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MATERNAL AND FETAL INDICATORS OF OXIDATIVE STRESS DURING INTRAUTERINE GROWTH RETARDATION (IUGR)

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ABSTRACT

The present study demonstrates the possibility of increased lipid peroxidation and protein oxidation in both maternal and fetal erythrocytes as markers of oxygen radical activity during intrauterine growth retardation. The erythrocyte MDA levels were significantly elevated in mothers of IUGR babies when compared to controls ($p<0.01$). The endogenous protein damage due to oxidative stress was significantly higher in IUGR mothers when compared to controls ($p<0.05$). Similarly the proteolytic activity in erythrocyte lysates against oxidatively damaged hemoglobin was significantly increased in mothers of IUGR babies compared to controls ($p<0.001$).

In fetuses born with IUGR, both lipid peroxidation and proteolytic activity were significantly increased when compared to normal newborns ($p<0.01$).

The result of this study indicates that oxidative stress was induced both in IUGR babies and their mothers which is manifested as increased lipid peroxidation and protein oxidant damage.

KEY WORDS

Intrauterine growth retardation, malondialdehyde, proteolytic activity

INTRODUCTION

In newborns, maturation in terms of gestational age may be a major element in the infant's tolerance against the harmful effects of free oxygen radicals (1). It has been observed that failure of maturation of antioxidant defence system in premature infants contributes to the onset and progression of bronchopulmonary dysplasia (2). Oxygen free radicals are highly reactive and are capable of damaging macromolecules like DNA, proteins, polyunsaturated fatty acids and carbohydrates (3, 4, 5, 6, 7). Polyunsaturated fatty acids upon peroxidation produce malondialdehyde. The presence of this oxidation product can be measured with thiobarbituric acid, which correlates with the extent of lipid peroxidation (8). Oxidant damage to proteins could result in changes in the secondary and tertiary conformation of the protein (9). An enzyme system exists in erythrocytes, which degrades oxidatively damaged proteins, thus preventing the accumulation of non-functional proteins and protein fragments (9).

In this study, our objective was to investigate the oxygen free radical activity in the maternal and fetal erythrocytes during intrauterine growth retardation (IUGR). Malondialdehyde (MDA) was measured in erythrocytes of maternal and cord blood to determine lipid peroxidation. Oxidant damage to protein was determined by estimating the amino groups released by proteolytic degradation of oxidatively damaged proteins of cord blood and maternal blood.

MATERIALS AND METHODS

Sample collection

This study included women with single pregnancies delivered at the department of Obstetrics and Gynaecology, Kasturba Medical College Hospital, Manipal. Test group consisted of thirteen pregnant females, who were diagnosed as having fetus with intrauterine growth retardation (IUGR). IUGR is a term applied to infants whose birth weights are much lower than expected. An infant is termed growth retarded or small for gestational age if infant's birth weight falls below the tenth percentile for the expected birth weight at a given gestational age (10). The maternal age

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ranged from 20 to 30 years (mean ± SD = 25.5± 3.41). Gestational age ranged from 33 to 39 weeks (mean ± SD = 36.2 ± 2.82). The maternal and cord blood were collected immediately after delivery in heparinised tubes and stored at 4°C. Erythrocyte malondialdehyde was estimated within 24 hours of blood collection. The hemolysates prepared from the above blood samples were stored at -25°C and proteolytic activities were measured within 2 weeks, during which time interval, the analytes were found to be stable. Similar estimations were done in women with normal healthy pregnancies (control subjects n=15).

Chemicals

DL-dithiothreitol (99%), o-phthalaldehyde (97%), thiobarbituric acid (98%) and N-(2-hydroxy-ethyl)piperazine-N'-(2-ethanesulfonic acid) sodium (NaHEPES;99%) were obtained from Sigma. Malondialdehyde (MDA) was prepared from 1,1,3,3-tetraethoxy propane (97%) from Sigma and used as standard for MDA estimation. DEAE-Sephadex A-50 was obtained from Pharmacia and phenylhydrazine hydrochloride (99%) from Loba Chem India. All other reagents were of analytical grade.

Preparation of oxidatively damaged hemoglobin

Oxidative damage to hemoglobin was induced by treating it with phenylhydrazine (9). Phenylhydrazine, in the presence of hemoglobin, autoxidizes to form both $O_2^{\cdot-}$ and H_2O_2. Hence, it was used to generate oxidatively damaged hemoglobin (11). This oxidatively damaged hemoglobin served as a substrate for the measurement of the proteolytic enzyme activity that degrades oxidatively damaged proteins in erythrocytes.

