DETERMINATION OF PROOXIDANT–ANTIOXIDANT BALANCE DURING UNCOMPLICATED PREGNANCY USING A RAPID ASSAY

ODREĐIVANJE RAVNOTEŽE PROOKSIDANSI/ANTIOKSIDANSI U TOKU TRUDNOĆE
BEZ KOMPLIKACIJA POMOĆU BRZOG TESTA

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Summary

Background: Oxidative stress is thought to be a major contributor to complications during pregnancy, for example preeclampsia. However, reports regarding prooxidant–antioxidant balance in uncomplicated pregnancy are inconsistent. In this study, we aimed to compare the levels of oxidative stress in non-pregnant women with apparently normal pregnant women during the first trimester and at delivery.
Methods: An assay for the determination of prooxidant–antioxidant balance (PAB) was used in this study, in which the prooxidant burden and the antioxidant capacity were measured simultaneously in a single assay. The levels of oxidative stress were determined in 85 non-pregnant and 64 primigravid pregnant women.
Results: Demographic data and biochemical indices did not differ significantly between the groups. Differences between PAB values were significant based on one-way ANOVA analysis (P<0.001). Using a *post hoc* test, we observed a statistically significant increase in PAB values during the first trimester and last trimester (P<0.001).
Conclusions: Normal pregnancy is associated with a change in the measure of redox status, as assessed by the PAB assay.

Keywords: antioxidants, oxidative stress, pregnancy, reactive oxygen species

Kratak sadržaj

Uvod: Veruje se da oksidativni stres u velikoj meri doprinosi komplikacijama u toku trudnoće, kao što je na primer preeklampsija. Međutim, izveštaji o ravnoteži između prooksidanasa i antioksidanasa u trudnoći bez komplikacija se razlikuju. U ovoj studiji, cilj nam je bio da uporedimo nivoe oksidativnog stresa kod žena koje nisu trudne sa ženama u naizgled normalnoj trudnoći tokom prvog tromesečja i na porođaju.
Metode: U studiji je za određivanje ravnoteže prooksidansi/antioksidansi (PAB) korišćen test u kom se istovremeno mere prooksidantni balast i antioksidantni kapacitet, u jednom testu. Nivoi oksidativnog stresa određeni su kod 85 žena koje nisu bile trudne i 64 trudnice u prvoj trudnoći.
Rezultati: Demografski podaci i biohemijski indeksi nisu se značajno razlikovali između grupa. Analiza ANOVA ukazala je na značajne razlike između vrednosti PAB (P<0,001). Pomoću *post hoc* testa uočili smo statistički značajan porast vrednosti PAB u toku prvog i poslednjeg tromesečja (P<0,001).
Zaključak: Normalna trudnoća povezana je s promenom u meri redoks statusa, utvrđenoj na osnovu PAB testa.

Ključne reči: antioksidansi, oksidativni stres, trudnoća, reaktivne kiseonične vrste

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Introduction

Oxidative stress occurs when the generation of reactive oxygen and other radical species exceeds the scavenging capacity by anti-oxidants (1). Free radicals are defined as molecules with one or more unpaired electrons in their outer electron orbit, and include hydroxyl radical, nitric oxide and superoxide. Toxicity of these free radicals is related to their ability to damage the structural integrity of proteins, lipids, and DNA (2). Oxidative stress has been implicated in the pathogenesis of cardiovascular and atherosclerotic disorders, cancer, rheumatoid arthritis and ageing (3, 4). There have been various reports suggesting an increased state of oxidative stress in pregnancy (5–7) as a consequence of the mitochondrion-rich placenta, increased lipid peroxidation (8), lower superoxide dismutase (SOD) activity (9), increased production of nitric oxide (NO), S-nitrosalbumin and S-nitrosothiols (10). However, few studies have investigated whether these changes have an effect on redox status, which may occur if free radical production exceeds the antioxidant capacity; several studies suggest that antioxidant defense mechanisms increase in pregnancy through enzymatic (catalase, SOD, etc.) and non-enzymatic protection (vitamin C, E, protein thiols, ceruloplasmin) (11, 12). An oxidant–antioxidant imbalance appears to be associated with complications during pregnancy including: preeclampsia, pregnancy-induced diabetes, and intrauterine growth restriction (7, 13, 14). There is little information about the baseline levels of prooxidant–antioxidant activity in healthy uncomplicated pregnancy.

