



Redox status of pregnant women with thrombophilia

Redoks status trudnica sa trombofilijom

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Abstract

Background/Aim. Since the role of oxidative stress in the pathogenesis of thrombophilia in pregnancy has still not been clarified, the aim of the study was to assess the redox status of pregnant women with thrombophilia. **Methods.** The study involved 120 pregnant women divided into two groups: pregnant women with thrombophilia ($n = 60$) and women with normal pregnancy ($n = 60$). Blood samples for biochemical analysis were collected at the end of the first, second, and third trimester of pregnancy. Concentrations of hydrogen peroxide (H_2O_2), nitrites (NO_2^-), and the index of lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS) were measured in plasma. Levels of reduced glutathione (GSH), activities of superoxide dismutase (SOD) and catalase (CAT) were measured in erythrocytes. **Results.** In women with thrombophilia, NO_2^- values were increased in the first and third trimester compared to healthy pregnant women ($p < 0.05$). The higher levels of TBARS and H_2O_2 were noticed in women with thrombophilia in the first trimester compared to healthy pregnant women ($p < 0.05$). The values of SOD and CAT were lower in women with thrombophilia in the third and GSH in the first trimester compared to the control group ($p < 0.05$). **Conclusion.** Our results suggest an increased generation of prooxidants in thrombophilia at the beginning of gestation, which declines as gestation progresses and reaches similar values as in normal pregnancy at the end of pregnancy. Generally viewed, pregnant women with thrombophilia was associated with impaired antioxidant capacity – activities of SOD and CAT were lower in the third and GSH in the first trimester compared to their values in healthy pregnant women.

Key words:

antioxidants; oxidation-reduction; pregnancy; pregnancy trimesters; thrombophilia.

Apstrakt

Uvod/Cilj. Imajući u vidu da uloga oksidacionog stresa u patogenezi trombofilije u trudnoći nije u potpunosti razjašnjena, cilj rada je bio da se proceni redoks status trudnica sa trombofilijom. **Metode.** Studija je obuhvatila 120 trudnica koje su bile podeljene u dve grupe: grupu trudnica sa trombofilijom ($n = 60$) i grupu sa trudnoćom bez pridruženih bolesti (zdravim) ($n = 60$). Uzorci krvi za biohemijske analize su sakupljeni na kraju prvog, drugog i trećeg trimestra trudnoće. U plazmi su određivane koncentracije vodonik peroksida (H_2O_2), nitrita (NO_2^-) i indeks lipidne peroksidacije meren kao reaktivne supstance tiobarbituric kiseline (RSTBK). U lizatu eritrocita određivan je nivo redukovano glutationa (GSH), aktivnosti superoksid dizmutaze (SOD) i katalaze (CAT). **Rezultati.** U grupi žena sa trombofilijom, vrednosti NO_2^- bile su povećane u prvom i trećem trimestru u poređenju sa zdravim trudnicama ($p < 0,05$). Viši nivoi RSTBK i H_2O_2 primećeni su kod žena sa trombofilijom u prvom trimestru, u poređenju sa zdravim trudnicama ($p < 0,05$). Vrednosti SOD i CAT bile su niže kod žena sa trombofilijom u trećem, a GSH u prvom trimestru trudnoće, u odnosu na kontrolnu grupu ($p < 0,05$). **Zaključak.** Naši rezultati ukazuju na to da je kod trudnica sa trombofilijom na početku trudnoće produkcija prooksidacionih parametara povećana, smanjuje se sa progresijom trudnoće i na kraju trudnoće dostiže vrednosti koje se beleže kod zdravih trudnica. Generalno posmatrano, trombofilija je bila povezana sa pogoršanjem antioksidacionog kapaciteta – aktivnosti SOD i CAT su bile niže u trećem, a nivo GSH u prvom trimestru, u poređenju sa njihovim vrednostima kod zdravih trudnica.

Ključne reči:

antioksidansi; oksidoredukcija; trudnoća; trudnoća, tromesečja; trombofilija.