Hemoglobin used was purified from erythrocyte hemolysates obtained from healthy individuals by anion exchange chromatography on DEAE-Sephadex A-50 (12). Phenylhydrazine hydrochloride, dissolved in water and neutralized with 2M NaOH, was added at a final concentration of 10mM to a solution containing 0.1mM EDTA, 50mM NaHEPES and purified hemoglobin at a concentration of 64 mg/ml (1 mM). After incubation on ice for 6 h, it was dialyzed at 4ºC with 10 volumes of dialysis buffer containing 20 mM $NaHCO_3$ and 20 mM NaCl (pH 8.0) with three changes of buffer. The phenylhydrazine-treated hemoglobin was adjusted to a final concentration of 50 g/l and stored at -25ºC. The above oxidatively damaged hemoglobin was used as substrate for the proteolytic enzymes of the erythrocyte cell-free extracts prepared from the study populations.

Preparation of erythrocyte cell-free extract

Erythrocytes obtained from maternal and cord blood were washed three times in ice-cold saline to remove plasma components and white blood cells. Cell-free extracts were prepared by lysing the erythrocytes in 1.5 volumes of freshly prepared 1 mM DL-dithiothreitol (DTT) (13). Intact cells and membranes were removed by centrifugation (16,000 x g for 20 minutes). The cell-free extracts were adjusted to a hemoglobin concentration of 50g/l using 1 mM DTT and stored at -25°C.

Estimation of erythrocyte proteolytic activity (13)

The erythrocyte contains several proteolytic enzymes, some of which are known to degrade oxidatively damaged hemoglobin. Under the experimental conditions used in this study, when a sample of erythrocyte lysate is incubated with phenylhydrazine-treated hemoglobin at 37°C, the enzymes in the erythrocyte degrade oxidatively damaged hemoglobin and simultaneously any other oxidant damaged protein present in the erythrocyte lysate. The end products of the degradation are a number of smaller peptides, which are TCA soluble and can be measured as an increase in the number of free amino groups. Estimation of free amino groups in erythrocyte lysates before incubation gives an indication of endogenous protein damage due to oxidative stress.

Proteolytic activity in the cell-free extracts was measured as follows. Aliquots of 0.1 ml of the cell-free extracts containing 20 mM phosphate buffer (pH 7.8) and 1mM DTT were incubated with 0.1 ml of phenylhydrazine-treated hemoglobin at 37°C for 3 hours after which the reaction was terminated by the addition of 0.2 ml of 10% trichloroacetic acid (TCA). In parallel, identical samples were treated with 0.2 ml of 10% of TCA before the incubations. Amino groups in the TCA supernatant were determined using o-phthalaldehyde (14). Fluorescence was measured using a SFM 25 Kontron spectrofluorimeter. Alanine was used as a standard for the estimation of amino groups released during proteolytic degradation of oxidatively damaged hemoglobin by the erythrocyte cell-free extract. Standard alanine (6 nmole per 10 □l) was used for calibration of the fluorimeter. Amino group concentrations in the TCA supernatants were calculated from the alanine standard graph. The difference in the amino group concentration before and after incubation was taken as a measure of proteolytic activity in the cell-free extract. Free amino groups (□moles/gram hemoglobin) present in the cell-free extracts before incubation was also measured and considered as the indicator of endogenous protein damage due to oxidative stress.

Estimation of MDA

MDA content of erythrocytes was estimated as thiobarbituric acid reactive substances by spectrophotometric method as described by Jain *et al*. (8). The MDA value was calculated from the MDA

standard graph and expressed as nanomoles/gram hemoglobin. Hemoglobin concentration was estimated as described in (15).

All statistics were calculated using SPSS/PC+, the statistical package for IBM PCs.

RESULTS

The various parameters of oxidative stress were determined in the erythrocytes taken from both maternal and fetal blood from women having normal healthy pregnancies (control group), and in pregnancy with IUGR (test group).

Parameters of erythrocyte oxidative stress which were estimated included malondialdehyde (MDA) and the proteolytic activity of the erythrocytes towards their own oxidatively damaged proteins and towards phenylhydrazine-treated hemoglobin used as substrate.

In the test group, the MDA levels, the amino groups in RBC lysates before incubation and the proteolytic activities in the maternal erythrocytes were significantly higher (Table 2) when compared to the control group ($p<0.01$, $p<0.05$ and $p<0.001$ respectively). Also the MDA levels in the maternal erythrocytes of this group correlated significantly with erythrocyte proteolytic activity ($r=0.54$). In this group, gestational age of the fetuses correlated significantly with proteolytic activity in the fetal erythrocytes ($r=0.52$).

In the test group, the MDA levels in fetal erythrocytes were significantly elevated when compared to controls ($p<0.01$). The proteolytic activity in the fetal erythrocytes was significantly higher than in controls ($p<0.01$).

