Full Length Research Paper

Maternal oxidative stress and enzymatic antioxidant status in premature rupture of membranes

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Premature rupture of the fetal membrane is a major cause of preterm birth and it is associated with infant morbidity. To examine the relationship between maternal oxidative stress (OS) and antioxidant statue and premature rupture of membranes (PROM). 30 pregnant women with PROM and 30 normal pregnant women were compared for maternal malonaldihyde concentration, erythrocyte glutathione (GSH) concentration, glutathione peroxidase (G-PX) and catalase (CAT) activities, using a cross sectional study and spectrophotometer method. Unpaired t test and Chi-square test were used for comparison of variables between controls and patient groups. A significant reduction was found in fthe GSH concentration of the PROM subjects when compared to the controls (6.40±0.43 µmol/g of Hb versus 7.28±0.40 µmol/g of Hb, P <0.001). The G-PX and CAT activities of the PROM population were significantly lower than controls (35±9.41 versus 44.17±11.92 U/g Hb, P=0.01 and 149.83±31.86 U/g of Hb versus 172.07±47.14U/g of Hb, P<0.01, respectively). The maximal rate of oxidation (Vmax) was shorter in the PROM group when compared to the control group (0.0068±0.00003 versus 0.008±0.00003, OD245 nm/min, P=0.01), whereas the maximal accumulation of absorbing products (ODmax) of the PROM subjects was higher than in the controls (0.73±0.013 versus 0.68±0.017 OD245 nm, P=0.01). The lag phase, reflecting resistance of serum lipids to oxidation, was different in the PROM group when compared to the control group (47.90±2.13 versus 53.53±2.54 min, P=0.01). The maternal malondialdehyde (MAD) percentage of the PROM group was higher than controls (75.62±3.73% versus 63.80±9.32%, P=0.001). Present study revealed that PROM is associated with decreased maternal erythrocyte antioxidant activities. As opposed to term labor, PROM is associated with increased maternal systemic OSwhen compared to normal pregnant women. The role for OSin preterm PROM warrants further studies.

Key words: Oxidative stress, antioxidant, premature, rubture, membrane.

INTRODUCTION

Premature rupture of the fetal membrane (PROM) is a major cause of preterm birth, accounting for 30 to 40% of all preterm births (Nourse and Steer, 1997) and its

association with infant morbidity and mortality (Nourse and Steer, 1997; Woods, 2001). PROM results initially from damage to collagen in the chorioamnion leading to a tear in the membrane (Nourse and Steer, 1997). Multiple epidemiological and clinical factors are considered to be promoters of PROM (Ramkumar and Stephen, 2007). Numerous risk factors are associated with PROM. There appears to be no single etiology of preterm PROM

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(Tanya et al., 2006). It has been shown that issue-damaging molecules called reactive oxygen species (ROS) are capable of damaging collagen in the chorioamnion that could lead to PPROM. This hypothesis was supported by epidemiological studies linking clinical conditions known to produce ROS or reduce antioxidant protection to Preterm premature rupture of membranes (PPROM), by *in vitro* studies in which membrane segments exposed to ROS exhibited tissue alterations consistent with PPROM, and by clinical studies showing that chorioamnion and amniotic fluid samples obtained from PPROM patients exhibit excessive collagen degradation (Nourse and Steer, 1997). It has been shown that (Ofer et al., 2007) oxygen free radicals, which are by products of inflammatory cells, increase the activity of matrix metalloproteinases in human fetal membranes. They suggested that oxygen free radicals, which are by products of inflammatory cells, increase the activity of matrix metalloproteinases in human fetal membranes.

This may cause degradation of the extracellular matrix, leading to rupture of the amniotic membranes. PROM is associated with elevated activity metalloproteinase in the amniotic fluid and amniochorionic membranes to which OSmay contribute (Nourse and Steer, 1997). It has also been shown (Fainaru et al., 2002) that active labor is associated with oxidative stress. However it is not known whether OStriggers labor or is an epiphenomenon and a byproduct of the labor process. The damage which is inflicted to tissues by reactive oxygen species can be widespread. ROS are capable of initiating lipid peroxidation, increasing intracellular free Ca^{2+}, damaging deoxyribonucleic acid (DNA), releasing destructive catalytic enzymes and damaging cell membranes (Nourse and Steer, 1997). Clinical conditions, in addition to infection, which frequently are linked to PROM fit with an ROS-induced mechanism of tissue damage. (Nourse and Steer, 1997). Lipid peroxidation is normal phenomenon that occurs continuously at low level in all animals. These peroxidation reactions are in part toxic to cells and cell membranes; however they are normally controlled by countervailing biological mechanisms (Akihito et al., 2006). Antioxidant enzymes G-PX, CAT, superoxidase dismutase (SOD) and GSH appear to be the key antioxidants that provide cell protection against oxidant agents generated in different conditions.

