



Journal of Medical Sciences

ISSN 1682-4474

science
alert

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JMS (ISSN 1682-4474) is an International, peer-reviewed scientific journal that publishes original article in experimental & clinical medicine and related disciplines such as molecular biology, biochemistry, genetics, biophysics, bio-and medical technology. JMS is issued eight times per year on paper and in electronic format.

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In vivo Oxidative DNA damage, Protein Oxidation and Lipid Peroxidation as a Biomarker of Oxidative stress in Preterm Low Birth Weight Infants

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Pregnancy while not a disease is often accompanied by a high energy demand of many bodily functions. Neonates are said to be more susceptible to oxygen radical injury. The objective of the present study was to measure cord blood levels of 8-OHdG, malondialdehyde, protein carbonyl and total antioxidant status in premature low birth weight infants to analyze the status of oxidative stress in relation to the degree of prematurity and birth weight of neonates. Umbilical cord blood samples were obtained at the time of delivery. 8-OHdG has been measured as oxidative DNA damage marker in preterm LBW newborns by competitive *in vitro* Enzyme-Linked Immunosorbent Assay (ELISA) along with malondialdehyde as marker of lipid peroxidation, protein carbonyl as marker of protein oxidation and total antioxidant status to study the oxidative stress. Significant elevation in the levels of 8-OHdG along with malondialdehyde, protein carbonyl has been noted in preterm LBW newborns. Serum 8-OHdG is found to be significantly and negatively correlated with birth weight ($r = -0.834, p < 0.001$) and gestational age of the newborn ($r = -0.626, p < 0.001$). These results provide evidence of increased oxidative stress in the form of DNA damage, protein oxidation and lipid peroxidation in premature LBW newborns which may be responsible for different complications associated with prematurity.

Key words: Preterm, low birth weight, 8-OHdG, oxidative stress

INTRODUCTION

According to World Health Organization (WHO), Low Birth Weight (LBW) is defined as a birth weight less than 2500 g. As per United Nations Children's Fund (UNICEF, 2003), the incidence of LBW delivery in India is 30% and it is the single most important determinant of neonatal deaths. Over 75-90% neonatal deaths occur among low birth weight infants. LBW includes both premature (gestational age ≤ 37 weeks) and small gestational age infants (birth weight < 10 th percentile).

The perinatal period and delivery in particular is a critical time for maintaining a balance between the production of free radicals and the incompletely developed antioxidative protection of the fetus and the newborn (Saker *et al.*, 2008). Human studies have consistently demonstrated that increased Reactive Oxygen Species (ROS)/ free radical production occurs in preterm and LBW infants and is associated with a relative lack of antioxidant enzyme concentration and activity (Trindade and Rugolo, 2007).

An imbalance between oxidant generating system and antioxidant in LBW infants is implicated in the pathogenesis of the major complications of prematurity including necrotizing enterocolitis (NEC) (Okur *et al.*, 1995), chronic lung disease (Saugstad, 1997), retinopathy of prematurity (Perrone *et al.*, 2009) and intraventricular haemorrhage (IVH) (Kelly, 1993). The antioxidant defense mechanisms are not fully developed in preterm infants which increase their vulnerability to oxidative stress (Trindade and Rugolo, 2007).

Increased oxidative stress can also lead to ROS/free radical attack on physiologically important molecules, such as lipids, proteins, various enzymes and DNA (Saugstad, 2001; Halliwell and Gutteridge, 1990). Oxidative stress presents numerous opportunities for tissue injury through formation of reactive oxygen/nitrogen species. Free radical i.e., any atom or molecule that has a single unpaired electron in an outer shell and other reactive species are constantly generated *in vivo* and cause oxidative damage to DNA and lipid. Evidence for oxidative injury comes from measurements of biochemical markers of lipid peroxidation and protein oxidation. Malondialdehyde and protein carbonyls are byproducts of oxidation of lipids and protein respectively (Winterbourn *et al.*, 2000).

Malondialdehyde (MDA) can be assessed biologically as a measure of lipid peroxidation (Marjani *et al.*, 2007a). DNA is probably the most significant biological target of oxidative attack and it is widely thought that continuous oxidative damage is a significant contributor to many neonatal diseases.

