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Short communication

Antioxidant defence in recurrent abortion

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Abstract

Increased free radical activity has been implicated in the pathogenesis of recurrent abortion. This investigation was conducted to determine if changes in some parameters of the antioxidant system contribute to this condition. Plasma ascorbic acid, α-tocopherol, total thiols, ceruloplasmin, uric acid, albumin, and erythrocyte glutathione (GSH) were assayed in 25 nonpregnant (NP) healthy women, 25 normotensive pregnants (NTP), and 120 women with recurrent abortion. Recurrent aborters were divided into four subgroups according to the etiology: autoimmune (AUTO, $n = 25$), luteal phase defect (LPD, $n = 25$), anatomical defect (AD, $n = 20$) and unexplained (UNEx, $n = 50$). Plasma levels of ascorbic acid, α-tocopherol, and erythrocyte GSH were significantly decreased in AUTO, UNEx and LPD subgroups than those in two control groups and the AD group (ANOVA). Plasma thiols of UNEx and AUTO aborters were diminished according to controls and other abortion subgroups (ANOVA). Ceruloplasmin levels showed a decline in AUTO and UNEx subgroups when compared to controls, AD and LPD aborters (ANOVA). When UNEx, AUTO and LPD recurrent abortion subgroups were compared with each other (Student's *t*-test) total thiols and erythrocyte GSH of UNEx and AUTO subgroups were diminished in comparison with LPD. We suggest that decreased concentrations of plasma ascorbic acid, α-tocopherol, total thiols and erythrocyte GSH in UNEx, AUTO and LPD reflect the increased oxidative stress, expressing a progress of the condition. Also, the imbalance between antioxidant defence and free radical activity is more evident in the AUTO subgroup. As a conclusion, although impaired antioxidant defence may be responsible for recurrent abortions, the recurrent abortions may also result in oxidative stress and depletion and weakness of antioxidant defence.

Keywords: Antioxidants; Recurrent abortion; Oxygen radicals

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1. Introduction

Damage from free radicals (oxidative stress) and lipid peroxidation has been etiologically involved in a variety of physiological, pathological and clinical conditions including pregnancy and its complications, mainly in preeclampsia [1,2]. Oxydative stress can arise through the increased production of reactive oxygen species (ROS) and/or because of deficiency of antioxidant defences. Antioxidant deficiencies can develop as a result of decreased antioxidant intake (such as vitamins C and E, ascorbic acid and α-tocopherol), synthesis of enzymes (such as superoxide dismutase and glutathione peroxidase) or increased antioxidant utilization [1]. In the rat there is evidence that increased lipid peroxidation (induced by vitamin E deprivation) reverses the normal pregnancy-related changes in expression of several enzymes that control eicosanoid synthesis [3]. Moreover, Wang et al. demonstrated that the imbalance between thromboxane and prostacyclin in pre-eclampsia is significantly correlated with the imbalance between lipid peroxides and antioxidants such as vitamin E [2]. The same imbalance between thromboxane and prostacyclin was found in women with recurrent abortion [4]. Maseki et al. reported elevated lipid peroxides in pregnant women according to nonpregnants, and attributed it to changes in total serum lipids. They also found that pre-eclamptic women, in comparison with normal pregnant women, displayed higher serum lipid peroxides [5]. In females undergoing induced or spontaneous abortions, Sane et al. found a maximum rise of serum lipid peroxide levels before the onset of abortion and significantly lower values after abortion [6].

Important plasma antioxidants appear to be ascorbic acid [5], α-tocopherol [2,7], uric acid [8], albumin bound bilirubin [8] and albumin itself [9]. Protein sulfhydryl groups have also been suggested to contribute significantly to the antioxidant capacity of plasma [10]. Ceruloplasmin [10] is considered to be a preventive plasma antioxidant because it sequesters transition metals, thereby preventing them from participating in free radical reactions. Finally, enzymes such as superoxide dismutase (SOD) [10] and glutathione peroxidase (GSHPx) [11] have been proposed to be involved in antioxidant defences in human plasma.

