



## A novel approach to study oxidative stress in neonatal respiratory distress syndrome



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### ABSTRACT

**Background:** Respiratory distress syndrome of the neonate (neonatal RDS) is still an important problem in treatment of preterm infants. It is accompanied by inflammatory processes with free radical generation and oxidative stress. The aim of study was to determine the role of oxidative stress in the development of neonatal RDS.

**Methods:** Markers of oxidative stress and antioxidant activity in umbilical cord blood were studied in infants with neonatal respiratory distress syndrome with reference to healthy newborns.

**Results:** Status of markers of oxidative stress (malondialdehyde, protein carbonyl and 8-hydroxy-2-deoxyguanosine) showed a significant increase with depleted levels of total antioxidant capacity in neonatal RDS when compared to healthy newborns.

**Conclusion:** The study provides convincing evidence of oxidative damage and diminished antioxidant defenses in newborns with RDS. Neonatal RDS is characterized by damage of lipid, protein and DNA, which indicates the augmentation of oxidative stress.

**General significance:** The identification of the potential biomarker of oxidative stress consists of a promising strategy to study the pathophysiology of neonatal RDS.

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### 1. Introduction

Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. It is a physiological event in the fetal-to-neonatal transition, which is actually a great stress to the fetus. These physiological changes and processes greatly increase the production of free radicals, which must be controlled by the antioxidant defense system, the maturation of which follows the course of the gestation. This could lead to several functional alterations with important repercussions for the infants. Adequately mature and healthy infants are able to tolerate this drastic change in the oxygen concentration. A problem occurs when the intrauterine development is incomplete or abnormal. Preterm or intrauterine growth retarded (IUGR) and low birth weight neonates are

typically of this kind [1,2]. An oxidant/antioxidant imbalance in infants is implicated in the pathogenesis of the major complications of prematurity including respiratory distress syndrome (RDS), necrotizing enterocolitis (NEC), chronic lung disease, retinopathy of prematurity and intraventricular hemorrhage (IVH).

In neonates born with respiratory distress syndrome, respiratory failure due to deficient alveolar development and surfactant production could be complicated by diminished antioxidant stores and enzymatic antioxidant inducibility. The premature newborn's lung is particularly susceptible to oxidant stress because there are many sources of reactive oxygen species (ROS) production and a relative lack of antioxidant defenses. A fully developed lung armed with sufficient defense is therefore critical in ensuring that the newborn lung is resistant to high O<sub>2</sub> tensions.

Infant respiratory distress syndrome (IRDS), also called neonatal respiratory distress syndrome or respiratory distress syndrome of newborn, previously called hyaline membrane disease (HMD), is a syndrome in premature infants caused by developmental insufficiency of surfactant production and structural immaturity in the lungs. IRDS affects about 1% of newborn infants and is the leading cause of death in preterm infants [3]. When born too early, infants are delivered with a very immature stage of lung development, the late canalicular stage for infants born at 24–26 weeks (i.e., for ELBW infants), and early or mild saccular stage for those born after 26 but before 32 weeks of

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gestation (in part ELBW and all very low birth weight (VLBW) infants) [4]. Preterm birth occurring during the late canalicular or early sacular stage is very likely to lead to severe respiratory distress syndrome (RDS). The poorly developed peripheral airways and immaturity of cells important for lung maturation are the major causes of poor surfactant production by type II cells and inadequate antioxidant responses to increased ambient oxygen. For instance, the superoxide dismutase (SOD) activity appears in the developing lungs concomitantly with the surfactant synthesis by type II pneumocytes [5].

When phagocytes such as neutrophils are stimulated by microorganisms or other means, they become activated and increase their oxidative metabolism; as a result, toxic oxygen and nitrogen derivatives, i.e., ROS/RNS, are formed. If these toxic products are not inactivated, their high chemical reactivity leads to damage to a variety of cellular macromolecules including proteins, carbohydrates, lipids and nucleic acid. This results in cell injury and may induce respiratory cell death [6]. Under these conditions, a surfactant deficiency may be aggravated by inactivation of the small amount of endogenous surfactant that is produced [7]. Furthermore, if exogenous surfactant is given this may also be destroyed [8,9].