Oxidative DNA modification occurs as a consequence of damage to purines and pyrimidines bases. Among the oxidative bases, 8-OHdG is the most abundant (McCord, 2000) and accepted as a sensitive marker for oxidative DNA damage (Ishikawa *et al.*, 2007). DNA damage has been assessed by measuring the steady state level of 8-OHdG in DNA in various tissues (Richter *et al.*, 1988). 8-OHdG is secreted in blood stream and also into urine providing an estimate of damaged DNA (Inoue *et al.*, 2003; Yin *et al.*, 1995). The study of oxidative DNA damage is recognized as a useful marker for the estimation of DNA damage produced by oxygen radicals generated endogenously or exogenously. DNA oxidative damage from Reactive Oxygen Species (ROS) is a common type of damage faced by cells in various diseases and it may also lead to many different mutations in DNA (Halliwell and Gutteridge, 1999).

Oxidative damage to macromolecules may have numerous negative health consequences so the present study was carried out to quantify the extent of oxidative DNA damage, protein oxidation, lipid peroxidation and total antioxidant status in premature LBW infants.

MATERIALS AND METHODS

Study participants and collection of sample: The prospective observational study was carried out in the Neonatal unit, Department of Pediatrics and department of Biophysics, Institute of Medical Sciences, Banaras Hindu University, Varanasi (India) over a period of 1 year (2009-2010). The study was approved by Institute Ethics Committee. Informed consent was taken from the parents or attendants in all cases. The study population comprised of 55 preterm (gestational age < 37 weeks) LBW newborns (birth weight < 2500 g) and 24 sex matched full term control (gestational age ≥ 37 weeks) newborns weighing ≥ 2500 g. LBW neonates were further divided into three groups for statistical calculation, group I (birth weight < 1500 g), group II (birth weight 1500-1999 g) and group III (birth weight 2000-2499 g).

Exclusion criteria of the study were Eclampsia, hypertension in the mother, Intrauterine Growth Retardation (IUGR), perinatal asphyxia, infection, hemolytic disease and major malformations. Newborns with history of difficult delivery, genetic disorder and fetal distress were also excluded. 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. Venous umbilical cord blood was collected from the placental end of the cord

immediately after the infant delivery. Blood samples were collected in tubes, centrifuged at 5000 rpm for 10 min and the serum was stored at -20°C. Hemolysed samples were excluded.

Laboratory analysis

Estimation of DNA damage marker: 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 (Shigenaga and Ames, 1991). 8-OHdG measurements were performed using microtiter ELISA plate precoated with antimouse IgG. 50 µL sample, 50 µL 8-OHdG AChE (Acetylcholinesterase) tracer and 50 µL 8-OHdG monoclonal antibody were added to each well and incubated at 4°C for 18 h. After the wells were washed five times, 200 µL 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 IC₅₀ (50% B/B₀) and IC₈₀ (80% B/B₀) values of approximately 100 and 30 pg mL⁻¹, respectively.

Estimation of malondialdehyde: Serum Malondialdehyde (marker of lipid peroxidation) levels in the cases and controls were assayed by Thiobarbituric Acid Reactive Substances (TBARS) technique of Philpot (1963).

Estimation of Total Antioxidant Status (TAS): The serum total antioxidant status was determined using Randox assay kit (Miller *et al.*, 1993).

Estimation of protein carbonyl: Protein carbonyl proteins were assayed by 2, 4-dinitrophenylhydrazine reaction (Levine *et al.*, 1990). 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⁻¹ protein. The total protein content was measured using colorimetric kit based on biuret method.

Statistical analysis: Data were expressed as the Mean±SD. One way Analysis of Variance (ANOVA) followed by Post Hoc Tukey's test was used to compare the results. Correlations between variables were studied by Pearson correlation test. Chi square test was used for comparison of non-parametric data. Independent sample t-test was used for comparison of parametric variables. p<0.05 was considered as statistically significant

(Hill, 1979). The index risk was calculated using the SPSS statistical software package (SPSS 16).

RESULTS

Low birth weight preterm newborns were included as test group and healthy full term newborns as control group. Table 1 shows the characteristics of the study subjects. Group I comprised of 14 preterm neonates born with a mean Gestational Age (GA) of 31.8±3 weeks having a mean birth weight 1220±233 g. Group II comprised of 22 preterm neonates born with a mean GA of 34.7±2 weeks having a mean birth weight 1852±129 g. Group III comprised of 19 preterm neonates born with a mean GA of 35.3±1.2 weeks having a mean birth weight 2271±133 g. Control group comprised of 24 healthy full term neonates born with a mean GA of 38±1 weeks having a mean birth weight 3149±347 g (Table 1). According the anthropometric results, we found significant difference in gestational age (p<0.001), weight (p<0.0001) and BMI (p<0.0001) of newborns (Table 1).