For the evaluation of the prooxidant–antioxidant balance, the determination of both the oxidant and the antioxidant status is necessary. Therefore, various methods for the separate measurement of the total oxidant or antioxidant status have been proposed (7, 15, 16). These methods are laborious; time consuming, expensive and imprecise. However, most of these methods investigate only one side of the oxidative stress balance (17) and there is no consensus for measuring oxidative stress; hence, estimation of PAB is indirect.

We have recently applied a simple, rapid and inexpensive method that can measure the balance of oxidants and antioxidants simultaneously in one assay (the PAB assay) that uses the TMB-TMB cation and provides a redox index (18), and has been validated (19). Furthermore, some previous studies have shown that the lipid profile changes during pregnancy and this may cause endothelial dysfunction that may play a role in generating oxidative stress. Also, Alamdari et al. (18) have shown that there may be a relationship between PAB and the lipid profile.

The main objective in this study was to compare this measure of redox status between pregnant and non-pregnant women in order to determine the impact of pregnancy on oxidative stress using a modified PAB assay. We have also investigated whether there are differences in the lipid profile between uncomplicated pregnancy and non-pregnant women, and whether there is a correlation between PAB and lipid profile laboratory values.

Materials and Methods

Subjects

A total of 75 apparently healthy pregnant women between 16–35 years of age were initially enrolled in the study from individuals referred to the Obstetrics and Gynecology Department of Om--Albanin Hospital (Mashhad, Iran) between June 2006 and August 2008. Eligible cases were singleton pregnancies with a gestational age up to 12 weeks. Eleven women were excluded because they were consuming medications other than routine supplements of folic acid and ferrous sulfate or had a history of previous medical condition. Considering the effect of iron on lipid oxidation and redox state, the dosage of iron supplement was 60 mg/d which is the recommendation of the International Nutritional Anemia Consultative Group and the World Health Organization for preventing iron deficiency anemia (20, 21). Eighty-five healthy non-pregnant women were selected as the control group in order to be compared with the case group for the prooxidant-antioxidant balance. None of the 160 subjects were tobacco smokers and alcoholics. The study protocol was approved by the Ethical Committee of the Research Council of Mashhad University of Medical Sciences (MUMS) and was performed in conformance with the Declaration of Helsinki ethical guidelines, as reflected in *a priori* approval by the committee. Written informed consent was obtained from each woman at enrolment.

Anthropometric measurements

For all subjects, anthropometric parameters including weight, height, waist and hip circumference were measured using a standard protocol. Subjects were asked to breathe normally, and to breathe out gently at the time of measurement. The hip circumference measurement was taken at the point yielding the maximum circumference over the buttocks. Height, body weight, waist and hip circumference were measured with the subjects dressed in light clothing after an overnight fast. Blood pressure was measured twice while the patients were seated and rested for 15 min, using a standard mercury sphygmomanometer calibrated by the Iranian Institute of Standards and Industrial Research. The interval between each blood pressure measurement was at least 30 min, and the average of the two measurements was taken as the blood pressure. The body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2).

Blood sampling

Blood samples were collected in the morning from each subject after an overnight fast into plain serum tubes. Blood was left to clot for 30–60 min and then centrifuged at 2500 rpm for 15 min at room temperature to obtain serum. Hemolysed samples were excluded from analysis. Serum was stored at –20 °C prior to analysis.

Biochemical measurements

A full fasted lipid profile comprising total cholesterol, triglycerides (TG), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) was determined for each patient after a 12-h fast. Serum lipid profile was measured by routine enzymatic methods using commercial kits.

PAB Assay chemicals

TMB powder (3,3',5,5'-tetramethylbenzidine, Fluka), peroxidase enzyme (Applichem: 230 U/mg, A3791, 0005, Darmstadt, Germany), N-chloro 4-methylbenzenesulfonamide, sodium salt (chloramine T trihydrate) (Applichem: A4331, Darmstadt, Germany), hydrogen peroxide (30%) (Merck), as well as all the other reagents used were reagent grade and were prepared in double distilled water.

PAB Assay

A modified PAB assay was applied based on a previously described method (18, 19). This method is based on two different oxidation–reduction reactions which take place simultaneously.

In the enzymatic reaction the chromogen TMB is oxidized to a colored cation by peroxides and in the chemical reaction the colored TMB cation is reduced to a colorless compound by antioxidants. The photometric absorbance is then compared with the absorbance given by a series of standard solutions that are made by mixing varying proportions (0–100%) of 250 µmol/L hydrogen peroxide – as a representative of hydroperoxides which is an indicator of total oxidant status, with 3 mmol/L uric acid – as a representative of the antioxidant capacity (in 10 mmol/L NaOH). It should be underlined that hydrogen peroxide and uric acid do not interact with each other, and do not neutralize the activity of each other. This photometric comparison is carried out using an ELISA reader.