Introduction

Thrombophilia may be defined as a disorder of hemostasis characterized by an increased tendency to many thrombotic events^{1,2}. This predisposition to form clots may be both inheritable and acquired, or more commonly, it appears as an interaction between genetic and acquired factors. The most frequent inherited thrombophilic defects include deficiencies of antithrombin, protein C, protein S, mutations in the genes for coagulation factor V (factor V Leiden), and prothrombin G20210A gene polymorphisms. On the other hand, antiphospholipid antibody syndrome is a very often acquired thrombophilia^{2,3}.

Epidemiological data have shown that thrombotic events are a significant cause of mortality and morbidity nowadays. There is a growing concern related to thrombophilia in pregnancy since it has been associated with an increased risk not only of pregnancy-related venous thromboembolism but also other complications, including severe preeclampsia/eclampsia, placental abruption, hemolysis, elevated liver enzyme levels, and low platelet levels HELLP syndrome, intrauterine growth restriction, and recurrent miscarriage^{4,5}.

Oxidative stress is a condition defined as a disturbed equilibrium between prooxidants/antioxidants in favor of prooxidants⁶. Numerous papers have proven that increased production of prooxidants can be present during a normal pregnancy due to the high energy demands of many body functions⁷⁻⁹. The relation between oxidative stress and adverse pregnancy outcome and pregnancy complications such as preeclampsia and diabetes has been established¹⁰. Literature data indicate that a high oxidative state of the mother corresponds to a high oxidative state of the newborn, thus suggesting the importance of protecting the fetus before the birth process¹¹.

Furthermore, it has been proposed that thrombophilia during pregnancy induces hemostatic response and microthrombi formation, which leads to the generation of prooxidants. Those events cause further pro-coagulation and consequently further prooxidant generation, which results in many harmful effects on the placenta and placental circulation^{12,13}. Unfortunately, there is a lack of studies referring to the oxidative stress markers and antioxidative defence system during the three trimesters of pregnancy in women with thrombophilia.

Considering the standpoint that the role of oxidative stress in the pathogenesis of thrombophilia in pregnancy has still not been clarified, the aim of our study was to establish the redox status of pregnant women with thrombophilia.

Methods

Study design

This study was designed as a longitudinal study, and it was conducted in 2016. The study protocol was approved by the Medical Ethics Committee of the University Clinical Center "Kragujevac" from March 2, 2016 (01/2862) and was

carried out according to the Declaration of Helsinki. All the participants were informed about the research protocol before giving their written consent to participate in the study.

Study population

A total of 120 pregnant women were included in the study. They were divided into two groups: thrombophilia group and normal pregnancy group. The thrombophilia group consisted of 60 pregnant women suffering from thrombophilia, while the normal pregnancy group included 60 physiologically healthy pregnant women. All 120 pregnant women were periodically examined at the Clinic of Hematology at the University Clinical Center "Kragujevac" and had an ultrasonic examination at the Clinic of Gynecology and Obstetrics at the University Clinical Center "Kragujevac" during the year 2016. Among 60 women with thrombophilia, half of them were methylenetetrahydrofolate reductase heterozygous, while the other half included plasminogen activator inhibitor homozygous. They received therapeutic doses of low molecular weight heparin for the treatment of thrombophilia. All participants of the study consumed multivitamin supplements that contained 100 mg of vitamin C and 5 mg of folic acid each day.

Our study involved pregnant women who were physiologically healthy, did not suffer from any chronic disease and have not used any medications, with no current complications, but who have had one or more pregnancies with complications – miscarriages, proven congenital or acquired thrombophilia, single and multiple pregnancies, pregnancy achieved by *in vitro* fertilization. None of the pregnant women had more serious complications during pregnancy and childbirth. All pregnant women gave birth to one child, while one pregnant woman gave birth to twins. Newborns of pregnant women with thrombophilia had a lower body weight at birth compared to healthy women.

Excluding criteria were the following: pregnancies that ended in miscarriage or missed abortion (the cessation of fetal cardiac activity before the 12th week of gestation) and pregnancies that ended with classic miscarriage (pregnancy loss occurring due to bleeding before the 12th week of gestation). Based on these criteria, 17 pregnant women were excluded from the study. Five pregnant women decided to leave the study, two pregnant women had an abortion due to pathologic fetal karyotype, and ten of them had a spontaneous abortion – nine of them had thrombophilia, and one was a physiologically healthy pregnant woman.