An attempt has been made to investigate the alterations in the biochemical parameters of oxidant/antioxidant by quantification of levels of 8-hydroxy-2-deoxy guanosine (8-OHdG), protein carbonyl and malondialdehyde (MDA) along with total antioxidant capacity in study subjects and healthy controls. Umbilical cord blood provides valuable information regarding the status of the infant at birth. Cord blood parameters can be considered as early predictors of some of the metabolic disorders in future adult life. Based on this concept, our present observational study aims at evaluating the cord blood oxidative stress parameters in newborns with respiratory distress syndrome and provides a concise view of their current status among the healthy newborns.

## 2. Materials and methods

A case–control study was conducted in the Department of Biophysics in collaboration with Department of Pediatrics, IMS, BHU, Varanasi. Cord blood samples from a total of 36 pregnant women were collected at the time of the delivery. The study group consisted of 16 preterm low birth weight newborns with diagnosis of RDS. The control group was composed of 20 preterm low birth weight newborns. All the subjects were selected from the Department of Obstetrics and Gynaecology, University Hospital, Banaras Hindu University (India). RDS was diagnosed on the presence of typical clinical and radiological signs of the disease in the preterm infants. Newborns were considered to have RDS if they have tachypnea, grunting and cyanosis with several hours of birth required mechanical ventilation and typical radiographic findings on the chest X-ray. The diagnosis was established from the clinical symptoms and needed for oxygen treatment.

Ethical approval and permission for the study was taken from the Ethical Committee of Institute of Medical Sciences, Banaras Hindu University (India). Informed consent was taken from the patients/attendants from all the studied subjects purely for of research purpose. With all aseptic precautions cord blood samples (with and without heparin) of all studied subjects were collected. The blood samples were centrifuged at 5000 rpm for 10 min and plasma/serum was separated and stored at  $-20^{\circ}\text{C}$ . The samples were assayed in triplicate for statistical purposes.

Exclusion criteria of the study were infection, hemolytic disease, major malformations, hypertension disorder and newborns with history of difficult delivery, genetic disorder and fetal distress. The weight of the newborn was recorded immediately after delivery in Seca weighing scale with an accuracy of 5 g. Maternal age, height, weight, date of last menstrual period, medical history and reproductive history were obtained from the hospital record.

### 2.1. Malondialdehyde—marker of lipid peroxidation

Plasma malondialdehyde (MDA) levels in the cases and controls were assayed by thiobarbituric acid reactive substances (TBARS) technique of Philpot [10]. The sample (1 ml) was mixed thoroughly with 2 ml of TCA–TBA–HCl (15% w/v TCA and 0.375% w/v TBA in 0.25 N HCl). The mixture was heated in a boiling water bath for 15 min. The samples were centrifuged at 1000 rpm for 10 min. The absorbance of the sample was determined at 530 nm in a spectrophotometer against a suitable blank. The malondialdehyde concentration of each sample was calculated by using extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

### 2.2. Protein carbonyl—marker of oxidative protein damage

Plasma protein carbonyl was measured using spectrophotometric DNPH method [11]. The assay is based on the spectrophotometric detection of the reaction between 2,4-dinitrophenyl hydrazine (DNPH) with protein carbonyl to form protein hydrazone. Carbonyl content was determined as nmol/mg protein. The intra- and inter-assay coefficients of variations were 4.7% and 8.5%, respectively. The total protein content was measured using colorimetric kit based on biuret method [12].

### 2.3. 8-OHdG—marker of oxidative DNA damage

Serum of all the cases and control samples was used for the measurement of 8-OHdG levels using competitive in vitro enzyme-linked immunosorbent assay (ELISA) kit obtained from Caymen Chemical Company U.S.A. [13]. 8-OHdG measurements were performed using microtiter ELISA plate pre-coated with anti-mouse IgG. 50  $\mu\text{l}$  sample, 50  $\mu\text{l}$  8-OHdG AChE (Acetylcholinesterase) tracer and 50  $\mu\text{l}$  8-OHdG monoclonal antibody were added to each well and incubated at  $4^{\circ}\text{C}$  for 18 h. After the wells were washed five times, 200  $\mu\text{l}$  Ellman's reagent was added to each well. The wells were incubated at room temperature in the dark for 100 min. The absorbance was read at wavelength of 420 nm. ELISA assay displays  $\text{IC}_{50}$  (50% B/B<sub>0</sub>) and  $\text{IC}_{80}$  (80% B/B<sub>0</sub>) values of approximately 100 and 30 pg/ml, respectively.