Table 1. Maternal age (in years), gestational age (in weeks) and birth weight (in kilograms) in normal pregnancies (control) and in patients with IUGR

(All values are mean ± SD)

	Control (n=15)	IUGR (n=13)
Maternal age (years)	26.27±4.23	25.5±3.41
Gestational Age (weeks)	37.9±1.04	36.2±2.82
Birth weight (Kg)	2.9±0.03	2.02±0.36*

* $p<0.05$ in comparison with controls.

Table 2. Indicators of oxidative stress in maternal and fetal erythrocytes. The product of lipid peroxidation, malondialdehyde is expressed in terms of nanomoles per gram of hemoglobin. Protein oxidant damage is estimated by the proteolytic degradation of phenylhydrazine-treated hemoglobin by erythrocyte lysates taken from controls and patients with IUGR. Concentration of free amino groups in the cell-free extracts before incubation indicates endogenous protein damage due to oxidative stress

(All values are mean ± SD)

	Control (n=15)	IUGR (n=13)
Maternal erythrocytes		
MDA (nmoles/g of Hb)	8.06±0.18	12.49±3.17**
Free amino groups present in the cell-free extracts before incubation (□moles/g of Hb)	17.79±5.70	22.98±4.13*
Proteolytic activity (□moles of amino groups/ g of Hb)	10.29±2.71	21.02±4.7***
Fetal erythrocytes		
MDA (nmoles/g of Hb)	7.91±3.94	12.23±2.54**
Free amino groups present in the cell-free extracts before incubation (□moles/g of Hb)	21.13±7.27	24.51±6.47
Proteolytic activity (□moles of amino groups/ g of Hb)	13.65±6.46	21.43±6.97**

*** $p<0.001$, ** $p<0.01$, * $p<0.05$ in comparison with controls.

DISCUSSION

Obstetric complications can result in oxidative stress to the mother and the fetus. We have previously reported increased lipid peroxidation and proteolytic activity in the erythrocytes of both mothers with gestational diabetes and their newborn infants (16) as well as during LSCS, premature rupture of membranes and prolonged second stage of labour (17). Often, fetuses born prematurely may have lowered antioxidant defenses (2). In normal pregnancy, the mean daily growth, as measured by weight, increases until 36 weeks of gestation. Deviation from this results in IUGR. IUGR is caused by maternal conditions like hypertension, chronic renal disease, congenital heart disease and anemia. Placental anomalies like chronic abruptio placenta, chorioangioma and nonspecific villitis can also cause IUGR (10). In 50% of cases, the etiology for IUGR is unknown. Studies have shown that the serum concentration of 8-isoprostane, one of the oxidative stress indices connected with vessel constriction is also increased in women diagnosed as having IUGR (18). It has been reported that during pregnancy complicated by IUGR, MDA concentration in amniotic fluid was almost three times more than in normal pregnancy (19). Studies have also suggested that gravidas with poor pregnancy have increased oxidative damage to their DNA (20). Oxidative DNA damage was reportedly increased in mothers with pregnancy complicated by IUGR (21). Erythrocyte antioxidants were lower and serum malondialdehyde (MDA) was higher in cord blood of term small for gestational age (SGA) newborn infants born to undernourished mothers as compared to term appropriate for gestational age (AGA) infants born to healthy mothers suggesting that intrauterine malnutrition is associated with significant oxidative stress in infants with IUGR (22). Parameters reflecting membrane damage such as MDA, lipid hydroperoxides and conjugated dienes were significantly increased in mothers with IUGR than in normal pregnancy (23). Our finding of increased MDA in erythrocytes of newborns with IUGR supports these findings. Free radical reactions have been evaluated in healthy term infants and critically ill preterm infants (4, 24). In premature newborns, both antioxidative enzymes and the level of scavengers are lower (25, 26). Complications of the newborns such as bronchopulmonary dysplasia, retinopathy of prematurity, subependymal and intraventricular hemorrhage and necrotizing enterocolitis are thought to be due to the increased susceptibility of the newborns to free radical injury (27). Oxidative protein damage measured as plasma carbonyl group levels was reportedly increased and the total antioxidative activity in serum was decreased in mothers with IUGR (28, 29). The present study has demonstrated increased proteolytic activity in erythrocytes of mothers as well as newborns with IUGR, which is also an indicator of excessive oxidative damage to proteins.

In our study, we have observed that lipid peroxidation was significantly increased in fetuses born to IUGR mothers. The birth weight of the babies in this group was lower than those in the control group. The mothers of this group showed elevated MDA, endogenous protein damage and proteolytic activity indicating increased oxidative stress.

In conclusion, oxidative stress is observed in the mother and fetus during intrauterine growth retardation, which has resulted in lipid peroxidation and protein oxidant damage. Further studies are required to examine the impact of this on fetal outcome.

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