biomarkers have been used as powerful tools in assessing neonatal asphyxia. Several diagnostic biomarkers have been used to identify neonatal asphyxia, such as lactate, lactate dehydrogenase (LDH), creatine kinase, adenylate kinase, pro-oxidant-antioxidant balance (PAB), interleukins, S-100 ß, neuron-specific enolase, brain-specific creatine kinase, neuroproteins, calcium combined with proteins, vasoactive factors and inflammatory mediators5,6,8, heat shock proteins (HSPs),9-11 and umbilical cord blood NRBC count.5,6,9-12

No marker is solely capable of effectively predicting perinatal asphyxia, and only combinations of different measures could be helpful in the

diagnosis of perinatal asphyxia2-4, 13. During the neonatal HIE, an inflammatory reaction occurs. This disorder will be determined using multiple inflammatory genes expression that produces chemokines and cytokines. Inflammatory cells, mainly macrophages and microglia, accumulate at the site of brain injury, and stimulating the production of amino acids, free radicals of oxygen, nitrous oxide, and pro-inflammatory cytokines can lead to brain damage.14 Various studies have indicated the possible role of inflammatory cytokines-mediated hypoxic-ischemic brain injury. According to studies, levels of IL-1ß, IL-8, and IL-6 increased in infants with neonatal asphyxia.15 IL6 cytokine stimulates a wide variety of cellular and physiological responses, including immune response, inflammation, hematopoiesis, and oncogenesis, through regulating cell growth, gene activation, cell proliferation, survival, and differentiation of cells. However, so far, its role as a significant mediator in brain damage is unknown.16 In addition, some researchers recently reported the relationship between PAB and severe traumatic brain injury in the perinatal period.8 Increase in NRBCs in response to neonatal hypoxia has been reported. NRBC counting 6-8 hours after brain damage has reached its peak and then returns to normal within 36 to 72 hours.14 The results showed that the number of NRBC per 100 leukocytes is a simple biochemical marker for detecting these verities and primary consequences of perinatal asphyxia.12 Many studies investigated the chemical biomarkers in diagnosing neonatal asphyxia. However, less has been done to compare these markers in diagnosing neonatal asphyxia and determine whether the simultaneous use of these together can help diagnose neonatal asphyxia. This study aims to compare the new biomarkers and their combination in the diagnosis of asphyxia for more precise and early identification of infants with asphyxia.

Material & methods

Population

This prospective study has been done with an available sampling of infants upper 35 weeks of gestational, which included neonates with asphyxia (case group) and healthy controls, 2014-2022 in Ghaem Hospital, Mashhad, Iran. Ghaem Hospital is a general referral hospital that possesses a NICU (12beds), Care Level 2 (25 beds), and maternity (care level1), that has done 3000 delivery throughout the year. Parents filled out an informed consent before recruitment into the study. The study was approved by the Ethical Committee of Mashhad University of Medical Sciences (IR.MUMS.fm.REC.1395.54).

Infants who had at least two of the following symptoms as neonatal asphyxia were assigned:

-Signs of fetal distress (heart rate less than 100, the lack of variability in HR, late deceleration).

-Meconium- stained amniotic fluid and hypotonia, decreased heart rate, or respiratory depression.

-In the first minute, Apgar scores less than four, and Apgar scores less than seven in the fifth minute.

-The need to restore more than a minute with positive pressure ventilation (PPV) and oxygen in the delivery room.

-PH<7/2 or 12>BE during the first hour after birth.

Exclusion criteria included: intrauterine growth retardation, congenital or perinatal infections, hemolytic anemia, and congenital malformations.

The control group included babies with no problem with delivery and the first week of life had shown a regular examination.

