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Evaluation of pro-oxidant antioxidant balance (PAB) in infants with respiratory distress syndrome (RDS) and their mothers compared to normal cases

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A – Study Design, **B** – Data Collection, **C** – Statistical Analysis, **D** – Data Interpretation, **E** – Manuscript Preparation, **F** – Literature Search, **G** – Funds Collection

Summary **Background.** Oxidative stress plays a key role in respiratory distress syndrome (RDS) in neonates. The pro-oxidant antioxidant balance (PAB) assay is a reliable and accurate method for determination of serum oxidants and antioxidants.
Objectives. Determination of the pro-oxidant antioxidant balance in preterm infants suffering from infant RDS and their mothers as patients in comparison with full-term infants and their mothers as normal controls.
Material and methods. The study was conducted on 54 neonates with infant RDS (sera pH < 7.2, Apgar score ≤ 5 and serious signs of RDS) and their mothers (n = 54), and the controls included 40 full-term infants (abnormal clinical sign were not seen until three days after delivery) and their mothers (n = 40). The samples were obtained from the infants' cord blood and mothers' venous blood immediately after delivery. The PAB assay was preformed on the subject's sera using a simple, rapid, and accurate method.
Results. The PAB values of preterm infants with respiratory distress syndrome and their mothers and the full-term normal controls and their mothers were (34 ± 21), (14 ± 8.1), (183 ± 29.3) and (174 ± 18.9), respectively. Our obtained results have demonstrated that the PAB level is significantly higher in infants suffering from RDS than that of normal infant (p < 0.01). SPSS version 16 was used for statistical analysis, based on the one-way ANOVA test. A p-value < 0.05 was considered statistically significant.
Conclusions. The PAB level of infants suffering from RDS and their mothers is significantly higher than those of the control group with a normal full-term delivery.
Key words: oxidative stress, respiratory distress syndrome, infant.

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Background

Respiratory distress syndrome (RDS) in infancy, also known as hyaline membrane disease (HMD), is considered a prevalent cause of death among the new-born infants worldwide [1]. RDS is usually associated with lung inflammation, which caused by free radicals and oxidative stress [2]. Due to the higher level of reactive oxygen species (ROS) in the trachea and the lungs of preterm infants and a lower antioxidant defence, the main cellular components, such as DNA, proteins and fatty acids, were not protected against oxidative damage and are consequently faced with severe oxidative damage [3]. Oxidative damage may cause cellular dysfunction and contribute to the pathogenesis of various disorders [4, 5]. Oxidative stress is defined as the condition where intracellular antioxidants outweigh extracellular antioxidants, leading to an imbalance in the generation of ROS [6]. Several investigations have demonstrated the significant role of oxidative stress in the progression and pathogenesis of cancers and various acute and chronic disorders in neonates and adults [7–10].

Studies have indicated that the level of pro-oxidants increases during pregnancy as a result of higher lipid peroxidation [11], nitric oxide generation [12] and lower enzymatic antioxidant activity, such as superoxide dismutase (SOD) and glutathione reductase [13]. Nevertheless, non-enzymatic antioxidants, such as vitamin E, C, A, thiol proteins and ceruloplasmin, increase in pregnancy [14, 15]. Oxidative stress is involved in various disorders associated with pregnancy, such as pregnancy-induced diabetes, preeclampsia, asphyxia and intrauterine growth restriction [16–18]. Moreover, the role of oxidative stress in the progression and pathogenesis of neonatal RDS and preterm delivery is poorly understood. Lack of a precise and reliable technique in the determination of pro-oxidant-antioxidant balance is a major limitation. In the present study, the accurate and rapid pro-oxidant-antioxidant balance (PAB) test, which provides simultaneous measurement of antioxidants and oxidants in the simple assay (PAB assay), was applied.