Biochemical analysis

Blood samples for biochemical analysis were collected in three specific periods of pregnancy, in each of three trimesters, at the end of the first, second, and third trimester of pregnancy. Blood samples were drawn from an antecubital vein into a Vacutainer test tube containing sodium citrate anticoagulant. Blood was centrifuged to separate plasma and red blood cells (RBCs). In plasma, the following parameters of redox balance were determined:

nitrites (NO_2^-), hydrogen peroxide (H_2O_2), and the index of lipid peroxidation (measured as thiobarbituric acid reactive substances – TBARS). Parameters of antioxidant defence systems such as superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) were determined in erythrocytes samples. Biochemical parameters were measured spectrophotometrically using UV-1800 Shimadzu UV spectrophotometer, Japan.

H₂O₂ determination

The protocol for measuring H_2O_2 is based on the oxidation of phenol red in the presence of horseradish peroxidase¹⁴. A 200 μL sample with 800 μL phenol red solution and 10 μL horseradish peroxidase were combined (1 : 20). The level of H_2O_2 was measured at 610 nm. Distilled water was used as a blank control.

NO₂⁻ determination

Nitric oxide (NO) decomposes rapidly to form stable metabolite nitrite/nitrate products. Nitrites were determined as an index of NO production with Griess reagent¹⁵. A mixture of 0.1 mL 3 N perchloric acid, 0.4 mL 20 mM ethylenediaminetetraacetic acid (EDTA), and 0.2 mL plasma were put on ice for 15 min, then centrifuged for 15 min at 6,000 rpm. After pouring off the supernatant, 220 μL K_2CO_3 was added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe¹⁶.

The degree of lipid peroxidation in plasma was estimated by measuring TBARS using 1% thiobarbituric acid (TBA) in 0.05 NaOH, incubated with plasma at 100 °C for 15 min, and read at 530 nm. Distilled water was used as a blank probe. TBA extract was obtained by combining 0.8 mL plasma and 0.4 mL trichloro-acetic acid. The samples were then put on ice for 10 min and centrifuged for 15 min at 6,000 rpm. This method was described previously. Distilled water served as a blank probe¹⁵.

Isolated RBCs were washed three times with three volumes of ice-cold 0.9 mmol/L NaCl and hemolysates containing about 50 g Hb/L (prepared according to McCord and Fridovich 1969) were used for the determination of CAT activity^{17, 18}. Then 50 μL CAT buffer, 100 μL sample, and 1 mL 10 mM H_2O_2 were added to the samples. Detection was performed at 360 nm. SOD activity was determined by the epinephrine method. A 100 μL lysate and 1 mL carbonate buffer were mixed, and then 100 μL of epinephrine was added. Detection was performed at 470 nm. Distilled water was used as a blank probe¹⁹.

The level of GSH was determined spectrophotometrically, and it is based on GSH oxidation via 5,5-dithiobis-6,2-nitrobenzoic acid. GSH extract was obtained by combining 0.1 mL 0.1 % EDTA, 400 μL hemolysate, and 750 μL precipitation solution (containing 1.67 g metaphosphoric acid, 0.2 g EDTA, 30 g NaCl, and filled with distilled water until 100 mL; the solution is stable for 3 weeks at +4 °C). After mixing in the vortex machine and extraction on ice (15 min), it was centrifuged at 4,000 rpm (10 min). Distilled water was used

as a blank probe. Measuring was performed at 420 nm. The concentration is expressed as nanomoles per milliliter of RBCs²⁰.

Statistical analysis

IBM SPSS Statistics 20.0 for Windows was used for statistical analysis. Descriptive statistics were used to calculate the arithmetic mean with dispersion measures [standard deviation (SD) and standard error (SE)]. Values were expressed as mean \pm SE. The distribution of data was checked by Shapiro–Wilk test. Data were analyzed using a one-way analysis of variance (ANOVA) and the *post hoc* Bonferroni test for multiple comparisons. Values of $p < 0.05$ were considered to be statistically significant.

