MATERNAL AND FETAL INDICATORS OF OXIDATIVE STRESS IN VARIOUS OBSTETRIC COMPLICATIONS

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ABSTRACT

The present study demonstrates the incidence of increased lipid peroxidation and protein oxidation in both maternal and fetal erythrocytes as markers of oxygen radical activity in different complications of pregnancy. In fetuses born after premature rupture of membranes, lipid peroxidation was significantly elevated as indicated by increased malondialdehyde levels ($p < 0.05$) as compared to controls. Proteolytic activity in the erythrocytes of mothers in this group was also significantly high ($p < 0.01$). In patients delivered by lower segment cesarian section, lipid peroxidation and proteolytic activity in maternal erythrocytes were significantly high ($p < 0.05$ and $p < 0.001$ respectively). In patients with prolonged second stage of labour, lipid peroxidation and proteolytic activity in maternal erythrocytes was significantly higher than in controls ($p < 0.001$ and $p < 0.05$ respectively). In this group, endogenous protein damage due to oxidative stress was significantly high both in the mother and the fetus ($p < 0.001$ and $p < 0.05$ respectively).

KEY WORDS

Lower segment cesarian section, malondialdehyde, premature rupture of membranes, prolonged second stage of labour, 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). The observation that the lower birth weight, more premature infants are most prone to develop bronchopulmonary dysplasia (BPD) is consistent with the idea that failure of maturation of antioxidant defence system contributes to the onset and progression of BPD (2). Neonates are said to be more susceptible to oxygen radical injury (19). Oxygen free radicals are highly reactive and potentially damaging to most macromolecules like polyunsaturated fatty acids, proteins, deoxyribonucleic acid and carbohydrates (3,4,5,6,7). Peroxidation of polyunsaturated fatty acids produces 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 proteins and they become increasingly susceptible to proteolytic degradation (9). In the red blood cell particularly, an enzyme system exists which degrades oxidatively damaged proteins, thus preventing the accumulation of non-functional proteins and protein fragments (9). In conditions of excess production of oxygen free radicals leading to oxidative damage to proteins, this enzyme system may become more active in the erythrocytes. Previous studies have reported increased lipid peroxidation in maternal and fetal blood in certain obstetric complications. But there have been no studies on the proteolytic activity in the maternal and cord blood in such conditions.

In this study, our objective was to investigate the oxygen free radical activity in the maternal and fetal erythrocytes in obstetric complications including premature rupture of membranes (PROM), prolonged second stage of labour and lower segment cesarian section (LSCS). Malondialdehyde (MDA) was

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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. Thirteen patients had premature rupture of membranes. Maternal age ranged from 20 to 30 years (mean $\pm$ SD = 26.03 ± 4.2). Gestational age ranged from 32 to 41 weeks (mean $\pm$ SD = 36.86 ± 3.4). Nine patients were delivered by LSCS. Maternal age ranged from 22 to 32 years (mean $\pm$ SD = 27.34 ± 4.1). Gestational age ranged from 34 to 42 weeks (mean $\pm$ SD = 39.61 ± 2.7). Nine patients had prolonged second stage of labour. Maternal age ranged from 22 to 35 years (mean $\pm$ SD = 25.62 ± 3.72) and gestational age ranged from 37 to 39 weeks (mean $\pm$ SD = 38.62 ± 1.2). In all cases, samples of 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. All the estimations were also done in control (n=15) subjects.

Chemicals:

DL-dithiothreitol (99%), o-phthalaldehyde (97%), thiobarbituric acid (98%) and N-(2-hydroxyethyl)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 and H_2O_2 . Hence, it was used to generate oxidatively damaged hemoglobin(10). 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(11). 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 (1mM). After incubation on ice for 6 h, it was dialyzed at 4°C with 10 volumes of dialysis buffer containing 20mM $NaHCO_3$ and 20mM 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 -25°C. 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) (12). 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

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 (13). Fluorescence was measured using a SFM 25 Kontron spectrofluorimeter. 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 (μ moles/gram hemoglobin) present in the cell-free extracts before incubation were 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) and hemoglobin concentration was estimated as described in (14).