There are only a few reports about antioxidant defences in pregnancy-related complications. Increasing serum antioxidant activity has been documented during normal pregnancy [12]. Also red blood cell (RBC) GSHPx activity increases although the level of selenium decreases [13]. Wisdom et al. observed that RBC cell lysate thiol levels in the pregnancy induced hypertension were lower than the normotensive pregnants' and that the ceruloplasmin levels were higher in the pregnancy induced hypertension with proteinuria than in the pregnancy induced hypertension without proteinuria [10].

Recurrent abortion is a complex syndrome in which many causative factors

appear to play an important role. The role of antioxidant activity in pregnant recurrent aborters remains unanswered as it was not ethical to test patients during their pregnancies while they were taking medications. Is etiology in some recurrent miscarriages or pre-eclamptic patients a real autoimmune activity or a deficiency of antioxidant activity? Whatever the reason is, there seems to be a reduction of antioxidant activity in some fateless pregnancies. So this study is designed to place emphasis on the probable imbalance between antioxidants and peroxides in recurrent abortion cases.

2. Materials and methods

In Istanbul Medical Faculty, Gynecology and Obstetric Department, 120 women with recurrent abortions (all of them experienced at least three consequtive abortions), 25 nonpregnant (NP) healthy women (Control I) in the productive era and 25 normotensive pregnant (NTP) women (Control II) within their first trimester, were taken into the study. None of the women were medicated and all of them were free from cardiovascular, hepatic, renal, endocrine and metabolic disorders. Recurrent aborters were divided into four etiological subgroups: autoimmune (AUTO), luteal phase defect (LPD), anatomical defect (AD) and unexplained (UNEx). LPD was defined according to Noyes criteria [14]. Autoimmune etiology was investigated by means of prolonged partial thromboplastin time activity and anticardiolipin (aCL) antibody presence. Low levels for aCL were accepted when aCL titres exceeded 16 GPL (gamma phospholipid) units. Between 48 and 80 GPL units medium levels were mentioned and when aCL levels were greater than 80 GPL units they were accepted as high levels. Prolongation of aPTT in test plasma for more than 4 s with respect to control plasma maintained the diagnosis of lupus anticoagulant activity (LAC) in all groups. Anatomical defect (cases with only mullerian abnormalities were included in the study) was diagnosed by routine hysterosalpingography and ultrasonography. When the etiology of recurrent abortion can not be explained by various diagnostic tests women were included in the UNEx subgroup. All participants were taking a free range diet. Venous blood samples were collected in heparinized tubes from the antecubital vein after an overnight fast beginning at 10.00 and centrifuged immediately at 2000 rpm (10 min, 4°C) to remove plasma and red blood cells. One milliliter of plasma was mixed with 2 ml 5% (w/v) trichloracetic acid, centrifuged at 2000 rpm and the upper phase was used for determination of ascorbate. The plasma samples were stored frozen (−20°C) until used. The RBC were washed with two 5-ml portions of physiological saline. After the last washing they were kept at −70°C until glutathione estimation.

Ascorbic acid was analyzed spectrophotometrically by means of Cu (II)

oxidation followed by condensation with 2,4-dinitrophenylhydrasine [15]. One milliliter of protein-free filtrate of plasma was placed into a tube together with 0.3 ml of DTCS (dinitrophenylhydrasine–thiourea–copper sulfate) reagent and incubated at 37°C in a water bath for 4 h. After incubation the solution mixture was chilled in an ice bath, 1.5 ml of 65% (v/v) H_2SO_4 (sulfuric acid) was added and absorbance was measured at 520 nm. The coefficient of variation (C.V.%) for this method was 5.7%.

