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ORIGINAL ARTICLE

Prooxidant-Antioxidant Balance in Perinatal Asphyxia

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Abstract
Objective To determine the prooxidant-antioxidant balance (PAB) in neonatal asphyxia and compare it with values for PAB in healthy neonates.
Methods In a prospective observational study, serum PAB of umbilical cord blood of 30 neonates with asphyxia [pH <7.2, low Apgar score, signs of respiratory distress syndrome (RDS)] as the case group and 35 healthy neonates (without an abnormal clinical event at birth and after the first week) as the control group were compared.
Results Among the 35 neonates in the control group, the average level of serum PAB was 20.00 HK units, which was significantly lower than for the 30 neonates within the case group (40.46 HK units; $p=0.019$). The blood pH in the case group was significantly lower than for control group ($P<0.001$). In controls, HCO_3^- and pCO_2 were 18.6 mmol/L and 38.5 mmHg respectively, whilst in the case group these values were 15.5 mmol/L and 45.7 mmHg respectively ($p<0.05$).
Conclusions Determination of PAB may be useful in the early diagnosis of perinatal asphyxia and is consistent with HCO_3^-, pCO_2 and Apgar score.

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Introduction

Perinatal asphyxia is a major cause of mortality and morbidity in neonates, and results from the lack of oxygen supply and perfusion of various organs [1–3]. With an incidence of about 5–10/1000 live births in developing countries [2, 3] and 1/1000 in developed countries [4, 5], perinatal asphyxia has a serious health impact [6]. The signs of asphyxial injury are often nonspecific and overlap with other illnesses [7]. Determining the severity of asphyxia before the establishment of signs and symptoms may significantly improve the outcome and survival in neonates with asphyxia. Although there is no gold standard for determining the presence of hypoxic-ischemic encephalopathy, there are various clinical signs suggesting asphyxia, including sentinel hypoxic event during labor; meconium-stained amniotic fluid, Apgar scores of 0 to 3 beyond 5 min after birth, low pH of the cord blood, onset of multisystem involvement within 72 h of birth, neonatal seizures, abnormal signs in the intra-partum electronic fetal monitoring or early imaging showing evidence of acute non-focal cerebral injury and encephalopathy [8–11]. These findings are non-specific and may occur in the absence of global hypoxic-ischemic brain injury or long-term neurologic sequelae. In order to identify perinatal brain injury due to birth asphyxia, the American College of Obstetricians and Gynecologists (ACOG) has suggested four criteria be applied to define an intra-partum hypoxic-ischemic state, that is severe enough to cause a neonatal encephalopathy. These include: profound metabolic acidosis [pH<7.00 and base deficit (BD) ≥12 mmol/L] on an umbilical cord arterial blood sample; early

onset of severe or moderate neonatal encephalopathy in infants born at 34 or more weeks of gestation; cerebral palsy of the spastic quadriplegic or dyskinetic type; exclusion of other identifiable etiologies such as trauma, coagulation disorders, infectious conditions, or genetic disorders [11].

These guidelines are arbitrary and do not allow the identification of the underlying causes. Therefore, further research is required to identify other early biomarkers of neonatal asphyxia.

Excess production of oxygen free radicals and lipid peroxidation are known to play important roles in perinatal asphyxia [12]. Oxidative stress can be defined as an imbalance between the amount of reactive oxygen species (ROS) and the intracellular and extracellular antioxidant protection systems [13]. ROS may be generated by different mechanisms such as ischemia–reperfusion with the potential of reacting with almost every type of molecules including lipids, proteins, polysaccharides and DNA [14, 15]. The contribution of oxidative stress to the pathogenesis and progression of perinatal asphyxia is not well understood. A major limitation in this area is the lack of an accurate and reliable method to measure prooxidant-antioxidant balance in these patients simultaneously. The authors have recently introduced a simple, rapid and inexpensive method that provides a redox index by using the 3, 3′, 5, 5′-tetramethylbenzidine (TMB)-TMB cation and measures the balance of oxidants and antioxidants simultaneously in one assay (the PAB assay) [16].

The main objective in the present study was to compare one measure of redox status for neonates suspected of asphyxia with normal healthy neonates and to compare this measure (the prooxidant-antioxidant balance) with other diagnostic methods available in this area. The authors also aimed to evaluate the efficacy of the modified PAB assay in the early diagnosis of neonatal asphyxia.