Biochemical analysis and anthropometric measurements

Sampling from subjects was done at the birth time from the umbilical cord, and serum samples to measure blood levels of IL6, IL1β, PAB, and HSP 70 were examined by using the ELISA method. For assay of PAB was performed according to Alamdari et al. (1).[17] For this purpose, 400 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB, Fluka), prepared in DMSP, was added in 20 mL of acetate buffer [0.05 M buffer, pH 4.5]. Afterward, 70 µL of fresh chloramine T (100 mM) solution was added to the mixture and incubated for two h for preparing standard control; different proportions of 250 µM hydrogen peroxide (Darmstadt, Germany) were prepared by mixing with three mM uric acid (in 10 mMNaOH) was used. Finally, the mixture was transferred to a 96 96-well, added 100 µL of 2 N HCl to wells and measured in an ELISA plate reader at 450 nm with a reference wavelength of 620 nm. A standard curve was drawn by using the values gained from standard samples. The PAB values were represented in optional HK units, which express the hydrogen peroxide percent in the standard solution.[18] Sandwich ELISA (The enzyme-linked immunosorbent assay) in-house was used for determining serum HSP 70 antigen concentrations. This assay was performed, as described by Boskabadi et al. [6] CBC and peripheral blood smear were prepared. A blood count using an automatic counter (Sysmex) was done in the routine laboratory. The next step is checking the results of the device; for this purpose, the peripheral blood smear was prepared, the cells were separated based on the morphology, and the observed NRBC number per 100 white blood cells was reported.

A neonatologist performed neonatal clinical examination and diagnosis. Neurological functions of the neonates were assessed at birth and on the second and seven days of life. These included a systematic assessment of mental status (level of alertness), cranial nerve function, and the motor and sensory systems. In particular, the motor examination assessed spontaneous movement and muscle tone. Posture and resistance of muscles to the passive movement were used to assess active tone. Newborn neurological examinations were performed by a single Neonatologist, experienced in neurological evaluation without knowledge of the cytokine concentration.

On physical examination, neurological function on the first, third and seventh was evaluated by the examiner and determined based on the severity of HIE Sarnat staging.[19] Hyper-vigilance, irritability, hyper reflex, and absence of seizures for at least 24 hours after birth as mild HIE or HIE grade 1 was divided. Lethargic, hypotonia, decreased reflexes, meiotic pupils, convulsions as moderate HIE or HIE grade 2, apnea, slackness, severe convulsions, or coma as severe HIE or HIE grade3 were considered. Evaluation of patients was performed based on clinical examination and laboratory studies, and if they have scientific indications imaging techniques such as X-ray of the chest, abdominal sonography, and CT scan of the brain were used.

The data collection tool was an in-house questionnaire that included data on the mothers(delivery type, complications of pregnancy, and delivery complications), infant(first minute Apgar score, fifth minute Apgar score, duration of IPPV, duration of oxygen therapy, degree of HIE, seizures, gender) and laboratory characteristics of newborns(using cord blood samples to measure at birth levels of IL6, IL1β, PAB, the number of WBC, the percentage of NRBC, PH, BE, HCO3,

HSP 70, the number of NRBC, and PAB level).

Statistical analysis

The collected data were analyzed by SPSS software v.20. Comparisons were made using the Mann-Whitney and Chi-square tests. The Chi-square test was used with a nominal scale to analyze the relationship between variables. Moreover, this research drew the receiver-operating characteristic (ROC) curve to evaluate the sensitivity and specificity of indicators for determining asphyxiated babies. To compare the diagnostic parameters based on indicators NRBC, PAB, IL6, IL1β, degrees of HIE, and HSP70 regression model (-2 log-likelihood, Cox & Snell R Square, Nagelkerke R Square, Hosmer, and Lemeshow Tests) were performed. A P-value of 0.05 was considered statistically significant.

Results

In the study, 596 infants enrolled. According to the exclusion criteria mentioned previously, intrauterine growth retardation (30 infants), congenital or perinatal infections (16 infants), hemolytic anemia (2 infants), and congenital malformations (7 infants). Finally, 541 children completed the study. Neonates included 266 (49.2%) newborns with asphyxia (case group) and 275(50.8%) healthy infants (control group). In the study, 266 newborns suffered asphyxia divided into the following groups: 37 infants (13.9%) without HIE, 116 infants (43.6%) with HIE grade 1, 69 infants (25.9%) with HIE grade 2, and 44 infants (16.5%) HIE grade 3.

Variables such as IL 6, IL1β, PAB, the percentage of NRBC, HSP70, PH, HCO3 and base excess, IPPV duration, and oxygen therapy time between two groups were statistically significant (P<0.001,Table1). This means that infants with asphyxia, IL6, IL1β, PAB, the percentage of NRBC, and HSP70, have higher values than the control group (Table 1).