### 2.4. Total antioxidant status (TAS)—marker of oxidative defense

The plasma total antioxidant status was determined using Randox assay kit [14]. The assay was based on the principle that ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) is incubated with a peroxidase and  $\text{H}_2\text{O}_2$  to produce the radical cation  $\text{ABTS}^+$ . This has a relatively stable blue green color, which is measured at 600 nm. Antioxidant in the cord blood causes the suppression of this color production to a degree which is proportional to their concentration.

### 2.5. Statistical analysis

Data were expressed as the mean  $\pm$  SD. A chi-square test was used for comparison of non-parametric data. An independent-sample *t*-test was used for comparison of parametric variables. Logistic univariate regression was chosen as inferential analysis, instead of multivariate logistic regression for the lack of data, and it checked the relation between the presence/absence at least one of disease as a dependent variable and each oxidative stress marker as an independent variable. Through the logistic model, it is possible to calculate estimated coefficients (B), standard error (SE), Wald statistic, significance (*p*) and exponential or odds ratio Exp(B) with the relative confidence interval (95% CI). *p* < 0.05 was considered as statistically significant. The index risk was calculated using the SPSS statistical software package (SPSS 16).

**Table 1**  
Mother and neonatal characteristics of studied subjects.

Characteristics	RDS group (N = 16)	Control (N = 20)	p
Maternal age (years)	27 ± 5 (24.51–29.61)* (24.51 – 29.61)	25 ± 4* (23.18–26.81)*	t = 1.427 (p = 0.163, NS)
Gravida			
Primigravidas	6 (37.5%)	9 (45%)	$\chi^2 = 0.206$ (p = 0.650, NS)
Multigravidas	10 (62.5%)	11 (55%)	
Mode of delivery			
Vaginal delivery	4 (25%)	7 (35%)	$\chi^2 = 0.419$ (p = 0.517, NS)
Cesarean section	12 (75%)	13 (65%)	
Sex			
Males	6 (37.5%)	4 (20%)	$\chi^2 = 1.357$ (p = 0.244, NS)
Females	10 (62.5%)	16 (80%)	
Gestational age(weeks)	29.6 ± 1.5 (28.8–30.4)*	34.6 ± 1.2 (34.1–35.2)*	t = –11.124 (p = 0.000)**
Birth weight(g)	1120 ± 156 (1037–1023)*	1725 ± 193 (1634–1815)*	t = –10.134 (p = 0.000)**
Body mass index (BMI) (kg/m <sup>2</sup> )	9.04 ± 2.06 (7.94–10.14)*	9.17 ± 1.01 (8.70–9.64)*	t = –0.249 (p = 0.805, NS)
Apgar score			
1 min	6.75 ± 1.34 (6.04–7.46)*	8 (8–8)*	t = –4.182 (p = 0.000)**
5 min	7.94 ± 1.12 (7.34–8.54)*	9 (9–9)*	t = –4.254 (p = 0.000)**

Data are presented as mean ± SD or as percentage.

p value-independent t or chi-square test as appropriate

NS = non-significant;  $\chi^2$  = Pearson chi-square; BMI = weight/height<sup>2</sup>.

\* 95% confidence interval for mean (lower bound–upper bound).

\*\* Significant at p < 0.001.

### 3. Results

The low birth weight infants with RDS were treated as cases and low birth weight infants without RDS, who presented with no respiratory manifestations served as control subjects.