Plasma was assayed for α-tocopherol by first diluting a 0.25 ml sample with 0.50 ml aqueus 2% (w/v) L-ascorbic acid and then deproteinizing it with 0.75 ml of absolute ethanol [16]. After thorough mixing 1.5 ml of hexane was added and the tube content was mixed for a further 1 min. The phases were separated by centrifugation (1000 rpm, 15 min). One milliliter of plasma extract was placed in a semimicrocuvette and mixed with 0.2 ml of 0.004 mol/l ethanolic 4,7-diphenyl-1,10-phenantroline. Absorbance was measured at 452 nm then 0.1 ml of 0.002 mol/l ethanolic ferric chloride was added. The reaction was stopped exactly 1 min after ferric chloride addition with 0.1 ml of 0.172 mol/l ethanolic orthophosphoric acid, and absorbance was measured at 532 nm. As stock standard was used 1 mg/ml α-tocopherol (in ethanol), from which were prepared working standards (1–10 μg/ml). The C.V. for α-tocopherol measurement was 12%.

Ceruloplasmin estimation was realyzed spectrophotometrically [17]. For each sample were prepared two tubes (blank and sample). To the tubes 0.75 ml of acetate buffer (pH: 5.0) and 50 μl plasma was added. The tubes were placed in a water bath at 30°C. At time intervals 0.2 ml of 8 mmol/l orthodianisidine dihydrochloride was added to each tube. Exacly 5 min after the addition of the dianisidine to the blank tubes, they were removed from the water bath and 2 ml of 9 mol/l H_2SO_4 was pipeted. Exactly 15 min after the addition of the dianisidine to the sample tubes, they were removed from the water bath and 2 ml of sulfuric acid solution was added. After cooling at room temperature absorbance was read at 540 nm against the blanks. The C.V. for ceruloplasmin estimation was 7.7%.

Determination of plasma thiol groups was realyzed with some modifications according to Sedlak and Lindsay's method [18]. Plasma (0.2 ml) was mixed with 0.6 ml of 0.2 mol/l Tris buffer (pH 8.2), and 0.04 ml of 0.01 mol/l DTNB (5,5′-ditiobis-(2-nitrobenzoic acid). The mixture was brought to 4 ml with methanol. The test tubes were stoppered and left to stand for 20 min, then centrifuged at 3000 rpm at room temperature for 10 min. The absorbance of the clear supernatants was read at 412 nm. The C.V. for the plasma thiol groups measurement was 4.5%.

Plasma uric acid and albumin were measured using an autoanalyzer.

RBC glutathione was assayed according to the method of Beutler et al. [19]. A 0.2 ml sample of washed RBC was mixed with 1.8 ml of bidistilled water for hemolysis, then 3 ml precipitating solution (including 1.67 g of glacial

metaphosphoric acid, 0.2 g of disodium-EDTA, and 30 g of NaCl, and brought to 100 ml with distilled water) was mixed with the hemolysate. The mixture was allowed to stand for 5 min and centrifuged at 2000 rpm for 10 min. To 1 ml of filtrate was added 4 ml of 0.3 mol/l disodium phosphate solution. Then 1 ml of 0.01 mol/l of DTNB solution was added, tubes were mixed and absorbance was read at 412 nm within 4 min of preparing cuvets. The C.V. for RBC glutathione measurement was 5%.

Data were given as means±S.D., one-way analysis of variance (ANOVA) and Student's *t*-tests were used for statistical evaluation. A *P*-value less than 0.05 after correction for multiple comparisons by Duncan was considered significant.

3. Results

The age and gravidity of recurrent aborters, NP and NTP women were comparable. The ages were 30 (range 19–38), 29 (range 22–35) and 28 (range 21–35), respectively. NP and NTP were multipara. Table 1 presents mean±S.D. values of plasma ascorbic acid, α-tocopherol, total thiols, ceruloplasmin, erythrocyte GSH, uric acid, and albumin in control and study groups.

Mean plasma ascorbic acid, α-tocopherol and erythrocyte GSH in AUTO, UNEx and LPD recurrent aborters were found to be significantly lower than those of two control and AD groups (ANOVA). Plasma total thiol concentrations

Table 1
Plasma ascorbic acid, α-tocopherol, total thiol groups, ceruloplasmin, uric acid, albumin and erythrocyte glutathione (GSH) levels in NP, NTP, UNEx, AUTO, LPD and AD women (mean±S.D.)[a]