The GPx and SOD enzymes acting as free radical scavengers limit the effects of oxidant molecules in tissues and oxidative injury. These enzymes work together to eliminate active oxygen species and small deviations in their physiological concentrations may have an adverse effect on the resistance of cellular lipids, proteins and DNA to oxidative damage (Fallah et al., 2011). The role of antioxidants to protect the

chorioamnion from ROS damage has been demonstrated in one *in-vitro* study. The result of a study conducted by Woods (2001) showed that a prospective, randomized blinded trial of antioxidant therapy during pregnancy is needed to evaluate this approach for the prevention of PPROM (Nourse and Steer, 1997). Normally a balance exists between production and elimination of ROS. OS occurs when prooxidants exceed antioxidants and the balance between antioxidant defenses is disturbed (Singh et al., 1998). SOD, G-Px, CAT and GSH constitute a team of antioxidant which provide defense against free radical mediated injury. The present study was conducted to assess the erythrocyte antioxidant enzymes G-Px, CAT activities and GSH concentration in PROM. In this study we tested "*in vivo*" the hypothesis that the antioxidant reduction and OS elevation associated with preterm PROM. In addition to measurement of the malonaldihyde levels as an OS index, we used an assay to determine serum lipid oxidizibility in pregnant women experiencing PROM and compared to pregnant women with intact membranes for investigation whether PROM is associated with OS.

PATIENTS AND METHODS

The study group consisted of 30 pregnant women aged 30±2.1 between 24 to 34 gestational weeks with PROM and 30 women aged 30±3.01 with normal pregnancy and intact membranes who had been admitted to the delivery unit of Shahid Akbar Abadi hospital of Southern Tehran because of a threatening preterm delivery with intact fetal membranes. Women in both groups were non-smokers, and were not taking any medications aside from iron supplementation, they were in good general health with no history of prior adverse pregnancy outcomes including preterm labor and delivery or premature rupture of the membranes, and known date of last menstrual period; more than 16 weeks of gestation at the beginning of the study. Women in both groups were not experiencing uterine contractions and did not show clinical or laboratory signs of chorioamnionitis (that is, uterine tenderness, systemic fever, fetal tachycardia or elevated white blood cell count). The groups were matched for age and gestational age and were of similar height and weight (Not shown). Gestational age was determined by the best obstetric estimate based on a combination of the last menstrual period dating and the earliest available ultrasonographic examination.

Exclusion criteria were as follows: Chronic diseases, (hypertension, diabetes, renal or cardiac diseases), genital tract anomalies of the mother, genetic or anatomical defects of the fetus, previous preterm deliveries. PROM diagnosis was identified by estimation the value of insulin-like growth factor-binding protein-1 (IGFBP–1) in cervical secretion in women with symptoms of preterm delivery and to investigate correlation of this test to the Bishop score in prediction of the preterm delivery. The concentration of IGFBP-1 in cervical mucus was estimated by an Actim Partus test (Medix Biochemica, Finland) according manufacturer's instructions. The test was considered positive if the concentration of IGFBP-1 in cervical mucus was higher than 10 µg/L. All experimental procedures were approved by the Human

Table 1. Antioxidant activities in women with PROM and women with normal pregnancy.

	Normal pregnancy	PROM	P
CAT (κ/g of Hb)	172.07±47.14	149.83±31.86	< 0.001
G-Px (U/g of Hb)	44.17±11.92	35.93±9.41	< 0.005
GSH (μmol/g of Hb)	7.28±0.40	6.40±0.43	< 0.001

Values are means±sd.

Table 2. Kinetic parameters of copper induced peroxidation in women with PROM and women with normal pregnancy.

	P	PROM (n=30)	Normal pregnancy (n=30)
Lag preceding oxidation (min)	0.01	47.90±2.13	53.53±2.54
t max (min)	0.01	90.67±3.48	97.80±3.13
V max (OD 245 /min)	0.01	0.0068±3×10-4	0.008±10-4
OD max (OD 245 nm)	0.01	0.73±0.013	0.68±0.017
OD initial (245 nm)	0.01	1.38±0.019	1.33±0.019

Values are mean±sd.

Ethics Committee of the Iran Health Ministry. Venous blood was drawn from each woman and serum was prepared, frozen immediately and stored at -80º C. Blood drawn from women in the PROM group was obtained no longer than 6 h after the onset of membrane rupture. The Hemoglobine (Hb) concentration was determined in 10-fold diluted hemolysate (by the cyanmethemoglubine) with Drabkin's reagent (200 mg $FeCN_6k_3$ + 50 mg CNk /Lit).