Material and Methods

This prospective, observational study was conducted between December 2010 and April 2011, in Ghaem Hospital, Mashhad, Iran. Based on clinical and laboratory signs suggesting of birth asphyxia, neonates with at least two of the following criteria were recruited to the study as the case group:

- Those with fetal distress [late deceleration, lack of heart rate variability, Fetal heart rate (FHR)<100].
- Those associated with thick meconial amniotic fluid plus hypotonia, bradycardia or respiratory distress.
- Neonates with an Apgar score <4 within the first min or an Apgar score <7 within the first 5 min.
- Neonates requiring cardiopulmonary resuscitation more than 1 min using oxygen and IPPV (Intermittent Positive Pressure Ventilation).
- Blood pH<7.2 and base deficit (BD)<−12.

There were a total of 34 neonates originally recruited to the case group, however for four of these data were incomplete, and were omitted from the final data analysis.

The control group included 35 neonates with normal delivery and stable condition for at least one week after birth.

Exclusion criteria for the control group included:

- Congenital malformations, metabolic disturbances, congenital or perinatal infections or maternal chorioamnionitis.
- Presence of maternal complications during pregnancy or delivery.

Blood samples were collected from cases and controls using umbilical blood sampling immediately after delivery. The PAB assay, pH, BD, pCO_2, PO_2, O_2 saturation measurement were done for all subjects. A comprehensive clinical examination was used to evaluate all subjects at birth and at 3 and 7 d post-natally. Neurologic examination was performed by the same neonatologist. According to the criteria of Sarnat, hypoxic ischemic encephalopathy (HIE) was classified as mild (Grade 1), if the neonate was hyperalert, hyperexcitable, with normal muscle tone and no seizures; as moderate (Grade 2), if the infant was hypotonic with decreased movements and often seizures; and as severe (Grade 3), if the infant was stuporous, flaccid without primitive reflexes and usually with seizures.

Informed parental consent was obtained for every neonate before recruitment into the study antenatally. The study protocol was approved by the Ethics Committee of Mashhad University of Medical Sciences.

A relatively simple, rapid method for evaluating the prooxidant burden and the antioxidant capacity simultaneously was used, based on a previously described method [16], in which the chromogen TMB (3, 3′, 5, 5′-tetramethylbenzidine, Fluka) is oxidized to a colored cation by peroxides. Many methods have been developed that can measure the prooxidant and antioxidant capacities separately. The only way to estimate prooxidant–antioxidant balance, other than PAB assay, is to perform two separate assays which is therefore more laborious, expensive and less precise. Although similar to other methods that evaluate the oxidant-antioxidant status, PAB assay does not assess all the oxidants and the antioxidants of the body, it may provide a reliable redox index which has been qualified by various previous researches.

The standard solutions were prepared by mixing varying proportions (0–100 %) of 250 μM hydrogen peroxide with 3 mM uric acid (in 10 mM NaOH). For the preparation of the TMB cation, 60 mg TMB powder was dissolved in 10 mL DMSO; then 400 μL of TMB/DMSO was added in 20 mL of acetate buffer (0.05 M buffer, pH 4.5), and then 70 μL of fresh chloramine T (100 mM) solution in distilled water was added

into this 20 mL, mixed well, incubated for 2 h at room temperature in a dark place; 25 units of peroxidase enzyme solution was added into 20 mL TMB cation, dispensed in 1 mL and stored at −20 °C. In order to prepare the TMB solution, 200 μL of TMB/DMSO was added into 10 mL of acetate buffer (0.05 M buffer, pH 5.8); the working solution was prepared by mixing 1 mL TMB cation with 10 mL of TMB solution, incubated for 2 min at room temperature in a dark place and immediately used. Ten microliters of each sample, standard or blank (distilled water) were mixed with 200 μL of working solution, in each well of a 96 well plate, which was then incubated in a dark place at 37 °C for 12 min; at the end of the incubation time, 100 μL of 2 N HCl was added to each well; and measured in an ELISA reader at 450 nm with a reference wavelength of 620 nm.

A standard curve was constructed from the values derived using standard samples. The values of the PAB were expressed in arbitrary HK units, which represent the percentage of hydrogen peroxide in the standard solution. The values of the unknown samples were then calculated based on the values obtained from the above standard curve.

All statistical analyses were performed with Statistical Package for the Social Sciences 15 (SPSS Science, Apache Software Foundation, and Chicago, IL, USA). Values were expressed as mean ± SD. Student *t* test, Kruskal-Wallis test and Mann–Whitney test were used as appropriate. Parametric and non-parametric correlations were assessed using Pearson correlation coefficients and Spearman correlation coefficients, respectively. A $P<0.05$ was considered significant. ROC curve was plotted to calculate sensitivity and specificity of the test and compare it to other parameters.

Results

The blood gas measurement and PAB assay were done for all cases and controls. The mean characteristic values of the neonates in both the groups are shown in Table 1. The mean values for PAB, pH, pCO_2, BD and HCO_3^- was significantly different between case and controls (Table 2). The results of PAB assay were consistent with the results of pH, pCO_2, BD and HCO_3^- comparing the cases and healthy controls.