Material and methods

Patient and samples

The current study was conducted on 54 preterm infants with infant respiratory distress syndrome (IRDS) and their mothers and 40 normal infants and their mothers hospitalised between

June 2016 and September 2017 in Taleghani Hospital, Arak, Iran. Due to the administration of medications besides routine supplementary drugs (B9 and ferrous sulphate), 6 mothers and their infants were excluded from our represented study. IRDS was diagnosed by clinical and radiological examinations based on Downes' score, which accompanied with an X-ray observation of the chest [19, 20]. Infants with a gestational age of < 36 weeks were considered as preterm. 2 ml arterial-venous mixed cord blood was collected after delivery, as well as 2 ml maternal venous blood. The collected blood samples were centrifuged at 3,600 rpm for 10 minutes after clotting to separate the sera. To measure the oxidative stress parameters using the rapid accurate method, the obtained sera were kept at -80°C until analysis. Haemolytic samples were excluding from the PAB assay. Informed consent was obtained from the mothers before participating in the study. The current study was confirmed by the ethical committee of Arak Medical University.

Demographic investigations

The obtained data from all participants, such as gestational time, type of delivery, mothers' age, mothers' BMI and any background disorders, as well as infants' Apgar scores and infants' weights, were measured and recorded based on standard protocols. The mothers' body mass index (BMI) was determined as follows: [weight (kg)/height^2]. All records were retained confidentially in the patient records room of Taleghani Hospital, Arak, Iran.

Chemicals

In the current study, the TMB powder (3, 3', 5, 5' – Tetramethylbenzidine, AppliChem), horse radish peroxidase practical grade, uric acid, dimethyl sulfoxide (DMSO) and chloramine T were purchased from AppliChem (USA).

Pro-oxidant antioxidant balance (PAB) assay

The pro-oxidant antioxidant balance (PAB) assay was performed based on the previous methods [21, 22]. In order to provide standard solutions, a certain proportion of 250 µM H_2O_2 (0–100%) and uric acid 3 mM was gently mixed and added to the NaOH (10 mM).

The TMB cation was prepared based on mixing the 60 mg TMB powder in a 10 ml dimethyl sulfoxide (DMSO) and 20 ml acetate buffer (0.05 M, pH 4.5). 70 µl freshly prepared chloramine T (100 mM) was then mixed well and added to the previously prepared solution. The prepared solution was thoroughly stirred in a dark environment and was incubated for 2 hours at room temperature (23–27°C).

The horse radish peroxidase working solution was prepared by blending the 25-unit peroxidase enzyme solution into the 20 ml TMB cation and was then aliquoted into 1 ml microtubes and stored at -20°C.

The TMB working solution was prepared by adding 200 µl TMB/DMSO into the 10 ml acetate buffer (0.05 M, pH 5.8). The 1 ml TMB cation solution was gently mixed with the 10 ml TMB/DMSO and immediately incubated at room temperature and in the dark for 100 seconds.

In order to determine the PAB values in the subjects' samples, 200 µl of working solution was added to each well of 96-well ELISA. 10 µl of the participants' sera, distilled H_2O (blank well) and standard solutions were then added and mixed gently and, along with the plates, were incubated in the dark at 37°C for 15 minutes. The 50 µl of 2 N HCl was recruited as the stop solution, and the PAB was measured at a 450 nm wavelength.

In the next step, 10 µl of the patients' sera, distilled water as the blank well, accompanied by five standard solutions, was incubated in the dark at 37°C for 12 minutes. 50 µL of 2 N HCl was then added to each well (stopper). Determination of pro-oxidant-antioxidant balance was done by an ELISA reader at a 450 nm wavelength. According to the absorbance of the standard samples' data, the standard curve was prepared. Meanwhile, any unknown samples were calculated by the values obtained from the standard curve.

Statistical analysis

The Statistical Package for Social Sciences (SPSS version 20.0, IBM, USA) was used for data analysis, while a *p*-value < 0.05 was considered statistically significant. The paired sample *t*-Test, Mann-Whitney U test and one-way ANOVA were applied for statistical analysis.