Table 1 summarizes the characteristics of mothers and newborns included in this study. The respiratory distress syndrome (RDS) group composed of 16 preterm low birth weight newborns born with a mean gestational age (GA) of 29.6 ± 1.5 weeks having a mean birth weight 1120 ± 156 g. The control group composed of 20 preterm low birth weight newborns born with a mean GA of 34.6 ± 1.2 weeks having a mean birth weight 1725 ± 193 g. The statistical analysis for comparison of non-parametric data between each group was performed by chi-square test. The significant difference of parametric data was evaluated with Student's t-test with equal variance. Newborns in RDS group were more premature and had lower birth weight in comparison to control group. There was no significant difference in maternal age (p = 0.163), gravida (p = 0.650), mode of delivery (p = 0.517), sex of newborn (p = 0.244) and BMI (p = 0.805) between RDS and control group. The mean of Apgar score in RDS group were significantly lower (p < 0.001) than the control.

Table 2 shows the concentration of oxidative damage markers and the total antioxidant status in the umbilical cord blood of newborn with respiratory distress syndrome in comparison to controls. The significant difference was evaluated with Student's t-test with equal variance. The extent of protein oxidation and oxidative DNA damage as evidenced by protein carbonyl and 8-OHdG, respectively, was significantly increased in the RDS group (p < 0.001) compared to control subjects. The product of lipid peroxidation–MDA was significantly increased 43.46% in RDS group (p < 0.005) than that of control. A

significant decrease in TAS (p < 0.001) was seen in the RDS group as compared to control.

Table 3 shows the results of univariate logistic regression analysis to estimate the association of oxidative damage markers and total antioxidant status with incidence of respiratory distress syndrome in newborns. The positive association of risk of RDS was observed with protein carbonyl (OR = 0.249, p < 0.005), MDA (OR = 0.404, p < 0.005) and 8-OHdG (OR = 0.682, p < 0.05) with partial regression coefficient 1.389, 0.907 and 0.383, respectively. A significant inverse correlation of risk of RDS in study subjects was found with TAS (OR = 1.039, p < 0.005) with partial regression coefficient –0.038. Logistic analysis showed that the elevated level of protein carbonyl, MDA and 8-OHdG might be risk factor, reduced level of total antioxidant status might lead to the RDS in newborn.

### 4. Discussion

Neonatal respiratory distress syndrome represents the major lung complications of newborn babies. Preterm neonates suffer from respiratory distress syndrome (RDS) due to immature lungs and require assisted ventilation with high concentrations of oxygen. The pathogenesis of this disorder is based on the rapid formation of the oxygen reactive species, which surpasses the detoxification capacity of anti-oxidative defense system [15,6]. The high chemical reactivity of free radical leads to damage to a variety of cellular macro molecules including proteins, lipids and nucleic acid. This results in cell injury and may induce respiratory cell death [8].

Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation. The present study showed increased concentration of MDA in neonates with respiratory disorders than that of control in consonance with the reported study [16].

**Table 2**  
Oxidative damage markers and total antioxidant status in different studied groups.

Damage markers	Protein carbonyl (nmol/mg protein)	MDA (mmol/l)	8-OHdG (pg/ml)	TAS (mmol/l)
RDS group (N = 16)	4.535 ± 1.602 (3.682–5.389)*	3.698 ± 1.516 (2.890–4.506)*	39.60 ± 11.153 (33.657–45.543)*	0.586 ± 0.281 (0.436–0.736)*
Control (N = 20)	2.663 ± 0.963 (2.212–3.114)*	2.091 ± 1.062 (1.594–2.588)*	23.35 ± 7.609 (19.789–26.911)*	1.241 ± 0.331 (1.087–1.396)*
t-value (p)	t = 4.345 (p = 0.000)**	t = 3.736 (p = 0.001)**	t = 5.187 (p = 0.000)**	t = –6.304 (p = 0.000)**

Data are presented as mean ± SD.

\* 95% confidence interval for mean (lower bound–upper bound).

\*\* Significant at p < 0.001.

\*\*\* Significant at p < 0.005.

**Table 3**  
Logistic regression analysis of the relation to biochemical parameters to respiratory distress syndrome (RDS) in newborn infants.

Parameters	B	Odd ratio (OR)	p	95% class interval (CI) of OR (lower–upper)
Protein carbonyl	1.389	0.249	0.004*	0.096–0.648
MDA	0.907	0.404	0.004*	0.219–0.747
8nOHdG	0.383	0.682	0.021**	0.492–0.945
TIBC	–0.038	1.039	0.017**	1.007–1.072

B = partial regression coefficient.

