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MATERNAL AND FETAL INDICATORS OF OXIDATIVE STRESS DURING PREGNANCY-INDUCED HYPERTENSION (PIH)

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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 pregnancy induced hypertension (PIH). The erythrocyte malondialdehyde (MDA) levels were significantly elevated in mothers with PIH when compared to controls ($p<0.001$). The endogenous protein damage due to oxidative stress was significantly higher in mothers with PIH when compared to controls ($p<0.01$). Similarly the proteolytic activity in erythrocyte lysates against oxidatively damaged hemoglobin was significantly increased in mothers with PIH compared to controls ($p<0.001$).

In babies born to mothers with PIH, erythrocyte MDA levels were significantly elevated in comparison those of normal newborns ($p<0.01$). Both the endogenous oxidative protein damage and erythrocyte proteolytic activity were significantly higher in newborns born to mothers with PIH than in newborns in the control group ($p<0.01$).

The results of this study indicate that oxidative stress is induced both in mothers with PIH as well as their babies which is manifested as increased lipid peroxidation and protein oxidant damage.

Key words: Pregnancy induced hypertension (PIH), malondialdehyde, proteolytic activity

INTRODUCTION

Hypertension in pregnancy is a leading cause of both maternal and fetal morbidity and mortality. Risks to the fetus include premature delivery, growth retardation and death (Garovic VD, 2000). The physiopathology of preeclampsia is still unclear, but an imbalance between reactive oxygen species (ROS) and antioxidants, also called oxidative stress, appears to be an important contributing factor (Boutet M et al, 2009). Preeclampsia was seen to negatively influence fetal lung maturity (Winn HN et al, 2000). It has been observed that failure of maturation of antioxidant defence system in premature infants contributes to the onset and progression of bronchopulmonary dysplasia (Frank L et al, 1987). Increased ROS interact with cellular macromolecules such as DNA, lipid, and proteins to produce oxidized DNAs, lipid peroxides, and oxidized proteins, respectively, and usually negatively affect their physiological functions (Song BJ et al, 2010). Polyunsaturated fatty acids upon peroxidation produce malondialdehyde (MDA). The presence of this oxidation product can be measured with thiobarbituric acid, which correlates with the extent of lipid peroxidation (Jain SK, 1989). Oxidant damage to proteins could result in changes in the structural conformation of the protein. In erythrocytes, an enzyme system exists, which degrades oxidatively damaged proteins, thus preventing the accumulation of non-functional proteins and protein fragments (Fagan JM et al, 1986).

In the present study, our objective was to investigate the oxygen free radical activity in mothers who were diagnosed as having PIH and their newborns. 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 maternal and cord blood and the proteolytic activity in the erythrocytes of maternal and cord blood towards an oxidatively damaged hemoglobin substrate.

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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. Study group consisted of fourteen pregnant females, who were diagnosed as having pregnancy-induced hypertension (PIH). Any increase over 140 mm of Hg systolic and 90 mm of Hg diastolic blood pressure must be viewed as an indicator of pre-eclampsia (Mudaliar et al, 1995). The maternal age ranged from 20 to 30 years (mean ± SD = 27.5± 3.71). Gestational age ranged from 34 to 39 weeks (mean ± SD = 37.6 ± 3.61). The maternal and cord blood were collected immediately after delivery in heparinised tubes and stored at 4°C. Erythrocyte MDA 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 (controls 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 (St Louis, MO). Malondialdehyde 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 (Uppsala, Sweden) 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 (Fagan JM et al, 1986). Phenylhydrazine, in the presence of hemoglobin, autoxidizes to form both $O_2^{\bullet-}$ and H_2O_2. Hence, it was used to generate oxidatively damaged hemoglobin (Jain SK et al, 1979). 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 (Riggs A, 1981). 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 4ºC with 10 volumes of dialysis buffer containing 20mM NaHCO3 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 -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) (Raghothama C et al, 1994). 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 (Peterson GL, 1983). 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 µl) 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 (µmoles/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 SK, 1989). The MDA value was calculated from the MDA standard graph and expressed as nanomoles/gram hemoglobin. Hemoglobin concentration was estimated as described in (Salvati AM et al, 1981).
All statistics were calculated using SPSS/PC+ statistical package.

RESULTS

The parameters of oxidative stress that were determined in the erythrocytes taken from both maternal and fetal blood from women having normal healthy pregnancies (control group), and pregnancy with hypertension (PIH group) 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 PIH 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 ($p<0.001$,$p<0.01$ and $p<0.001$ respectively). In the newborns born to mothers with PIH, the MDA levels in the erythrocytes were significantly elevated when compared to controls ($p<0.01$). The level of free amino groups before incubation and the proteolytic activity in the fetal erythrocytes were significantly higher than in controls ($p<0.01$) (Table 2). Also the birth weight of the babies born to mothers with PIH was significantly lower than those born to mothers in the control group ($p<0.05$) (Table 1).