Results

Our data normality was confirmed by Kolmogorov-Simonov statistical analysis. The higher range and lower range of participants' PAB level amounts to 246.03 – 85.28 in mothers with RDS infants, 189.01 – 79.61 in normal mothers, 65.94 – 0.846 in infants suffering from RDS and 50.07 – 0.491 in normal infants. The PAB values of cases and controls is illustrated in Table 1. As Table 1 shows, there was a significant difference between PAB values in infants suffering from respiratory distress syndrome and the normal group ($p = 0.01$). Furthermore, there was a statistically significant difference between mothers with pre-term infants with RDS and mothers with full-term normal neonates ($p = 0.005$) (Figure 1).

Table 1. PAB values (there is a significant difference between infants with RDS and normal infants ($p = 0.0131$) and mothers with preterm infants with RDS and mothers with normal full-term infants ($p = 0.0050$)

Participants	Number	Mean	Std. Deviation	*p*
Preterm infants with RDS (IRDS)	54	34.61	21.04	0.0131
Normal infants	40	14.85	8.189	
Normal mothers	40	174.85	18.91	0.0050
Mothers with preterm IRDS	54	183.54	29.30	

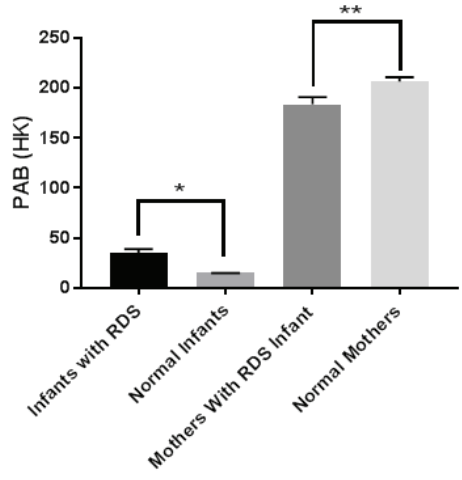

Figure 1. PAB values of participants

* – ($p < 0.01$), ** – ($p < 0.005$).

The obtained demographic records illustrated that the mean age of mothers with an IRDS infant was 27.3 ± 5.67, while the mean age of mothers in the control group was 27.7 ± 5.61 years. The mothers' BMI in the patients and controls was 30.73

± 4.73 and 27.9 ± 3.68, respectively, which shows a significant difference between the cases and controls ($p = 0.48$). The causes of early delivery in mothers with IRSD was considered as the rupture of the placenta (40%), pain and bleeding, while in the control, this was related to pain and bleeding. Moreover, the level of the mothers' education is illustrated in Table 2.

Table 2. Educational level between cases and controls

Educational level	Patients' valid percent	Controls' valid percent
Primary school	28.1	9.4
Middle school	31.3	37.5
High school	25	50
University degree	15.6	3.1

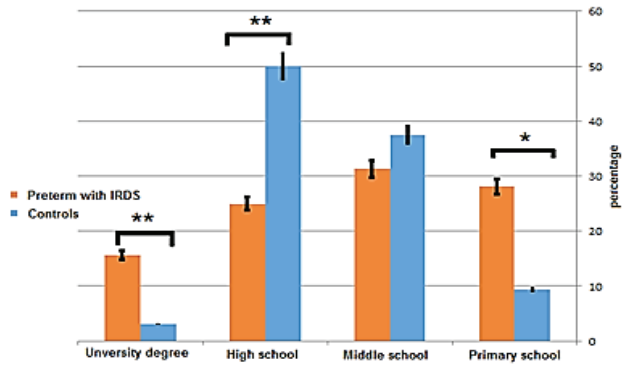

Figure 2. The educational level percentages showed a significant difference in mothers' educational degree between the preterm group with IRDS and the healthy controls.

* – $p < 0.05$, ** – $p < 0.01$.

Discussion

It has been thought that preterm infants are very susceptible to oxidative damages caused by free radicals, oxidative stress and insufficient enzymatic and/or non-enzymatic antioxidant defence. Respiratory distress syndrome (RDS) usually occurs in preterm neonates and is defined as seriously progressive disorders with oxygen depletion among the first few days after delivery, which is considered the main cause of neonatal brain damage and death [23–25]. Since premature lungs need a higher amount of O_2 ventilation, the rate of ROS production raises and surpasses the antioxidant defence [26]. The increase in oxidants damages the components of respiratory cells, such as DNA, proteins, and lipids, which then triggers both the intrinsic and extrinsic apoptosis pathways, resulting in respiratory cell death [27].