TMB powder (60 mg) was dissolved in 10 mL dimethyl sulfoxide (DMSO). For preparation of the TMB cation, 400 µL of the TMB/DMSO solution was added to 20 mL of acetate buffer (0.05 mol/L buffer, pH 4.5), and then 70 µL of fresh (chloramine-T) (100 mmol/L) solution was added to this 20 mL.

The solution was mixed well and incubated for 2 hours at room temperature in a dark place. Then 25 units of peroxidase enzyme solution were added to 20 mL of TMB cation solution, dispensed in 1 mL and stored at –20 °C. In order to prepare the TMB solution, 200 µL of TMB/DMSO was added to 10 mL of acetate buffer (0.05 mol/L buffer, pH 5.8) and the working solution was prepared by mixing 1 mL TMB cation with 10 mL of TMB solution. This working solution was incubated for 2 min at room temperature in a dark place and immediately used. Ten µL of each sample, standard, or blank (distilled water) were mixed with 200 µL of working solution in each well of a 96 well plate, which was then incubated in a dark place at 37 °C for 12 minutes. At the end of incubation, 100 µL of 2 normal hydrochloric acid (2 N HCl) was added to each well, and the optical density (OD) was measured in an ELISA reader at 450 nm with a reference wavelength of 620 or 570 nm. A standard curve was provided from the values relative to the standard samples. The values of the PAB are expressed in an arbitrary HK unit, which is the percentage of hydrogen peroxide in the standard solution (calibration mixture) multiplied by 6. The values of the unknown samples were then calculated based on the values obtained from the above standard curve.

For the determination of the precision of the modified PAB method, the intra- and inter-assay coefficient of variation (CV %) were determined in our previous study (19). The CV% of the intra-assay for 28 samples analyzed in triplicate was between 1.4 and 3.5%, with a mean of 2.1%. The CV% of the inter-assay for 20 samples, analyzed over three days, was between 4.1 and 8.5%, with a mean of 6.1%. Serum PAB was not affected by storage at 4 °C for one day or at –20 °C for one week, or at –80 °C for three months (19).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Two–tailed P-values<0.05 were considered as significant. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software, 15th edition. Statistical analyses were carried out using the paired t-test and Mann-Whitney U test (for two independent groups), or paired samples t-test and Wilcoxon test (for two related groups). One-way Analysis of Variance (ANOVA) and *post hoc* tests were used to determine the significance of difference between 3 groups.

Results

The demographic data for pregnant and control groups are displayed in *Table I*. There were no differences in age, anthropometric indices, past medical history (miscarriage and infertility history) and family

Table I Comparison of clinical and biochemical parameters between pregnant and non-pregnant groups.

Parameter	Non-pregnant (n=85)	Pregnant (n=64)	Significant correlation with PAB (n=149)
SBP (mm Hg)	103.24±7.00	101.86±11.41	No
DBP (mm Hg)	65.12±8.73	62.9±11.5	No
Cholesterol (mmol/L)	3.82±0.59	4.30±0.29	No
TG (mmol/L)	0.99(0.47–1.16)	0.90(0.82–1.16)	No
LDL-C (mmol/L)	2.35±0.5	2.70±1.29	No
HDL-C (mmol/L)	1.00±0.18	1.22±0.22*	No

Values are expressed as mean ± SD (for normally distributed data) or median and interquartile range (for non-normally distributed data). Comparison between the two groups' values was made using paired t-test or Wilcoxon test for normally and non-normally distributed data, respectively (* $P<0.001$). Correlation between PAB and other parameters was assessed using Pearson Correlation test.

SD: standard deviation, SBP: systolic blood pressure, DBP: diastolic blood pressure, TG: triglycerides, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, PAB: prooxidant–antioxidant balance.

Table II Comparison of PAB values between pregnant and non-pregnant groups.

Parameter	Non-pregnant	Pregnant	
		First trimester	Delivery
Serum PAB (arbitrary HK unit)	129.079 (64.13–168.82)	167.7 (122.5–221.0)*	221 (162–223.3)*

Values are expressed as median and interquartile range. Between group comparisons were assessed by the Mann-Whitney test for pregnant and non-pregnant groups and Wilcoxon test for two pregnant groups (*$P<0.001$).