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), from patients having premature rupture of membranes, from patients following LSCS and from patients with prolonged second stage of labour.

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. All parameters were correlated with

gestational age and birth weight of the fetus.

In the group of patients with premature rupture of membranes, the MDA levels in the fetal erythrocytes were significantly higher ($p<0.05$) when compared to the control group (Table 2). The proteolytic activity in the maternal erythrocytes was significantly increased compared to controls ($p<0.01$). The birth weight of the babies in this group was significantly lower ($p<0.05$) than the babies in the control group (Table 1).

In the group of patients who underwent LSCS, the MDA levels and the proteolytic activities in the maternal erythrocytes were significantly higher (Table 2) when compared to the control group ($p<0.05$ and $p<0.001$ respectively). Also the MDA levels in the maternal erythrocytes of this group correlated significantly with erythrocyte proteolytic activity ($r=0.74$). In this group gestational age of the fetuses correlated significantly with proteolytic activity in the fetal erythrocytes ($r=0.54$).

In the group of patients with a prolonged second stage of labour, the MDA level in the maternal erythrocytes was significantly elevated (Table 2) when compared to controls ($p<0.001$). The MDA levels in the maternal erythrocytes correlated significantly with the MDA levels in the fetal erythrocytes ($r=0.64$) and with proteolytic activity in the maternal erythrocytes ($r=0.65$). The quantity of free amino groups in the erythrocytes before incubation was significantly higher than in controls, both in the maternal and fetal erythrocytes ($p<0.001$ and $p<0.05$ respectively) of this group. The proteolytic activity in the maternal erythrocytes was significantly higher than in controls ($p<0.05$). The quantity of free amino groups in the fetal erythrocytes before incubation correlated significantly with MDA levels in the fetal erythrocytes ($r=0.88$, $p<0.001$). The birth weight of the fetuses in this group correlated significantly with proteolytic activity in the fetal erythrocytes ($r=0.75$).

DISCUSSION

Various 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 (15). Often, fetuses born prematurely may have lowered antioxidant defenses (2). In previous studies, free radical reactions have been evaluated

in healthy term infants and critically ill preterm infants (4,16). Preterm infants expressed a higher rate of lipid peroxidation during the first week of life than did term infants. In premature newborns, both antioxidative enzymes and the level of scavengers are lower (17,18). Some complications in the newborns, like bronchopulmonary dysplasia, retinopathy of prematurity, subependymal and intraventricular hemorrhage and necrotizing enterocolitis are thought to be mainly due to the increased susceptibility of the neonates to oxygen radical injury (19).

An increase in lipid peroxidation in the maternal and cord blood after premature rupture of membranes has been reported (20). It has also been reported that generation of reactive oxygen species may be a potentially reversible pathophysiologic pathway leading to premature rupture of membranes (21). In our study, we have observed that lipid peroxidation was significantly increased in fetuses born after premature rupture of membranes which agrees with previous reports. The birth weight of the babies in this group was lower than those in the control group. The mothers of this group showed elevated proteolytic activity which might reflect increased oxidative damage to proteins.

In the LSCS group, fetal oxidative stress was minimal. This is consistent with the previous findings that high levels of free oxygen radical activity are a function of the labour process and that there is no increase in lipid peroxidation levels in the cord blood

of fetuses delivered by LSCS (22). However, maternal oxidative stress levels were higher as indicated by increased MDA and proteolytic activity. Gestational age correlated well with proteolytic activity in the fetal erythrocytes, which may indicate a certain degree of enzyme maturity.

Oxygenation of both maternal and fetal tissues oscillates frequently during labour. As a consequence of tissue reoxygenation during labour, reactive oxygen species are produced which might explain the oxidative stress reported in mothers and fetuses in cases of prolonged second stage of labour (23,24). In our studies involving the group of patients with prolonged second stage of labour, lipid peroxidation and erythrocyte proteolytic activity in the mothers was significantly high. Endogenous protein damage due to oxidative stress in this condition was significantly increased both in the mother and the fetus as indicated by increased quantity of free amino groups before incubation. Birth weight of the babies in this group correlated well with their proteolytic activity, which may indicate a certain degree of enzyme maturity.