Parameter	NP ($n=25$)	NTP ($n=25$)	UNEX ($n=50$)	AUTO ($n=25$)	LPD ($n=25$)	AD ($n=20$)
Ascorbic acid (mg/dl)	1.33±0.45	1.25±0.39	0.87±0.23	0.90±0.30	0.85±0.30	1.11±0.37
α-Tocopherol (mg/l)	9.90±2.51	10.21±1.55	6.15±2.57	5.70±1.50	6.80±2.50	8.55±3.47
Total thiols (mmol/l)	0.45±0.07	0.55±0.05	0.36±0.05	0.37±0.07	0.44±0.06	0.50±0.07
Ceruloplasmin (U/l)	220±42	233±49	179±67	165±71	216±84	217±58
Erythrocyte GSH (mg/dl Ery)	70.90±4.00	72.74±6.09	56.88±5.82	58.77±5.10	65.21±8.27	70.22±7.05
Uric acid (mg/dl)	3.50±0.80	3.40±0.62	3.54±0.82	3.88±0.67	3.47±0.53	3.44±0.60
Albumin (g/dl)	4.80±0.50	4.30±0.51	4.41±0.53	4.45±0.60	4.70±0.62	4.70±0.69

[a] NP (nonpregnant healthy women), NTP (normotensive pregnants), UNEx (unexplained etiology), AUTO (autoimmune subgroup), LPD (luteal phase defect), AD (anatomical defect).

Table 2
Plasma ascorbic acid, α-tocopherol, total thiol groups, ceruloplasmin and erythrocyte GSH in UNEX, AUTO and LPD subgroups (mean±S.D. and P value) (Student's t-test)[a]

Parameter	UNEX ($n = 50$)	AUTO ($n = 25$)	LPD ($n = 25$)
Ascorbic acid (mg/dl)	0.87±0.23 NS[c]	0.90±0.30 NS[b] NS[d]	0.85±0.30
α-Tocopherol (mg/l)	6.15±2.57 NS[c]	5.70±1.50 NS[b] NS[d]	6.80±2.50
Total thiols (mmol/l)	0.36±0.05 $P < 0.001$[c]	0.37±0.07 NS[b] $P < 0.01$[d]	0.44±0.06
Ceruloplasmin (U/l)	179±67 NS[c]	165±71 NS[b] NS[d]	216±84
Erythrocyte GSH (mg/dl Ery)	56.88±5.82 $P < 0.001$[c]	58.77±5.10 NS[b] $P < 0.001$[d]	65.21±8.27

[a] UNEx (unexplained etiology), AUTO (autoimmune subgroup), LPD (luteal phase defect).
[b] When AUTO were compared with UNEx.
[c] When UNEx were compared with LPD.
[d] When AUTO were compared with LPD.

of UNEx and AUTO subgroups were decreased in comparison with controls (NP and NTP) and other abortion subgroups. Mean ceruloplasmin values of AUTO and UNEx subgroups showed significant decline according to controls and other recurrent aborters (ANOVA).

Plasma albumin and uric acid concentrations exposed no changes among all groups. As can be seen from the results, no significant difference was encountered between controls and recurrent abortion with anatomic etiology. When abortion etiology was considered, LPD, UNEx and AUTO subgroups were compared with each other by Student's t-test and the results expressed some differences (Table 2).

4. Discussion

Antioxidant status in normal pregnancy and pregnancy related diseases is gathering increasing interest in recent years [6,20]. The significant rise of antioxidant activity during normal pregnancy is documented in the literature so far [3,12]. Greater elevation of lipid peroxidation markers has been found in

pregnancy induced hypertension [2]. Also, a rise in serum lipid peroxide levels before the onset of abortion and significantly lower values after abortion have been observed by Sane et al. [6].

The antioxidant status is known to be a physiological barrier against free radical attack in certain body compartments [10]. The tendency of free radicals by way of lipid peroxidation which contribute to the development of destructive processes leads ones attention to antioxidant activity. Although toxic actions of lipid peroxides are opposed by this system, under certain conditions the protective mechanisms may be overwhelmed resulting in oxidative stress of tissues.