Based on this study's findings, in predicting the diagnosis of asphyxia PAB more than 9HK had 81% sensitivity and specificity of 56%, IL6 higher than nine pg/ml had 81% sensitivity, and 85% specificity, as well as sensitivity and specificity of the absolute number of NRBC more than 200 was 85% and 64% respectively. IL1β values of more than 2.39 pg/ml have 89% sensitivity and 54% specificity. In addition, HSP 70 upper than 0.23 ng/ml showed 89% sensitivity and 77% specificity in predicting the diagnosis of asphyxia (Figure 1).

The results of data analysis based on regression models showed that variables such as IL6 (P= 0.000), IL1β (P= 0.000), PAB (P= 0.000), and NRBC percent (P= 0.000), including diagnostic markers are neonatal asphyxia.

In addition, data analysis showed that the combination of indicators HSP + PAB and IL6 + lL1β + PAB + NRBC percent, respectively, have a power to prediction upper than 93.2% and 87.3% compared to the combination of other criteria for diagnosis of asphyxiated newborns (Table 2).

The current study's results indicated a correlation between the severities of asphyxia by means of diagnostic biomarkers and indicated that PAB, PH, and first-minute Apgar scores have an inverse relationship with the severity of asphyxia. As within the creasing severity of asphyxia, the PAB was increased and decreased in the first-minute Apgar score (Table 3).

Table 1. comparison of the maternal and neonatal clinical parameter means between two groups

Groups Variables*	Case group (asphyxiated neonates) 266 (49.2%) Median(IQR)	Control groups (healthy neonates) 275 (50.8%) Median(IQR)	Significance level (Mann-Whitney)
IL6 (pg/ml)	64.20 (177.42)	0.000 (3.10)	0.000
IL1β (pg/ml)	7.70 (8.05)	2.30 (2.62)	0.000
PAB (33)	21.30 (21.10)	7.70 (11.62)	0.000
WBC	21.00 (28.70)	14.50 (12.46)	0.000
NRBC /100WBC	14.00 (29.00)	2.00 (7.00)	0.000
HSP70 (ng/ml)	0.19(0.08)	0.40(0.16)	0.000

* Values are based on Interquartile Range± Median

Table 2. comparison of combination of diagnostic indicators for neonatal asphyxia

Diagnostic methods	-2 log likehod	Cox & Snell R Square	Nagelkerke R Square	Hosmer and Lemeshow Test	Predicted Percentage Correct
HSP	97.584	0.475	0.640	0.000	87/00
IL6	88.723	0.454	0.606	0.351	83.1
lL1b	245.919	0.287	0.383	0.000	77.9
PAB	578.882	0.169	0.225	0.000	69.5
NRBC percent	260.066	0.199	0.267	0.000	63.7
HSP + PAB	49.903	0.623	0.841	0.001	93.2
IL6 + NRBC percent	121.710	0.467	0.627	0.000	85.5
IL6 + lL1b	176.466	0.464	0.619	0.000	84.1
lL1b + NRBC percent	144.326	0.380	0.510	0.000	83.4
IL6 + PAB	148.594	0.458	0.611	0.017	82.3
lL1b + PAB	174.415	0.358	0.477	0.435	78.9

Diagnostic methods	-2 log likehod	Cox & Snell R Square	Nagelkerke R Square	Hosmer and Lemeshow Test	Predicted Percentage Correct
IL6 + lL1b + PAB + NRBC percent	94.700		0.562	0.007	87.3
lL1b + PAB + NRBC percent	107.108	0.365	0.493	0.008	85.00
IL6 + PAB + NRBC percent	97.609	0.425	0.573	0.073	83.6
IL6 + lL1b + PAB	139.870	0.460	0.614	0.689	82.4
PAB + NRBC percent	165.821	0.345	0.461	0.636	77.00

Table 3. comparison of clinical and lab characteristic means of asphyxiated infants according to HIE severity.

HIE grade variables	normal	No HIE	HIE grade 1	HIE grade 2	HIE grade 3	(chi-square)
PAB	10.61±13.31	12.30±19.47	16.40±24.06	22.55±31.62	40.84±42.35	0.000
First **hour pH**	0.06±7.33	0.09±7.25	0.06±7.18	0.12±7.15	0.17±7.09	0.000
first-minute Apgar score	0.62±8.65	1.50±5.72	1.44±4.54	1.54±4.39	1.30±4.31	0.000

Discussion

This study revealed that the new biochemical markers (HSP70, IL6, IL1ß, and PAB) and NRBC count in the cord blood could be reliable markers to identify neonates with asphyxia. In addition, the composition of the HSP + PAB indicators and IL6 + lL1β + PAB + NRBC percent, respectively, of the power of prediction 93.2% and 87.3% compared with other combined indicators for the diagnosis of asphyxiated neonates.