Results

Values of NO_2^- were statistically significantly increased in the second trimester compared to the first and third trimester in the normal pregnancy group ($p < 0.05$). In women with thrombophilia, the level of this parameter was increased when compared to the levels in healthy pregnant women in the first and third trimester ($p < 0.05$) (Figure 1a).

In women with thrombophilia, the release of H_2O_2 was significantly higher in the first trimester compared to the second and third trimester ($p < 0.05$). On the other hand, there was no statistically significant change in H_2O_2 generation during the period of gestation in normal pregnancy. The only difference in the level of this parameter between healthy and thrombophilic mothers was noticed in the first trimester, where the level was higher in thrombophilic women ($p < 0.05$) (Figure 1b).

During the observed pregnancy period, in women with thrombophilia, the level of TBARS was higher in the first trimester compared to the second and third trimester ($p < 0.05$). In addition, the level of TBARS in the third trimester was increased compared to the second trimester ($p < 0.05$). In the normal pregnancy group, an increase in the level of this parameter was found in the third trimester compared to the level in the first trimester ($p < 0.05$). A higher level of TBARS was noticed in women with thrombophilia in the first trimester compared to healthy pregnant women ($p < 0.05$) (Figure 1c).

The activity of SOD in the first and second trimester was similar and higher than in the third trimester in women with thrombophilia ($p < 0.05$). During the followed period, lower activity of this enzyme was found in the first compared to the second trimester in healthy pregnant women ($p < 0.05$). The activity of SOD was lower in pregnancy with thrombophilia compared to normal pregnancy in the third trimester ($p < 0.05$) (Figure 2a).

In a group of pregnant women with thrombophilia, the highest activity of CAT was in the third trimester, significantly higher than in the first and second trimester ($p < 0.05$). The activity of CAT was increased in the first trimester compared to the second and third in normal pregnancy ($p < 0.05$). In addition, the lower activity of CAT

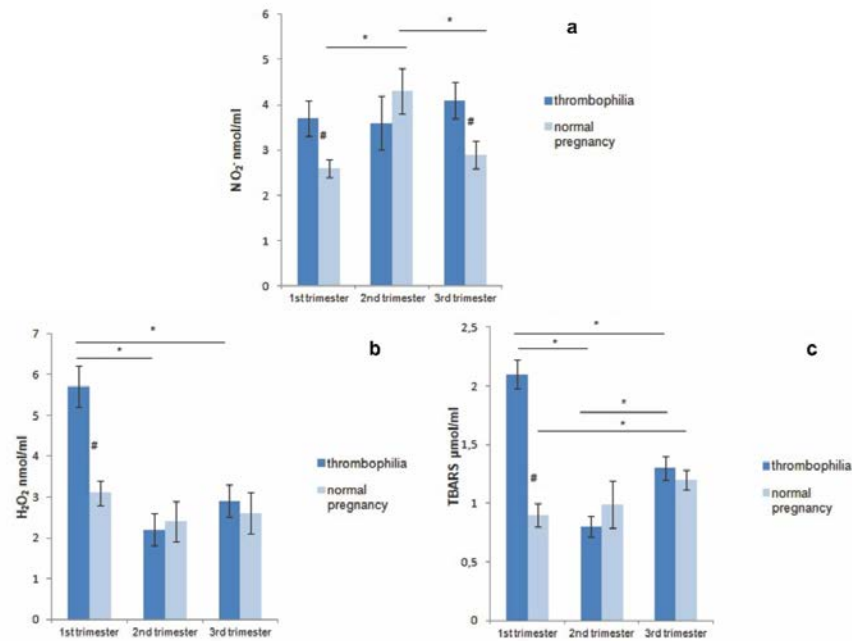


Fig. 1 – Prooxidants in healthy pregnant women and pregnant women with thrombophilia: a) NO₂; b) H₂O₂; c) TBARS. * statistical significance at the level of $p < 0.05$, which refers to the comparison of the 1st vs 2nd vs 3rd trimester; # statistical significance at the level of $p < 0.05$, which refers to the comparison of normal pregnancy vs thrombophilia. NO – nitrites; H₂O₂ – hydrogen peroxide; TBARS – thiobarbituric acid reactive substances.