The covariate correlation was statistically significant between pH and PAB in controls ($P=0.05$) and between BD and PAB and HCO_3^- and PAB in total ($P=0.013$, 0.03 respectively). The PAB values were not significantly different between males and females in two groups.

For the case group, 4 (13.3 %) patients were included without hypoxic ischemic encephalopathy, 13 (43.3 %) in grade I, 10 (33.3 %) in grade II and 3 (10 %) patients in grade III hypoxic ischemic encephalopathy group. Regarding the association between PAB values and HIE, the median/interquartile range of PAB values for 39 neonates without encephalopathy was 17.83 (11.32, 28.82); whereas it was 22.04 (11.45, 40), 17.93 (12.25, 30.3) and 46.78 for the hypoxic ischemic encephalopathy grade I, II and III respectively.

A ROC curve was plotted for PAB, pH, pCO_2, HCO_3^- and BD and area under curve were calculated (Fig. 1). Sensitivity and specificity for PAB were calculated as 47.1 and 85.7 respectively with the criterion value of 28.82.

Discussion

Although the presence of oxidative stress has been documented in perinatal asphyxia, the determination of prooxidant–antioxidant balance is not yet a routine clinical laboratory test in these patients mainly because of the lack of a simple and accurate method that can evaluate oxidative stress and antioxidant potential simultaneously. In the present study, the authors have determined PAB status in neonates with asphyxia and healthy controls using a simple and rapid procedure. They have found that PAB values were significantly higher in neonates with perinatal asphyxia.

In the index study, the prevalence of asphyxia was higher among boys than in girls ($P=0.04$) but the authors could not state a conclusion as they did not collect a representative

Table 1 Clinical characteristics of the studied population

Group	Case ($n=30$)	Control ($n=35$)	*P*-value
Birth weight (g)[a]	2910±467	2918±461	0.943
Gestational age (wk) [b]	37.00 (35, 38)	37.00 (37, 39)	0.153
First min Apgar score [b]	4.0 (3, 5)	9.0 (8, 9)	<0.001*
Fifth min Apgar score [b]	8.0 (6, 8)	10.0 (10, 10)	<0.001*
Mode of delivery (ND/CS)[c]	12 (40) / 22 (73)	20 (57) / 15 (43)	0.069
Gender (male/female) [c]	21 (70) / 13 (43)	13 (37) / 22 (63)	0.041
Length (cm) [a]	48.70±3.35	49.11±2.22	$P=0.565$
Head circumstance (cm) [a]	34.01±1.36	34.16±1.32	$p=0.668$
Maternal age (y) [a]	27.55±6.14	28.37±5.85	$p=0.598$

ND Normal delivery; *CS* Cesarean section

Values expressed as:

[a] Mean ± SD

[b] Median/Interquartile range

[c] Number (%)

*Significant

Table 2 Laboratory characteristics of the studied population

Group	Case	Control	*P* value
pH[a]	7.14±0.13	7.32±0.6	<0.001*
HCO_3^- (mmol/L)[a]	15.52±8.07	18.59±4.22	<0.001*
BD (mmol/L)[a]	−13.14±5.7	−4.70±4.27	<0.001*
pCO_2 (mmHg)[a]	45.67±16.22	38.52±10.64	0.034*
PAB (HK)[b]	27.70 (15.36, 46.9)	15.52 (10.32, 27.5)	0.019*

BD Base deficit; *PAB* Prooxidant-antioxidant balance (HK Unit)

Values expressed as:

[a] Mean ± SD

[b] Median/Interquartile range

*Significant

sample of all births whether they entered the study or not. Moreover after adjusting this variable, the same results were observed.

Although maternal age is not considered as a major risk factor for developing asphyxia in neonates, maternal age of <16 or >45 is thought to be associated with neonatal asphyxia [17]. In this study, maternal age was not significantly different between the case and control groups. In addition, birth weight, gestational age and method of delivery were not shown to be associated with perinatal asphyxia and this is in line with previous findings. The relationship between mode of delivery and asphyxia is controversial. The differences found between various deliveries modes are, probably, due to the expression of causes related to the obstetric choice. Normal labor may cause a mild degree of acidosis [18]. Another reason is that the presence of risk factors such as macrosomia in normal delivery would increase the risk of asphyxia. On the other hand, cesarean is indicated as a hazardous condition that also could be associated with asphyxia.