Table 2 shows that the levels of 8-OHdG in cord blood, the biomarker of DNA damage, was significantly higher in preterm LBW newborns compared to control group (p<0.001). There is a significant increase in serum 8-OHdG in group I (46.321±13.994 pg mL⁻¹), group II (31.205±9.902 pg mL⁻¹), group III (23.0263±11.673 pg mL⁻¹) compared with control group (4.15±2.976 pg mL⁻¹). Table 2 also shows the biomarker of protein damage in different studied groups. There was significant rise in the cord blood protein carbonyl value of preterm LBW groups in comparison to control group (p<0.001). The present study demonstrates increased oxidative DNA damage and protein oxidation in very low birth weight newborns which indicates that preterm low birth weight newborns are subjected to oxidative stress.

Cord blood 8-OHdG was significantly and negatively correlated with birth weight (r = -0.834, p<0.001 Fig. 1a) and GA of the newborn (r = -0.626, p<0.001 Fig. 1b).

MDA levels, the biomarker of lipid peroxidation was significantly higher (p<0.001) in premature LBW neonates compared to controls, whereas total antioxidant status was significantly lower in these neonates (p<0.001) (Table 2). Cord blood Protein carbonyl was significantly and negatively correlated with birth weight (r = -0.572, p<0.001) and GA of the newborn (r = -0.397, p<0.001). A positive correlation was demonstrated between 8-OHdG and MDA (r = 0.585, p<0.001), 8-OHdG and Protein carbonyl (r = 0.503, p<0.001) and Protein carbonyl and MDA (r = 0.400, p<0.001) in umbilical cord blood.

Table 1: Mother and neonatal characteristics

Characteristics	LBW(<1500g) Group-I	LBW(1500-1999g) Group-II	LBW(2000-2499g) Group-III	Control (=2500 g)	p-value
Newborns No.	14	22	19	24	NS
Maternal age (years)	27±4	26±4	25±4	25±4	NS
Gestational age (weeks)	31.8±3	34.7±2	35.3±1.2	38±1	p<0.001
Birth weight (g)	1220±233	1852±129	2271±133	3149±347	p<0.0001
BMI (kg m ⁻²)	7.95±0.46	9.69±1.04	10.79±0.59	13.30±1.53	p<0.0001
Mode of delivery					
Vaginal delivery	3 (21.4%)	5 (22.7%)	5 (26.3%)	6 (25%)	NS
Cesarean section	11 (78.6%)	17 (77.3%)	14 (73.7%)	18 (75%)	NS
Sex					
Males	5 (35.7%)	8 (36.4%)	7 (36.8%)	9 (37.5%)	NS
Females	9 (64.3%)	14 (63.6%)	12 (63.2%)	15 (62.5%)	NS

BMI (Body Mass Index) = weight/height², NS: Non-significant

Table 2: Oxidative stress in different study groups

Neonatal factor	Oxidative stress markers			
	8-OHdG (pg mL ⁻¹)	MDA (mMol L ⁻¹)	Protein carbonyl (nMol mg ⁻¹)	Total antioxidant status (mMol L ⁻¹)
Group-I (n = 14)	46.321±13.994	3.469±1.577	4.380±1.173	0.801±0.504
Group-II (n = 22)	31.205±9.902	2.039±1.278	3.744±0.954	1.412±0.357
Group-III (n = 19)	23.026±11.673	1.317±0.644	3.457±1.127	1.585±0.477
LBW (I+II+III) (n = 55)	32.421±14.446	2.169±1.445	3.343±1.259	1.312±0.544
Control (>2500g) (n = 24)	4.15±2.976	0.663±0.201	2.281±0.948	2.128±0.340
ANOVA (F-value)	60.542 (p<0.001)	18.902 (p<0.001)	10.91 (p<0.001)	22.634 (p<0.001)

Unpaired t-test p<0.001 for I vs. III, I vs. control, II vs. control, LBW (I+II+III) vs. control. p<0.005 for I vs. II, III vs. control. p<0.05 for II vs. III