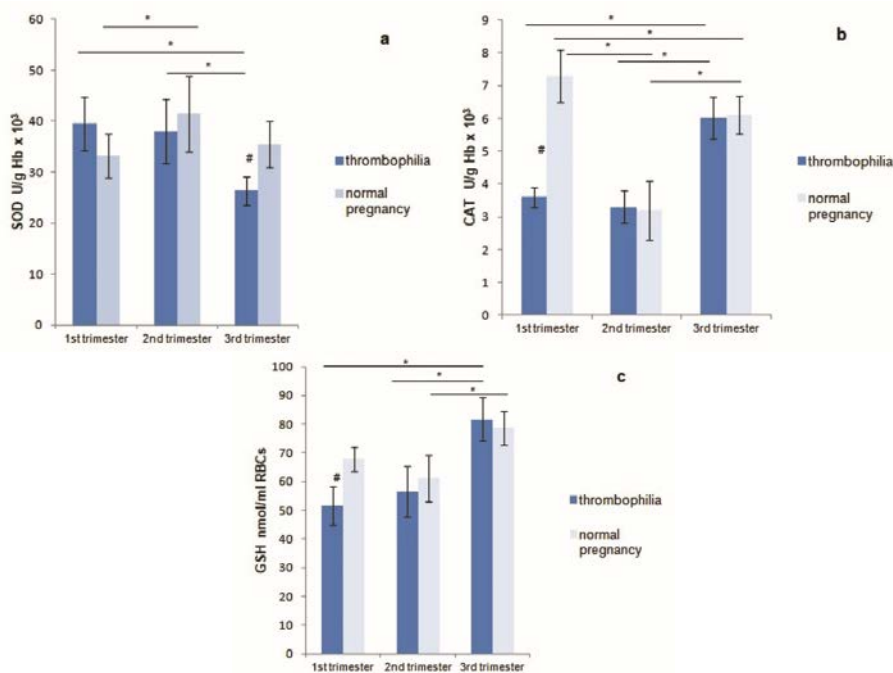


Fig. 2 – Antioxidants in healthy pregnant women and pregnant women with thrombophilia: a) SOD; b) CAT; c) GSH. * statistical significance at the level of $p < 0.05$, which refers to the comparison of the 1st vs 2nd vs 3rd trimester; # statistical significance at the level of $p < 0.05$, which refers to the comparison of normal pregnancy vs thrombophilia. SOD – superoxide dismutase; CAT – catalase; GSH – reduced glutathione.

was revealed in the second compared to the third trimester in healthy pregnant women ($p < 0.05$). The values of this enzyme were higher in the normal pregnancy group compared to the thrombophilia group in the first trimester ($p < 0.05$) (Figure 2b).

Among women with thrombophilia, the value of GSH was similar in the first and second trimester, and those values were significantly lower compared to the value in the third trimester ($p < 0.05$). During the gestation period in healthy women, there was an increase in the level of GSH in the

third trimester compared to the second trimester ($p < 0.05$). The difference in the level of GSH was noticed in the first trimester between normal pregnancy and pregnancy with thrombophilia, where the level was lower in the thrombophilia group ($p < 0.05$) (Figure 2c).

Discussion

This research was designed to estimate the potential differences in dynamics of production of prooxidative and antioxidative markers during the gestation period in normal pregnancy and pregnancy with thrombophilia. In order to complete the picture of redox status in whole pregnancy, we have chosen to follow up on values of oxidative parameters in three periods, at the end of the first, second, and third trimester.

Our results indicate that in normal pregnancy, the release of H_2O_2 did not differ during the gestation period. In addition, NO_2^- levels raised in the second trimester compared to the first and then in the third returned to similar values from the first trimester. Lipid peroxidation was the lowest in the first trimester, then started increasing until the end of the third trimester. Other authors revealed that in the first trimester of pregnancy, lipid peroxidation was the lowest and then increased during pregnancy^{21–23}, which is in correlation with our findings. Few studies showed the different dynamics of lipid peroxidation during the gestation period, which may be a consequence of applying a different methodology from ours since they measured conjugated dienes or lipid hydroperoxides as a marker of peroxidation^{24, 25}.