Table 1: Maternal age (in years), gestational age (in weeks) and birth weight (in kilograms) in normal pregnancies (control) and in patients with PIH. (All values are mean ± SD).

	Control (n=15)	**PIH (n=14)**
Maternal age (years)	27.36±5.64	27.5± 3.71
Gestational Age (weeks)	37.8±1.25	37.6 ± 3.61
Birth weight (Kg)	2.96±0.22	2.12±0.39*

* $p<0.05$ 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 mothers with PIH. 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)	**PIH** (n=14)
Maternal erythrocytes		
MDA (nmoles/g of Hb)	8.06±0.18	15.28±3.44***
Free amino groups present in the cell-free extracts before incubation (µmoles/g of Hb)	17.67±3.06	22.07±4.00**
Proteolytic activity (µmoles of amino groups/g of Hb)	10.29±2.71	18.86±3.5***
Fetal erythrocytes		
MDA (nmoles/g of Hb)	7.91±3.94	11.75±2.39**
Free amino groups present in the cell-free extracts before incubation (µmoles/g of Hb)	18.7±3.02	22.03±3.44**
Proteolytic activity (µmoles of amino groups/g of Hb)	13.25±3.26	17.35±4.21**

*** $p<0.001$, ** $p<0.01$ 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 (Kamath U et al, 1998), during LSCS, premature rupture of membranes and prolonged second stage of labour (Guruprasad Rao et al, 2003) and during intrauterine growth retardation (Kamath U et al, 2006). Often, fetuses born prematurely may have lowered antioxidant defenses (Frank L et al, 1987). Preeclampsia can have significant impact on health of both mother and fetus. It has been proposed that maternal endothelial cell dysfunction is the key event resulting in the diverse clinical manifestations of preeclampsia. Research in recent times is indicative of the role of oxidative stress in the endothelial cell dysfunction. It has been reported that MDA levels were increased and antioxidant enzymes activities were decreased in erythrocytes with increase in age in both preeclamptic and normal pregnant women suggesting that an increase in the risk of preeclampsia with maternal age could be due to an increase in oxidative stress with age (Haque SK et al, 2010).

Uncontrolled lipid peroxidation may play an important role in the pathophysiology of preeclampsia and eclampsia by causing vascular endothelial cell dysfunction (Patil SB et al, 2009). An earlier study has reported increased plasma MDA and decreased levels of nitric oxide and adrenomedullin in pregnant women with preeclampsia suggesting it might contribute to the pathophysiology of preeclampsia through the lack of a paracrine vasodilatory effect on uteroplacental blood flow (Dikensoy E et al, 2009). Increased levels of MDA and reduced enzymatic antioxidants activities were reported in pregnant women with preeclampsia demonstrating the presence of oxidative stress (Patil SB et al, 2007). In the present study, we have observed a significant increase in malondialdehyde levels in the erythrocytes of mothers with PIH indicating increased peroxidation of lipids and this agrees well with previous reports.

The present study has also demonstrated increased endogenous oxidative protein damage in erythrocytes of mothers diagnosed as having PIH as well as their newborns compared to controls. In addition, the proteolytic activity in erythrocyte lysates against oxidatively damaged hemoglobin was significantly increased in mothers with PIH as well as their newborns compared to controls. This is the first such report of increased proteolytic activity in erythrocytes and is an evidence for accumulation of oxidative damage to proteins in the erythrocytes of both mothers with PIH and their newborns demonstrating the presence of oxidative stress. Earlier workers have reported a decrease in total antioxidant status and an increase in protein oxidation markers in pregnant women with preeclampsia compared to normal controls (Karacay O et al, 2010).

In our study, we have observed that lipid peroxidation was significantly increased in fetuses born to mothers with PIH. The birth weight of the babies in this group was lower than those in the control group. It has been reported earlier that expression of stress proteins such as heme oxygenase 1 and Hsp-70 and the antioxidant enzyme glutathione peroxidase was significantly higher in both fetal and maternal circulations of the preeclamptic group indicating that preeclampsia is associated with a specific antioxidant response in both maternal and fetal circulations, likely in response to the deleterious oxidative stress observed in this syndrome (Boutet M et al, 2009). The demonstration of oxidant damage to proteins and lipids in both maternal and fetal erythrocytes seen in the present study supports the view that oxidative processes are involved in the pathophysiology of preeclampsia.

In conclusion, oxidative stress is observed in the mothers with PIH and their newborns which results in lipid peroxidation and protein oxidant damage. Further studies are required to examine the impact of this on fetal outcome.

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