In conclusion, oxidative stress is observed in the mother and fetus in certain obstetric complications. This oxidative stress results in lipid peroxidation and protein oxidant damage. Further studies are required to examine the impact of this on fetal outcome.

Table 1

Maternal age (in years), gestational age (in weeks) and birth weight (in kilograms) in normal pregnancies (control), in patients having PROM, in patients following LSCS and in patients with prolonged second stage of labour.

(All values are mean $\pm$ S.D.)

	Control (n=15)	PROM (n=13)	LSCS (n=9)	Prolonged second Stage of labour (n=9)
Maternal age (years)	26.27 $\pm$ 4.23	26.03 $\pm$ 4.2	27.34 $\pm$ 4.1	25.62 $\pm$ 3.72
Gestational Age (weeks)	37.87 $\pm$ 1.36	36.86 $\pm$ 3.4	39.61 $\pm$ 2.7	38.62 $\pm$ 1.2
Birth weight (Kg)	2.85 $\pm$ 0.13	2.42 $\pm$ 0.19*	2.65 $\pm$ 0.48	2.98 $\pm$ 0.56

* 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 having PROM, patients who underwent LSCS and from patients with prolonged second stage of labour. Concentration of free amino groups in the cell-free extracts before incubation indicates endogenous protein damage due to oxidative stress.

(All values are mean $\pm$ S.D.)

	Control (n=15)	PROM (n=13)	LSCS (n=9)	Prolonged second Stage of labour (n=9)
Maternal erythrocytes				
MDA (nmoles/g of Hb)	8.06 $\pm$ 0.18	9.12 $\pm$ 3.84	10.98 $\pm$ 0.94*	13.45 $\pm$ 2.24***
Free amino groups present in the cell-free extracts before incubation (μ moles/g of Hb)	18.34 $\pm$ 4.86	19.54 $\pm$ 9.85	20.84 $\pm$ 7.87	32.56 $\pm$ 5.6***
Proteolytic activity (μ moles of amino groups/g of Hb)	10.18 $\pm$ 1.84	14.56 $\pm$ 4.7**	19.63 $\pm$ 1.2***	14.48 $\pm$ 3.4*
Fetal erythrocytes				
MDA (nmoles/g of Hb)	6.82 $\pm$ 3.75	10.56 $\pm$ 2.6*	6.45 $\pm$ 2.36	9.65 $\pm$ 2.45
Free amino groups present in the cell-free extracts before incubation (μ moles/g of Hb)	21.94 $\pm$ 6.37	22.32 $\pm$ 9.7	24.35 $\pm$ 15.6	33.71 $\pm$ 1.8*
Proteolytic activity (μ moles of amino groups/g of Hb)	14.64 $\pm$ 6.21	16.5 $\pm$ 8.31	21.3 $\pm$ 8.92	18.34 $\pm$ 3.2