The mean Apgar score in the first and 5th min, pH and BD were significantly lower in the case group. Apgar score is a

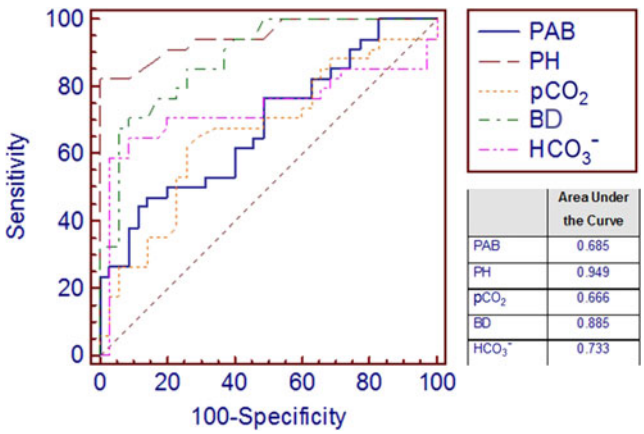

Fig. 1 ROC curve with area under the curve for PAB and the blood gas parameters

quantitative marker for evaluating the condition of a neonate at birth. While Apgar score <3 is used for defining asphyxia, other causes such as preterm labor can decrease Apgar score. Hence, it is not specific for diagnosis of neonatal asphyxia and the criteria of sole Apgar score is replaced by multi-index diagnostic criteria. In a recent collaborative study [19], the range of the umbilical artery blood pH and BD was clinically considered <7.00 to <7.20 and <−8 to <−18, respectively for neonatal asphyxia and the authors suggested that in the presence of the other indexes for diagnosing neonatal asphyxia, the blood gas index should be used flexibly in the above ranges. In another study by Gao et al., umbilical blood pH value concomitant with Apgar score has been shown useful in the assessment of severity and prognosis of neonatal asphyxia [20].

Perinatal asphyxia is a major cause of death and acquired brain injury in infants [21] and a majority of infants with severe encephalopathy after perinatal asphyxia become handicapped [4]. Oxidative stress is thought to play an important role in the pathogenesis of neonatal asphyxia and infants have limited protection mechanisms against oxidative stress [22]. Kumar et al. concluded that despite the increased activities of antioxidant enzymes in perinatal asphyxia, the increased levels of these enzyme activities are not able to scavenge the free radicals and these neonates experience higher degrees of oxidative stress, as evidenced by increased levels of malondialdehyde in plasma and cerebrospinal fluid indicating the significant role of oxygen free radicals in the pathophysiology of perinatal asphyxia [12]. Furthermore Aydemir et al. showed that the degree of oxidative stress is related to severity of neurological involvement in the first days of life [23]. The PAB assay has been used for assessing the oxidative status of subjects with a number of conditions associated with oxidative stress; including diabetes mellitus, coronary artery disease, acute coronary syndrome, exfoliative glaucoma, and stroke [24–28]. Tara et al. suggested that selenium supplementation may reduce oxidative stress (PAB) associated with pregnancy [29]. In a previous study, Parizadeh et al. concluded that serum PAB values are elevated in patients with stroke, indicating a heightened state of oxidative stress [30]. Alamdari et al. reported a significant increase of the PAB value in patients with angiographically defined coronary artery disease in comparison to control group [24]. In the present study, the PAB assay method was applied to neonates with perinatal asphyxia as well as healthy infants and a significant increase of the PAB value was observed in case group in comparison with the control group. The information provided by the PAB assay in asphyxiated neonates was consistent with other diagnostic measures and was significantly different comparing the patients with healthy neonates. Therefore the PAB assay may be useful in assessing neonates and providing a more definite diagnosis. However, comparing the sensitivity and specificity of the parameters shows that PAB still does not appear to be

sufficiently powerful on its own to replace blood gas testing. PAB values in the neonates without encephalopathy were higher in comparison with grade 1 and 2 of HIE and the highest values of PAB were observed in neonates with HIE grade 3. Although the number of patients in each grade was small, it appears that the PAB values may not be an index for severity of neonatal asphyxia. This should be studied in greater detail in the future.

More studies for determining cut-off values and comparing blood gas examination with the PAB assay and evaluating the prognostic utility of the PAB values are suggested.

Conclusions

The PAB assay is a simple and rapid test that may be useful for risk prediction in perinatal asphyxia when used with other forms of assessments. The authors admit that in this study it did not show high sensitivity and specificity in comparison with other tests thus, we need other tests to identify the perinatal asphyxia definitely. PAB could help identify neonates with high levels of oxidative stress, in order to introduce interventions for the prevention of hypoxic ischemic injury. However, further clinical research is required on larger populations, as well as on various physiological and pathological correlates of oxidative stress and parameters of asphyxia.

Conflict of Interest None.

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