PAB: prooxidant–antioxidant balance.

history (incidence of preeclampsia, diabetes, hypertension and hyperlipidemia) between groups. Systolic and diastolic blood pressure, lipid profile did not significantly differ between both groups at baseline. A significant difference was observed in high density lipoprotein between pregnant and non-pregnant groups, but there was no significant relationship between TG, total cholesterol, LDL, HDL and PAB values in either group (*Table I*).

In regard to the prooxidant–antioxidant balance, data analysis revealed a statistically significant increase in PAB values in pregnant women. Furthermore, PAB values at parturition were significantly higher compared with non-pregnant and first trimester sampling values (*Table II*). PAB values of pregnant in comparison with non-pregnant values were approximately 30% higher and 70% higher at the end of pregnancy compared with non-pregnant individuals.

Discussion

Our results indicate that PAB values are significantly higher in women with a normal pregnancy compared with non-pregnant women.

In a previous study using this method, PAB values showed a linear response against a series of oxidants (hydrogen peroxide, tert-buthylhydroperoxide, chloramine T and HClO) and antioxidants (vitamin C, trolox, glutathione, uric acid, bilirubin, albumin and ceruloplasmin) (18).

PAB values change during normal pregnancy due to oxidant and antioxidant alterations.

Other studies have shown that oxidative stress is increased during pregnancy (6, 11, 22). When this increase is not associated with an optimal antioxidant status, harmful effects could arise that include hypertension, diabetes, preeclampsia, intrauterine growth retardation, abortion and teratogenic processes (7, 23). Most of these are considered to be secondary to endothelial cell dysfunction. However, a conclusive consensus of systemic oxidant–antioxidant balance in pregnancy is lacking.

There are several studies that have compared healthy pregnant and non-pregnant women and most of them have reported higher lipid peroxide levels and oxidative stress in the pregnant women (5, 24). Reported ratios of pregnant to non-pregnant values

ranged from 1.08 to 3.04. Lipid peroxide levels in the first trimester of pregnancy were sometimes reported to be higher and sometimes reported to be lower than the level of the non-pregnant control group (25). By the second trimester, increases of 10 to 50% over first trimester values were usually seen. PAB levels in the third trimester sometimes, but not always, declined. Only one investigation reported decreasing lipid peroxides during pregnancy. Takehara et al. (26) reported a 50% decrease by the end of pregnancy compared with the first trimester and a reduction of antioxidant enzymes such as glutathione peroxidase, suggesting the beneficial role of antioxidant balance in normal pregnancy.

Whilst there are several methods for assessing oxidative stress, there is as yet no consensus on a simple method to use in routine clinical practice. Most methods currently measure the prooxidant and antioxidant capacities separately. We have recently developed a modified, simple, rapid and inexpensive method (the prooxidant–antioxidant balance (PAB) assay).

Our previous clinical investigations support the validity of the PAB assay for the estimation of oxidative stress in patients with type 2 diabetes (18), angiographically defined coronary artery disease (19) and patients with acute coronary syndrome (27). We have now extended the application of our modified PAB assay to assess the extent of oxidative stress during pregnancy.

Although there are few studies on the prooxidant–antioxidant balance during pregnancy, our results are consistent with other studies in which oxidant stress has been studied during pregnancy. PAB was found to be generally higher during pregnancy. The increase in PAB persists until parturition, but the cause of their change in the course of pregnancy is unclear.

Prooxidant–antioxidant imbalance should be considered serious because the relentless production of toxic species may cause oxidative stress and endothelial tissue injury and further clinical diseases, for instance, this may suggest an increased risk for cardiovascular and thromboembolic events in multigravida women or consequent pregnancies. So, measuring the prooxidant–antioxidant balance with a rapid, easy and cost-effective test could be potentially useful to find high risk groups and start antioxidant therapy in order to prevent the progression of oxidative stress and consequent complications. Further prospective longitudinal investigations have to be carried in order to shed some light on this relevance.

Limitation and Suggestion

The main limitation of the current study was that a number of specific antioxidants have not been measured. Several antioxidants such as uric acid, albumin, creatine and bilirubin are known to change during pregnancy, and this is probably of little clinical consequence. Our subjects were not followed-up for assessing the long-term prevalence of pathologies and perhaps the pregnant women should have been assessed, before pregnancy, after pregnancy and in the third trimester, with a more detailed analysis of diet, activity level and other variables which are known to affect oxidative stress.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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