In the current study, the PAB values of preterm infants with RDS and their mothers (cases) were compared with healthy full-term infants and their mothers (controls). The obtained results illustrate the significant difference between both groups. The higher level of PAB values in preterm infants with RDS showed a higher rate of oxidant generation and inadequate antioxidant protective mechanisms. On the other hand, at birth, the transition from a relatively hypoxic to a relatively hyperoxic condition in infants can lead to severe oxidative stress. Due to the late induction of an antioxidant mechanism and late upregulation of foetus antioxidant enzymes during the exposure of preterm infant lungs to excess O_2, preterm infants with RDS are typically vulnerable to oxidative damage [28].

Dizdar et al. demonstrated a lower level of total antioxidant capacity (TAC) and higher level of total oxidant status (TOS) in preterm infants (gestational age < 28 weeks) in comparison to those more than 28 weeks of gestation age [21]. According to our data, the higher level of PAB values illustrates the higher amount of generated oxidants and inadequate antioxidant defence. Georgeson et al. in 2002 showed that the level of enzymatic antioxidant activity in the cord blood of preterm infants was significantly lower than infants with full-term gestational age [29]. Although the results in this field are controversially different, the site, time and the kind of measured compounds may affect the assessments and results. Therefore, the rapid, reliable pro-oxidant-antioxidant balance assay reliably shows the status of oxidants and antioxidants in an infant's body. However, the higher levels of PAB values measured in preterm infants with RDS, and consequently in their mothers, illustrate the insufficient enzymatic and non-enzymatic antioxidants, along with a higher level of ROS and resembling oxidants.

It has been reported that PAB values rise during pregnancy, though the cause of this elevation is under investigation. Based on a study by Little and Gladen, the body temperature and the level of lipid peroxidation raises during pregnancy [30]. Most of the energy needed for foetus development, reconstruction of sex hormones and regeneration of tissues is supplied by lipid metabolism and fatty acid peroxidation. Boskabadi et al. showed higher PAB values during normal pregnancy due to an alteration of the status of oxidants and antioxidants [23]. Moreover, several studies showed that PAB values increased during the pregnancy period [31].

Our results indicate higher PAB values in mothers with preterm infants with RDS compared to mothers with normal full-term delivery. Takehara et al. showed a 10–50% increase in PAB values during the first trimester, and this gradually reduced over the second and third trimester [32]. Based on the study by Takehara et al., the level of enzymatic antioxidant activity reduced after delivery. In other words, PAB values increased with foetus progression over the first trimester because of higher the metabolism needed for maternal tissues and foetus development. In the second and third trimester, the level of lipid peroxidation fell, which illustrates the beneficial role of enzymatic and non-enzymatic antioxidant balance concerning foetus development, tissue regeneration, pregnancy and delivery.

The imbalance between oxidants and antioxidants is a serious condition due to the role of oxidative stress in severe clinical disorders. In the presented study, preterm infants showed a higher level of PAB values, which may be connected with the higher generation of ROS in their premature lungs in comparison to full-term infants. Furthermore, mothers with RDS infants showed a higher level of PAB values due to higher lipid peroxidation for foetus development during pregnancy compared to normal mothers with full-term delivery. The measurement of pro-oxidants-antioxidants balance with a reliable, rapid test could be considered a useful method for discovering high-risk groups and preventing oxidative damage and its subsequent complications.

Conclusions

The PAB values in the pre-term infants suffering from RDS might be helpful to distinguish high-risk cases. The information obtained from the PAB assay in combination with the other serum parameters might be predictive for any prognostic and antioxidant treatment response to be elucidated. More detailed studies are essential for the evaluation of PAB values in the various stages of the prenatal and post-natal period and maternal status, which may develop a new strategy for better prognosis and treatment. Overall, the PAB level of infants suffering from RDS and their mothers is significantly higher than those of the control group with a normal full-term delivery.

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Tables: 2
Figures: 2
References: 32

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