\* Significant at  $p < 0.005$ .

\*\* Significant at  $p < 0.05$ .

Protein carbonyls represent markers of early protein oxidation. The present study observed elevated level of protein carbonyl in umbilical cord blood of neonates with respiratory syndrome (RDS) compared to control. Some studies also reported that prematurely born babies with RDS showed high concentrations of protein carbonyls [17,18]. The amount of oxidatively modified protein may provide a quantitative assessment of oxygen toxicity and of pulmonary antioxidant defenses [19]. Lung proteins are attacked by oxygen reactive species. When RDS is present, pulmonary oedema occurs because of increased permeability of cell membranes. The fluid in the oedema is rich in proteins, which represent the ideal target for oxygen reactive species. In order to initiate the oxidative attack, oxygen reactive species inactivates alpha-1 protease, thus causing an imbalance in the lung protease–antiprotease system [20,21]. Reactive oxygen species also interact with pulmonary surfactant as well as with other protein and lipid structures, thus delaying the normal functioning of the lung. Therefore, surfactant administration before the initiation of mechanical ventilation diminishes the severity of lung lesions by providing consistent ventilation [22]. This protein oxidation process activated by oxygen reactive species has been proven to contribute to pathogenesis in newborns with RDS [23,24]. The present study shows that neonates with RDS had higher protein damage compared to control. Statistically significant correlations were observed between protein carbonyl value and the occurrence of RDS [25].

In present study, 8-OHdG was used as a sensitive marker for oxidative DNA damage. 8-OHdG was higher in the umbilical cord blood of neonates with RDS compared to control. Oxidative DNA damage could be the crucial mechanism in the pathogenesis of respiratory disorder [26].

Oxidative damage is important in the pathogenesis of respiratory distress syndrome (RDS). It can be assumed that these newborns might be at risk of an oxidative stress. One previous study [27] also observed oxidative stress in neonates with respiratory distress syndrome. Reactive oxygen is generated by several inflammatory and structural cells of the airways. These oxidant species have important effects on a variety of lung cells as regulator of signal transduction, activators of key transcription factors and modulators of gene expression and apoptosis. Thus, increased oxidative stress accompanied by reduced antioxidant defenses may play a role in the pathogenesis of a number of inflammatory pulmonary diseases including RDS in the newborn [28].

The present study observed reduction in total antioxidant status in newborns with RDS compared to healthy newborns. One study also reported decreased level of total antioxidant activity in newborn with RDS [29]. Oxidant–antioxidant balance shifts in favor of the oxidative damage in premature newborns with RDS due to diminished antioxidant activity. Antioxidant defenses of the immature lung will be prepared neither for the hyperoxic environment nor the inflammation found in association with respiratory distress. The lung depends on a delicate balance between oxidant and antioxidant systems to maintain normal cellular function. The lungs of prematurely born infants suffering from respiratory distress syndrome may be ill-adapted for protection against ROS. Antioxidants clearly have an important role in the

defense against free radical induced lung injury in newborns with respiratory distress syndrome (RDS).

The present study showed that the elevated level of protein carbonyl, MDA and 8-OHdG, might be risk factor, reduced level of total antioxidant status might lead to the RDS in newborn. Total antioxidant status might serve as prognostic marker in newborns with RDS and might help distinguish high risk infants. Neonatal respiratory distress syndrome is accompanied by inflammatory processes with free radical generation and oxidative stress [30,31]. The imbalance between the oxidative forces and the antioxidant defense systems was suggested to predispose the lungs to the development of RDS [32,33]. Many studies have shown increased oxidant stress markers and/or reduced antioxidant defense in preterm infants with RDS [34,35].

## 5. Conclusion

The result of this study indicates that oxidative stress was induced in newborns with RDS which is manifested as increased lipid peroxidation, protein oxidant damage and oxidative DNA damage. Thus, an increased oxidative stress accompanied by reduced antioxidant defenses may play a role in the pathogenesis of respiratory distress in newborns.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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