In order to maintain the physiological conditions in body compounds like the case in normal pregnancy, there should be a balance between pro-oxidant and antioxidant forces. Impairment of this balance which normally favors the antioxidant activity leads to some pathological clinical conditions such as pre-eclampsia. Today it is not clear whether the identity of this imbalance could lead to some other pathophysiological events of pregnancy like abortions. Microthrombosis, vasospastic changes, and necrosis are common in the placentas of recurrent abortion and pre-eclamptic patients, which suggests that imbalance of prostacyclin/thromboxane production (seen in both disorders) is responsible for activated lipid peroxidation and depleted antioxidant amounts. Provided that the same pathological conditions may take place in recurrent abortions, like hypertensive disorders of pregnancy, the antioxidant status is under investigation in recurrent abortions. In this study plasma total thiols and plasma vitamin C, E concentrations decreased with respect to values obtained in control groups. In likewise fashion RBC glutathione decreased significantly. These alterations are compatible with oxidative stress. The measured fall in plasma ascorbic acid, vitamin E and thiol amounts probably reflects increased lipid peroxidation reactions in recurrent abortions. These findings indicate that there are ascorbate-dependent and glutathione-dependent tocopherol regenerating mechanisms in plasma of recurrent aborters. Increased lipid peroxidation causes consumption of those vitamins and thiols. In addition, plasma ceruloplasmin values depleted with a significant pattern in UNEx and AUTO aborters according to normotensive pregnants. Although the ceruloplasmin level was higher in the pre-eclampsia group than in pregnancy induced hypertension without proteinuria [10], the significance of this difference obtained in recurrent aborters was found to be on a borderline value. This study reflected depleted values for ceruloplasmin, especially in UNEx and AUTO groups. Because ceruloplasmin is a plasma protein with ferrooxidase enzymatic activity and not consumed by oxidative stress, the decreased levels might reflect insufficient production or impaired metabolism or some kind of pathological consumption.

One other explanation of the decline of some antioxidants in recurrent aborters probably is the dietary regiment. All participants of this study were taking a free range diet. The diet of study groups is probably different from

controls by way of calories and content. The fact that the increased vitamin E intake correlates with high plasma levels [20] and that activated lipid peroxidation (induced by vitamin E deprivation) in rat reverses the normal pregnancy-related changes [3] may support our hypothesis.

Plasma albumin and uric acid concentrations expressed no changes between study and control groups because these factors are influenced by many other factors except oxidative stress (e.g., some pathological conditions). The steady state of their concentrations suggests that the problem is related to a more sensitive state.

When the results were investigated with regards to abortion etiology, no significant difference was found in the AD subgroup. The most striking results were encountered in LPD, UNEx and especially in AUTO subgroups. Almost all of the parameters investigated reached significant differences in those groups. Alterations implicate an imbalance in favor of pro-oxidant activity. Decreased antioxidant activity gives way to increasing levels of circulating lipid peroxides. When lipid peroxide levels are elevated, they inhibit the synthesis of the endothelial cell-derived vasodilator prostacyclin. Disruption of platelet cell membranes is another action of peroxides which results in thromboxane elevation [2]. This abnormal prostaglandin action is supposed to be the triggering factor of endothelial cell damage of vasculopathic diseases of pregnancy, like pre-eclampsia. Altogether, pregnancy related changes encountered in the decidua–placental interface specimens of recurrent abortion cases might draw attention to the same triggering point as the pathophysiology, that is the decreased antioxidant activity. Again, this reduction will induce membrane peroxidative reactions resulting in membrane instability and hemolysis [12]. Elevations of serum iron with hiperlipidemia favors lipid peroxide levels in circulation [21]. Perhaps the question of the art should be the reason of depleted or decreased steady state concentration of antioxidants in recurrent abortion cases which might be under some kind of genetic control, like familiarity to autoimmune diseases or due to an idiopathic immune malfunction. Antioxidant changes of variable pregnancy conditions are under investigation by many authors. The altered balance of oxidative activity in recurrent abortion cases observed in this study seems to need further study and new comments in order to reach a conclusion. However, we can conclude that although increased oxidative stress probably is one of the main reasons of recurrent abortions, the recurrent abortions should be seen to be responsible for depletion and weakness of antioxidant defence.

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