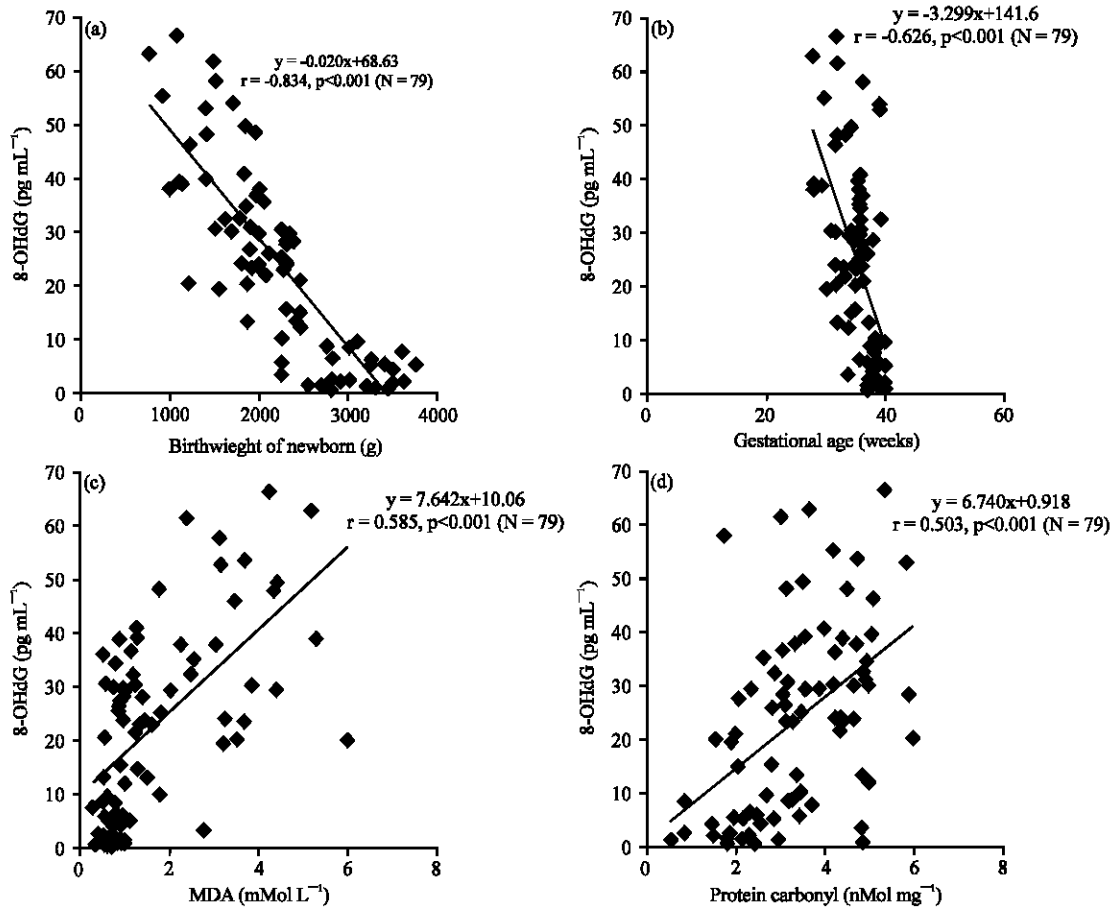


Fig. 1 (a-d): Scatterplot with regression line showing correlation (a) between the birth weight of newborn and levels of cord blood 8-OHdG, (b) between the gestational age of newborn and levels of cord blood 8-OHdG, (c) between the level of cord blood MDA and 8-OHdG and (d) between the level of cord blood Protein carbonyl and 8-OHdG

DISCUSSION

In a healthy body, ROS and antioxidants remain in balance. Impairment in antioxidant enzymes may result in increased risk for the development of oxygen radical diseases (Saugstad, 1996; Balkis *et al.*, 2008). The present study demonstrates increased oxidative stress in premature LBW neonates using specific biomarker of DNA damage, protein oxidation and lipid peroxidation. At the same time, total antioxidant status was also found to be decreased in these neonates. We studied oxidative stress of the fetus immediately after birth, measuring the stress biomarker of DNA damage, protein oxidation and lipid peroxidation in the umbilical cord blood.