The erythrocyte antioxidant enzymes G-PX and CAT activities were assayed as described by Pagila and Valentin (1967) and Aebi (1984), respectively. The enzyme activities were reported as unit per gram of Hb. For G-PX activity, the decrease in nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) absorbance was followed at 340 nm using a spectrophotometer (Cecil CE 9050 Modle, Germany). CAT activity was determined at 25°C by decrease in absorbance of HO at 240 nm Aebi (1984). The erythrocyte GSH concentration was measured spectrophotometrically by the method of Buteler et al. (1963). GSH concentration was measured in 5- sulphosalicylic acid-deproteinized samples. The rate of 2-nitro-5-thiobenzoic acid formation, which is proportional to the GSH present, is followed at 412 nm. Samples were assayed rapidly to minimize GSH oxidation (Beutler et al., 1963).

For OS assay, kinetics of copper-induced oxidation of serum lipids *ex vivo* for evaluating of oxidizibility of blood lipids (Ofer et al., 2007). The kinetic profiles were analyzed in terms of the lag preceding oxidation reflecting the resistance of serum lipids to oxidation, the maximal rate of accumulation of absorbing products (Vmax) as computed from the first derivative of the time course of absorption and the maximal accumulation of absorbing products (OD max) (Ofer et al., 2007). A method based on measurement of the oxidation products such as MAD as a plasma index of lipid peroxidation (Purnima et al., 2006) was also used for OS measurement. The absorbance of the sample was determined at 535 nm (using a spectrophotometer Ultrosec 300, Pharmacia Biotech England double-beam) against a blank that contained all the reagents minus the serum. The MAD concentration of the samples could be calculated using an extinction coefficient of

$1.56×10^5 M^{-1} cm^{-1}$ (Purnima et al., 2006).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used to assess the normality of data distribution. Normally distributed variables were presented as mean and standard deviation (±SD). The t test for independent samples was used for comparison of continuous variables, as appropriate. The difference of proportions between COC users and non-COC users was assessed by $\chi 2$ test. A p value ≤0.05 was considered significant.

RESULTS

Results of present investigation are shown in Tables 1 to 3. As it shown in Table 1 the erythrocyte G-PX activity of the PROM population was decreased significantly (-18%, P<0.005) as compared to controls. The mean G-PX activity of the PROM and normal pregnant groups was 35.93±9.41 and 44.17±11.92 U/g of Hb, respectively. The results of same comparison for erythrocyte CAT activity of the controls (172.07±47.14 U/g of Hb) and PROM group (149.83±31.86 U/ g of Hb) revealed a significant decrease (-20%, P≤0.001) in the PROM subjects. The erythrocyte GSH level of PROM subjects (6.40±0.43 μmol/g of Hb) was significantly lower (-12%, P≤0.001) than that in the normal pregnant group (7.28±0.40 μmol/g of Hb).

As shown in Table 2 the maximal rate of oxidation (Vmax) was shorter in the PROM group when compared

Table 3. Malonaldihyde concentration in women with PROM and women with normal pregnancy.

	Normal pregnancy (n=30)	PROM (n=30)	P
MDA-I(nmol/gofHb)	395.47±89.84	561.63±76.54	0.001
MDA-C(nmol/gof Hb)	257.73±85.94	427.17±76.54	0.001
MDA%	63.80±9.32	75.62±3.73	0.001

Values are means ± sd. MAD-I = MAD in the presence of CAT inhibitor (sodium azide as CAT inhibitor) MAD-C = MAD in the absence of CAT inhibitor. MAD%= the maximum of released MAD.

to the control group (0.0068±0.00003 versus 0.008±0.00003, OD245 nm/min, P=0.01), whereas the maximal accumulation of absorbing products (ODmax) of the PROM subjects was higher than in the controls (0.73±0.013 versus 0.68±0.017 OD245 nm, P=0.01). The lag phase, reflecting resistance of serum lipids to oxidation, was different in the PROM group when compared to the control group (47.90±2.13 versus 53.53±2.54 min, P=0.01). The maternal MAD percentage (Table 3) of the PROM group was higher than in the controls (75.62±3.73% versus 63.80±9.32%, P=0.001). All the data were analyzed by "SSP software" (version 16, for Windows). Continuous variables were compared by the two-tailed t test and two-tailed X². P<0.05 was considered significant.