In this study, the IL6 and IL1β levels included diagnostic markers of neonatal asphyxia. While IL6 levels of more than nine pg/ml have 81% sensitivity and specificity of 85%, and IL1β more than 39/2pg/ml indicated a sensitivity of 89% and specificity of 54% in diagnosing asphyxia. Another representative pro-inflammatory cytokine, IL-6, is also induced by hypoxia-ischemia and follows a similar time course of expression as IL-1β in neonatal rats with HIE.20 According to the results of experimental studies, cytokines that regulate the inflammatory response are essential in the incidence of hypoxic-ischemic brain injury. Interleukins are produced and secreted by lymphocytes, monocytes, and macrophages in response to stimuli.16 Cytokines are predominantly released from immune cells, including monocytes, macrophages, and lymphocytes. Pro- and anti-inflammatory cytokines facilitate and inhibit inflammation, respectively21. Increased serum levels of IL-6 in the first 24 hours after HIE raises a possible link between IL-6 in the pathogenesis of brain damage. This means that IL-6 may be secreted as a protective response after HIE and

plays a role in the possible recovery mechanisms in the HIE sub-acute phase.9

Boskabadi et al. (2019) in diagnosing neonatal asphyxia reported that sensitivity and specificity IL6 > 41 Pg/dl, rates of 84.88% and 85.43%, respectively, and for IL1β > 4.7 Pg/dl were 78% and 83%. For neonatal asphyxia, a combination of IL6 and IL-1β had the highest sensitivity (92.9%).22

An association was found between asphyxia and IL-6 and IL-1β levels in neonates. IL-6 and IL-1β levels were increased in neonates with moderate and severe asphyxia, with the extent of the increase significantly higher in the latter. 23

In another study (2015) on the sensitivity and specificity of IL6 in diagnosing perinatal asphyxia, rates of 80.6% and 81.6%, respectively, and for IL1β, 71% and 89% were determined.24

IL-1β level was associated with stage of asphyxia. Level of IL -1β in severe asphyxia significantly higher than moderate asphyxia.25

According to this study, the PAB values in asphyxiated newborns were about three times healthy infants. Besides, PAB of more than 9HK has 81% sensitivity and 56% specificity in diagnosing asphyxia. In asphyxia and stress, PAB changes in the body could be an asphyxia indicator. 26 In Boskabadi's study (2017), PAB in combination with HIE grade had a better predictive value for the prognosis of asphyxiated babies and predicting future neurologic problems in asphyxiated term infants27.

In the present study, the percentage of NRBC in infants with asphyxia is about seven times more compared to healthy neonates. In addition, the absolute number of NRBCs of more than 200 has a sensitivity of 85% and specificity of 64% in the diagnosis of asphyxia. In response to hypoxia, the fetal liver increases erythropoietin production and even causes some degree of increased erythrocytosis. In response to erythropoietin, NRBC is primarily produced in the bone marrow and stored as a precursor of reticulocytes and mature erythrocytes. Thus, the number of NRBCs reflects the high production of erythropoietin. Since NRBC could change the size and expansibility of its shape, it is a release from the bone marrow into the peripheral blood. Many acute and chronic stimuli increase NRBC production due to increased activity of EPO or sudden release from the bone marrow reserves.28-30 In another study of NRBC upper 70 cells per cubic millimeter for predicting perinatal asphyxia, the diagnosis has a sensitivity of 83.4% and 73.5%, respectively.12 In another study, an NRBC count of more than 11 per 100 WBC had a sensitivity of 85% and specificity of 90% in predicting complications of asphyxia.31