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

REFERENCES

1. Frank, L. and Sosenko, I. (1991) Failure of premature rabbits to increase antioxidant enzymes during hyperoxic exposure. Increased susceptibility to pulmonary oxygen toxicity compared with term rabbits. *Pediatr. Res.* 29, 292-296.
2. Frank, L. and Sosenko, I. (1987) Development of lung antioxidant enzyme system in late gestation: Possible implications for the prematurely born infant. *J. Pediatr.* 110, 9-14.
3. Wang, W., Pang, C., Rogers, M. and Chang, A. (1996) Lipid peroxidation in cord blood at birth. *Am. J. Obstet. Gynecol.* 174, 62-65.
4. Pitkanen, O., Haliman, M. and Andersson, S. (1990) Correlation of free oxygen radical induced lipid peroxidation with outcome in very low birth weight infants. *J. Pediatr.* 116, 760-764.
5. Halliwell, B. and Gutteridge, J.M.C. (1989) *Free radicals in biology and medicine*. 2nd ed. Oxford: Clarendon Press.
6. Dizdaroglu, M. (1993) Chemistry of free radical damage to DNA and nucleoproteins. In: Halliwell, B. and Aruoma, O.I. eds. *DNA and free radicals*. Chichester: Ellis Harwood, 19-39.
7. Wolff, S.P., Garner, A. and Dean, R.T. (1986) Free radicals, lipids and protein degradation. *Trends Biochem. Sci.* 11, 27-31.
8. Jain, S.K. (1989) Hyperglycemia can cause membrane lipid peroxidation and osmotic fragility in human red blood cells. *J. Biol. Chem.* 254, 21340-21345.
9. Fagan, J., Waxman, C. and Goldberg, A.C. (1986) Red blood cells contain a pathway for the degradation of oxidant damaged hemoglobin that does not require ATP or ubiquitin. *J. Biol. Chem.* 261, 5705-5713.
10. Jain, S.K. and Hochstein, P. (1979) Generation of superoxide radicals by hydrazine: its role in phenylhydrazine induced hemolytic anemia. *Biochim. Biophys. Acta* 586, 128-36.
11. Riggs, A. (1981) Preparations of blood hemoglobins of vertebrates. *Methods Enzymol.* 76, 5-29.
12. Raghothama, C. and Rao, P. (1994) Increased proteolysis of oxidatively damaged hemoglobin in erythrocyte lysates in diabetes mellitus. *Clin. Chim. Acta* 225, 65-70.
13. Peterson, G.L. (1983) Determination of total protein. *Methods Enzymol.* 91, 95-119.
14. Salvati, A.M. and Tentori, L. (1981) Determination of aberrant hemoglobin derivatives in human blood. *Methods Enzymol.* 76, 715-731.
15. Kamath, U., Rao, G., Raghothama, C., Rai, L. and Rao, P. (1998) Erythrocyte indicators of oxidative stress in gestational diabetes. *Acta Paediatr.* 87, 676-9.
16. Wispe, J.R., Bell, E.F. and Roberts, R.J. (1985) Assessment of lipid peroxidation in newborn infants and rabbits by measurements of expired ethane and pentane: influence of parenteral infusion. *Pediatr. Res.* 19, 374-379.
17. Smith, C.V., Hansen, J.N., Martin, N.E., McMicken, H.W. and Elliot, S.J. (1993) Oxidant stress responses in premature infants during exposure to hyperoxia. *Pediatr. Res.* 34, 360-365.
18. Sullivan, J.N. and Newton, A.B. (1988) Serum antioxidant activity in neonates. *Arch. Dis. Child* 63, 748-57.
19. Sullivan, J.L. (1988) Iron, plasma antioxidants and the "oxygen radical disease of prematurity". *A.J.D.C.* 142, 1341-1344.
20. Yin, B. and Zhen, M. (1995) Lipid peroxidation in plasma and the activity of superoxide dismutase (SOD) in pregnant women with premature rupture of membrane. *Chung-Hua-I-Hsueh-Tsa-Chih* 75(8), 463-5.
21. Woods, J.R. Jr., Plessinger, M.A. and Miller, R.K. (2001) Vitamins C and E: missing links in preventing preterm premature rupture of membranes ? *Am. J. Obstet. Gynecol.* 185(1), 5-10.
22. Rogers, M.S., Mongelli, J.M., Tsang, K.H., Wang, C.C. and Law, K.P. (1998) Lipid peroxidation in cord blood at birth : the effect of labour. *Br. J. Obstet. Gynaecol.* 105(7), 739-44.
23. Nakai, A., Oya, A., Kobe, H., Asakura, H., Yokota, A., Koshino, T. and Araki, T. (2000) Changes in maternal lipid peroxidation levels and antioxidant enzymatic activities before and after delivery. *J. Nippon Med. Sch.* 67(6): 434-9.
24. Mongelli, M., Wang, C.C., Wang, W., Pang, C.C. and Rogers, M.S. (1997) Oxygen free radical activity in the second stage of labor. *Acta Obstet. Gynecol. Scand.* 76(8), 765-8.