CAT and GSH values were the lowest, while the value of SOD was the highest in the second trimester of normal pregnancies. On the other hand, the activity of CAT decreased in the third trimester compared to the first. It was previously reported that CAT and SOD activities in placental tissues increased as the pregnancy progressed, while glutathione peroxidase activity (GPx) remained unchanged during the whole pregnancy²². In addition, other researchers confirmed unaltered activities of CAT and GPx in maternal erythrocytes during gestation²³. Antioxidant activity in placental tissues is a significant indicator of the oxidative state of mother and fetus since it has been discovered that a high mother oxidative stress corresponds to an even higher oxidative stress of the newborn¹¹.

In women with thrombophilia, NO_2^- did not significantly change during the pregnancy period, while TBARS and H_2O_2 levels were the highest in the first trimester, followed by a significant decrease in the second and third trimester. Enhanced activity of CAT, which catalyzes the decomposition of H_2O_2 to water and oxygen at the end of gestation, may explain obtained results for H_2O_2 .

The special focus of our investigation was the potential difference in redox status between normal pregnancy and pregnancy with thrombophilia. We noticed higher levels of most of the measured prooxidants in

pregnant women with thrombophilia during the first trimester, while during the second trimester, differences in observed values practically did not exist. Furthermore, NO_2^- was increased in the third trimester in the thrombophilia group as well. In addition to these changes, we revealed lower CAT and GSH values in the first trimester and lower activity of SOD in the third trimester in pregnancy with thrombophilia. Generally viewed, the difference between normal pregnancy and pregnancy with thrombophilia in the second trimester was not observed.

Lower antioxidative protection during pregnancy has been reported, but most studies were focused on the redox status of healthy pregnant women or women with diabetes, preeclampsia, etc. However, data referring to the antioxidative defence system during pregnancy with thrombophilia is limited. One research aimed to evaluate the redox status of the placental tissue after Caesarean delivery in pregnancy with thrombophilia and healthy pregnancy, with a focus on the investigation of the origin of oxidative stress by using both fetal blood and amniotic fluid as samples¹³. The obtained findings suggest that higher activities of CAT and GPx are present in the placental tissue of thrombophilic mothers compared to healthy mothers, and the major prooxidant affecting the placental tissue is H_2O_2 , probably distributed from the mother's blood and endothelium. This research also showed that antioxidative activity in the fetal blood and oxidative status of amniotic fluid was not changed in thrombophilia, thus suggesting that H_2O_2 is most likely distributed to placental tissue from the mother's blood and endothelium¹³.

We noticed an increased level of H_2O_2 in thrombophilia compared to normal pregnancy only in the first trimester. When H_2O_2 passes membranes of placental cells, the placenta tends to defend itself in thrombophilia by antioxidative system activation, particularly CAT, which is indirectly noticed in our study^{11, 13}. It is of great importance given the fact that H_2O_2 can cause dysfunction of placental cells²⁶.

An association between oxidation markers in plasma and antioxidative defence enzymes *ante partum* and *post partum* in thrombophilia was reported as well. Unlike healthy women, increased oxidative stress in thrombophilic women was noticed after delivery, thus suggesting that the placenta plays a role in antioxidative defence for maternal circulation *ante partum*²⁷. Moreover, a very recent study from the same authors showed that SOD, CAT, and GSH are lower in thrombophilic than in healthy women, which is similar to our findings. Moreover, according to the dynamics of these antioxidative molecules through pregnancy, they highlighted oxidative stress increases with the progress of pregnancy²⁸.

Conclusion

Our results suggest an increased generation of prooxidants in thrombophilia at the beginning of gestation, which declines as gestation progresses and reaches similar

values as in normal pregnancy at the end of pregnancy. Generally viewed, thrombophilia was associated with impaired antioxidant capacity – SOD and CAT were lower in the third and GSH in the first trimester compared to healthy women. The connection between thrombophilia and oxidative stress suggests the possibility of implementing antioxidant therapy as an adjuvant in the treatment of thrombophilia in pregnant women.

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Conflict of interest

The authors declare no conflict of interest.

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