According to the obtained findings, the amount of antigen HSP70 in asphyxiated neonates was twice compared to healthy ones. In addition, HSP70 higher than 23.0 ng/ml has a sensitivity of 89% and specificity of 77% in predicting the diagnosis of asphyxia. A few studies have been done on HSP in human infants. The role of this protein is to prevent changes in the composition of cells under stress. This protein exists in all cells of living organisms and is made by various types of stress, including fever, alcohol, inflammation, oxidative stress, heavy metals, and circumstances causing damage and necrosis.32 In another research, the HSP70 value was higher in infants with asphyxia. In addition, antigen HSP70 was reported as a useful marker for the early detection of prenatal hypoxia. Besides, HSP70 higher than 31.0 ng/ml has a sensitivity of 58% and specificity of 77% in predicting the diagnosis of asphyxia.6 Jiang et al. (2004) examined HSP70, HSP27, c-Fos, c-Jun, and

calpain activation in 42 rats seven days aged with ischemic-hypoxic encephalopathy. The results showed that calpain in the cerebral cortex during 24 hours and 12 hours after hippocampus injury regulates the expression of c-Fos and c-Jun, whereas their expression, reaches their peak at two and four hours after injury. HSP70 and HSP27 expression in the cortex, at 12 and 24 hours after injury reaches its peak, while the expression of HSP70 in1 hour post-traumatic reached its peak and then reduced to 24 hours after the ischemic-hypoxic encephalopathy. HSP 27 expression in the hippocampus increases after Hypoxic ischemic encephalopathy. So hypoxic-ischemic encephalopathy seems that causes apoptosis through activation of calpain and increased expression of HSP 70 to protect against brain injury after the HSP 27 expression in the brain of rats.33

Comparing the sensitivity and specificity of biomarkers in the diagnosis of asphyxia suggest that HSP and IL1β most sensitive, while the highest specificity in the diagnosis of asphyxia was related to the IL6. In other studies, IL1β had been the most sensitive marker (96%) in predicting the prognosis of asphyxia, and IL6 has shown the most specific marker (93%).34 In comparing the predictive power of biomarkers for the diagnosis of asphyxia, the highest percentage was related to HSP (87%). Evaluation of biomarker values shows that the combined amounts of HSP + PAB has the most power to determine asphyxia (93%). After comparing the biomarker with the severity of asphyxia, it indicated that PAB values correlate with the severity of asphyxia, as asphyxia intensity increases with increasing PAB. On the flip side, PAB values are significantly higher in asphyxiated infants. The highest values of PAB were observed in neonates with HIE grade 3.18 Investigating new diagnostic biomarkers in neonatal asphyxia using a simple and minimally invasive method was an essential step in treatment in the study strengths.

In Conclusion

Based on this study's results, in asphyxiated infants group, IL6, IL1β, PAB, the percentage of NRBC, HSP70, complications of pregnancy and delivery complications have higher values, and PH first hour of birth, BE the first hour of birth, HCO3, Apgar score and the score fifth minute Apgar, were lower values. According to the findings of this study, PAB more than 9HK had a sensitivity of 81% and specificity of 56%, IL6 more than nine pg/ml showed a sensitivity of 81% and specificity of 85%, the absolute number of NRBC more than 200, a sensitivity of 85% and specificity of 64%, IL1β higher than 2.39 pg/ml, 89% sensitivity, and 54% specificity in predicting the diagnosis of asphyxia. In addition, HSP 70 higher than 0.23 represented a sensitivity of 89% and specificity of 77% in predicting the diagnosis of asphyxia. These variables included IL6, IL1b, PAB, and NRBC percent may be new biomarkers in diagnosing asphyxia in newborns. Besides, the composition of the HSP + PAB and IL 6 + lL1b + PAB + NRBC percent, respectively, the power to predict the top 93.2% and 87.3% compared with other combined indicators for diagnosing neonatal asphyxia.

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All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work: IR.MUMS.fm.REC.1395.54

Author's Contribution

Hassan Boskabadi: conceptualized and designed the study, drafted the initial manuscript, and

approved the final manuscript as submitted.

Fatemeh Bagheri: carried out the initial analyses, reviewed and revised the manuscript, and approved the final manuscript as submitted.

Maryam Zakerihamidi: designed the data collection instruments, and coordinated and supervised data collection at two of the four sites, critically reviewed the manuscript, and approved the final manuscript as submitted.

Majid Ghayour Mobarhan: designed the study and carried out the initial analyses, reviewed and revised the manuscript, and approved the final manuscript as submitted.

Ali Moradi: designed the study, performed data analyses, drafted the initial manuscript, reviewed, revised, and approved the manuscript.

Mehran Beiraghi Toosi: drafted the initial manuscript, reviewed, revised, and approved the manuscript.

Conflict of interest

None declare

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