There are only a few reports on profiles of oxidative damage in cord blood. Currently, 8-OHdG has been used as a sensitive marker for oxidative DNA damage in different studies (Shoji *et al.*, 2003). 8-OHdG may be used as true biomarker of oxidative stress mediated diseases in LBW infants (Matsubasa *et al.*, 2002). In this study, we confirmed that blood 8-OHdG in preterm LBW newborns was significantly higher than full term newborns having birth weight more than 2500 g. Increased elevation of urinary 8-OHdG in very low birth weight infants has already been reported by some investigators (Matsubasa *et al.*, 2002; Scholl and Stein, 2001).

The primary cause of LBW is premature birth. The premature infant has poorly developed antioxidant defense systems and therefore may be at increased risk of ROS/free radical damage (Pitkanen *et al.*, 1990). We found the biomarker of DNA damage to be significantly elevated in the preterm group compared with full term group. This is in agreement with previous study (Nassi *et al.*, 2009). Significant increase in urinary 8-OHdG excretion in women giving preterm birth (<37 completed weeks) or giving birth to a child with a body weight below 2500 g or growth restriction (<10th percentile) was also reported (Ames *et al.*, 1993). The concentration of 8-OHdG in cord blood can be considered as momentary steady state levels of DNA damage which is sustained by many factors like nature and concentration of ROS, the overall efficiency of the antioxidant defense systems and the efficiency of the DNA repair system directed against 8-OHdG (Ames *et al.*, 1993; Demple and Harrison, 1994).

Lipids and proteins are the most susceptible to oxidation damage. Reaction of ROS with lipid is a highly damaging reaction (lipid peroxidation). Estimation of lipid peroxidation in cord blood has been proposed as a reliable marker of ROS activity in the fetus and a measure of perinatal outcome (Rogers *et al.*, 1997). MDA estimation is accepted as routine method of assessing lipid peroxidation (Del Rio *et al.*, 2005; Mahmoud *et al.*, 2006). We observed significant higher levels of MDA, protein carbonyl along with decreased levels of total antioxidant

status in premature LBW newborns. This observation is in agreement with the pattern observed in previous studies (Saker *et al.*, 2008; Inder *et al.*, 1994). Possible sources of elevated free radicals include increased production of radical oxygen species, especially from lipid peroxidation, protein oxidation processes and decreased antioxidant defense systems (Marjani *et al.*, 2007b).

In this study, we found a significant positive correlation between 8-OHdG and MDA, 8-OHdG and protein carbonyl in cord blood. We also observed a significant negative correlation between biomarkers of oxidative stress (8-OHdG and MDA) and total antioxidant status in cord blood. These data indicate that antioxidants are possibly consumed by the enhanced level of ROS/free radical. El-Bassyouni *et al.* (2006) also emphasized the importance of antioxidant to minimize the effects of oxidative stress. More premature infants with lower birth weight were found to be more vulnerable to oxidative stress in the form of increased DNA damage, protein damage and lipid peroxidation.

The present results demonstrate that the oxidant/antioxidant balance was spoiled in favor of DNA damage, protein damage and lipid peroxidation in preterm low birth weight newborns. The significant increase in the levels of 8-OHdG protein carbonyl and MDA and decrease in the levels of total antioxidant status in cord blood suggest the possible involvement of oxidative stress in preterm low birth weight newborns.

Our findings suggest the possibility that increased oxidative stress (elevated level of 8-OHdG, protein carbonyl and MDA) and decreased total antioxidant status in umbilical cord blood may be associated, at least in part, with neonatal diseases faced by premature LBW newborns. Formation of 8-OHdG is regarded as a useful marker of oxygen radical induced DNA damage. The oxidative DNA damage should be importantly considered because it may change genes and results neonatal diseases.

CONCLUSION

In summary, we found that serum 8-OHdG, protein, carbonyl protein and malondialdehyde, oxidative products of cellular DNA and lipid, were increased with degree of prematurity in LBW newborns along with deficiency in antioxidant defense systems. Antioxidants encounter the increased production of free radicals and to minimize the bad effects of oxidative stress produced (Al-Menabbawy *et al.*, 2006; Uma Devi and Chinnaswamy, 2008). These findings may be helpful to improve the understanding of oxidative stress in neonates and means to combat the situation. Further studies should be carried out to demonstrate the

role of oxidative stress and DNA damage in individual systemic diseases in newborns and the role of antioxidative treatment in prevention of such diseases.

ACKNOWLEDGMENTS

This study was supported by Banaras Hindu University, Varanasi (India). The authors would like to acknowledge Department of Pediatrics, Institute of Medical Sciences, Banaras Hindu University, Varanasi (India) for providing us the sample for the study.

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