DISCUSSION

The rupture of the fetal membranes before the onset of regular uterine contraction at preterm labor takes place in some 8 to 10% of pregnancies. This can result in an increased rate of maternal and fetal infection (Bryant-Greenwood and Millar, 2000). The results of present study demonstrated that the maternal antioxidant levels in erythrocyte decrease significantly in PROM subjects as compared to the normal pregnant population. Several studies have attempted to evaluate the antioxidants of maternal healthy pregnant and PROM pregnant women (Akihito et al., 2006). There are conflicting reports regarding to antioxidants levels changes throughout the gestation. G-PX enzyme, a component of antioxidant system, is decreased during pregnancy (Akihito et al., 2006). However, Akihito et al. (2006) showed an elevation in SOD activity throughout the normal pregnancy. In a study, Purnima et al. (2006) revealed that the maternal GSH concentration at PROM labor was significantly lower than those at normal labor (Purnima et al., 2006; Longini et al., 2007). It has been seen that lipid peroxidation products are more in case of preterm labor. GSH/G-PX system is very important in catabolizing H2O2 (Purnima et al., 2006).

They demonstrated that the G-PX activity of patients with preterm labor was higher than that in the controls. A rise in the G-PX activity was reported in the case of preterm labor patients which may be predominant cause of GSH depletion (Purnima et al., 2006). They also showed that the SOD and CAT activities of preterm labor patients were lower than those in the control group significantly (Purnima et al., 2006). Support for the concept that ROS are involved in the pathogenesis of PROM is derived from several lines of investigation. Generation of tissue-damaging molecules, called ROS, may impair the physical integrity (elasticity and strength) of amniotic epithelium and collagen in the amnion and chorion, thus resulting in PROM (James et al., 2001). Results of the present investigation are shown in Table 1. A significant decrease was found in the erythrocyte GSH concentration of PROM subjects as compared to the normal pregnant group. The mean erythrocyte G-PX and CAT activities in PROM group were lower than those in controls. The protective antioxidant mechanisms are complex and multifactorial. The susceptibility of cells to OS is a function of overall balance between the degree of the OS and oxidant defense capability (Akihito et al., 2006).

It is suggested that the decrease in antioxidant activities probably occur in response to an increase in the OS due to an elevation in the maternal lipid peroxidation levels, which might be resulting in PROM. Present study, which examined maternal lipid peroxidation in the healthy pregnant and PROM subjects, demonstrated that the MAD concentration of PROM group was significantly higher than those in the normal pregnant. These findings make it likely that the uncontrolled lipid peroxidation caused by ROS, which are produced in the consequences of the tissue reoxygenation that may occur during pregnancy, which caused PROM. It seems that in PROM, a possible OS is responsible for the induction of protective mechanisms through an increased consumption of antioxidants. Under physiological conditions, antioxidant defense systems have evolved to counterbalance their toxic actions by limiting the amount of lipid peroxides that can be formed (Martin et al., 2008). It is suggested that the PROM probably caused by oscillated oxygenation of both maternal and fetal tissue during pregnancy. Furthermore, it has also been confirmed that the ischemic-reperfusion in human and

other species lead to production of free radicals (Akihito et al., 2006) and increased levels of ammonia may have toxic effects by generating free radicals (Fainarno et al., 2007; Loverro et al., 1997). The results of the present study indicate a disturbed antioxidant balance in case of PROM.

Conclusion

In this study we defined that the OS in pregnancy may enhance the risks of PROM. We indicated that women experiencing PROM are subject to OS. We propose that when an OS develops early in pregnancy, intrauterine growth restriction or PROM or both may occur depending on its entity and length. A topic for future prospective clinical trials is whether dietary supplementation with antioxidants may protect the fetal membranes and decrease the risk of PROM and preterm delivery As opposed to term labor, PROM is associated with increased maternal systemic OS when compared to normal pregnant women. The role for OS in preterm PROM warrants further studies. Our results also revealed a significant decrease in antioxidant enzymes (GP-X and CAT) activities and GSH concentration of PROM group when compared to the controls. It is proposed that an imbalance existed between production and elimination of ROS in the PROM subjects and suggested the need for further study.

Study limitations

A few limitations of the present study deserve comment. First a common limitation of this study was small sample size and these observations must be confirmed in a larger sample of patients with more analysis works. Other postulated limitation involving antioxidants and OS levels, variability between individuals in the diet feeding and cellular utilization and endogenous synthesis of them, the associations observed may still to some degree reflect the effect of varying metabolic responses to dietary fat and environmental situation. The other point might be considered in interpretation of these results as the role of genetic variations in adipose tissue fatty acid percentages, which we have not studied in the current project and might have affected our results. Our study is also limited by the non-representative nature of our study sample, because the pregnant subjects of southern Tehran who referred to Shahid Akbarabadi hospital have low social and economic status. This fact together with the promising results of experimental studies suggests the possibility that antioxidant replacement might become a new pharmacological approach to protect probably elevation of lipid peroxidation, antioxidant reduction and

resultant PROM of pregnant women. Therefore further studies are required to elucidate mechanism underpinning PROM risk associated with decrease antioxidant and OS increase. Further, this observation interplay between genetic and environmental factors must be thoroughly considered in order to evaluate the etiological role of OS and antioxidants in PROM.

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