THE MATERNAL LEUCOCYTES IN THROMBOPHILIA AND HYPOTHYROIDISM AND THEIR INFLUENCE ON FETAL CELLS

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LEUKOCITI MAJKE U TROMBOFILIJI I HIPOTIREOIDIZMU I NJIHOV UTICAJ NA FETALNE ĆELIJE

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ABSTRACT

The literature data show that thrombophilia and maternal dysfunction of thyroid gland during pregnancy are associated with an increased risk of miscarriage, placental abruption, hypertensive disorders and fetal growth retardation. It was shown that thyroid hormones and hypercoagulable states influence onto a leucocyte activity. The aim of this study has been to investigate maternal leucocytes changes and their correlation with frequency of fetal cells micronuclei in pregnant women with thrombophilia and hypothyroidism. The samples of blood and amniotic fluid were collected from healthy pregnant women and pregnant women with inherited thrombophilia and hypothyroidism (16 - 18 weeks of gestation). Hematological characteristics were determined by using standard hematological methods. The frequency of micronuclei was determined in fetal cells after amniocentesis by using standard cytogenetic methods. The results of this study showed significant higher levels of β-hCG, number of monocytes and eosinophils in blood of pregnant women with thrombophilia. A large number of eosinophils was documented in blood of pregnant women with hypothyroidism. Increased percentage distribution of eosinophils and basophils is shown in both investigated groups of pregnant women. The increased fetal cells micronuclei frequency and their correlation with percentage distribution of eosinophils and basophils were indicated in pregnant women with hypothyroidism. The obtained results suggest that an increased percentage of eosinophils and basophils in pregnant women with hypothyroidism contribute to a formation of micronuclei in fetal cells.

Keywords: *thrombophilia, hypothyroidism, leukocytes, fetal cells, micronuclei*

SAŽETAK

Podaci iz literature pokazuju da su trombofilija i disfunkcija štitaste žlezde majke tokom trudnoće povezane sa povećanim rizikom od pobačaja, odlubljivanjem placente, hipertenzivnim poremećajima i zastojem rasta fetusa. Pokazano je da tireoidni hormoni i stanje hiperkoagulacije utiču na aktivnost leukocita. Cilj ove studije je bio da se ispitaju promene leukocita majke i njihova veza sa učestalošću mikronukleusa u fetalnim ćelijama kod trudnica sa trombofilijom i hipotireoidizmom. Uzorci krvi i amnionske tečnosti sakupljeni su od zdravih trudnica i trudnica sa naslednom trombofilijom i hipotireoidizmom (16 - 18 nedelja gestacije). Hematološke karakteristike su određene korišćenjem standardnih hematoloških postupaka. Učestalost mikronukleusa je određena u fetalnim ćelijama nakon amniocenteze upotrebom standardnih citogenetičkih metoda. Rezultati ove studije pokazuju da je u krvi trudnica sa trombofilijom značajno veća koncentracija β-hCG, broja monocita i eozinofila. U krvi trudnica sa hipotireoidizmom pokazan je veći broj eozinofila. Kod obe grupe ispitivanih trudnica povećan je procenat zastupljenosti eozinofila i bazofila. Kod trudnica sa hipotireoidizmom pokazana je povećana učestalost mikronukleusa u fetalnim ćelijama i njihova korelacija sa procentom zastupljenosti eozinofila i bazofila u krvi majke. Dobijeni rezultati pokazuju da povećani procenat eozinofila i bazofila kod trudnica sa hipotireoidizmom doprinosi formiranju mikronukleusa u fetalnim ćelijama.

Ključne reči: *trombofilija, hipotireoidizam, leukociti, fetalne ćelije, mikronukleus*

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INTRODUCTION

Thrombophilia is inherited or acquired condition that predisposes patients to venous and/or arterial thrombosis and thromboembolic events. In pregnancy and postpartum period, the risk of thromboembolic events increased at a 4- to 5-fold compared to non-pregnant women (1). The main reason for the increased risk of thromboembolism in pregnancy is hypercoagulability, which is a result of hormonal changes and increase of pressure of pelvic veins (2). The literature data show that thrombophilia in pregnancy is associated with many complications such as severe preeclampsia, intrauterine growth retardation, abruption placentae, still birth and recurrent miscarriage (3). Thrombophilia in pregnancy is characterized by microthrombi generation, which induces mechanical damage of endothelium and leukocytes activation. On the other hand, inflammatory stimuli activate the coagulation (4). Wirstlein and coworkers (5) showed that the concentrations of pro-inflammatory factors are increased in the new-borns cord blood of mothers with thrombophilia.

The optimal function of thyroid gland during pregnancy is very important for a mother and a fetus development. The most common thyroid disorder in pregnancy is hypothyroidism, which occurs with a frequency of 3-5% of all pregnant women (6). Maternal dysfunction of thyroid gland during pregnancy is associated with an increased risk of miscarriage, placental abruption, hypertensive disorders and growth restriction (7). Thyroid hormones have a significant role in regulation of activation of leucocytes, which are important sources of reactive oxygen and nitrogen species (8). In hyperthyroid patients have been found increase number of eosinophils and mononuclear cells as well as the reduction in the number of neutrophils (9). Activated leucocytes produce large amount of reactive oxygen and nitrogen species that can damage DNA, lead to genomic instability and consequence to a formation of micronuclei (10).

Micronuclei frequency in cells cytoplasm is the sensitive biomarker of DNA damage by endogenous and exogenous toxins. Micronuclei are the structures formed as a result of DNA fragmentation or lagging of acentric chromosome or chromatid during mitosis. The increased frequency of micronuclei as biomarkers of genetic instability is shown in cancer, diabetes, autoimmune, neurodegenerative and cardiovascular diseases (11-13).

The aim of this study was to investigate maternal hematological changes and their correlation with frequency of fetal cells micronuclei in pregnant women with thrombophilia and hypothyroidism.

MATERIAL AND METHODS

Patients

The study protocol was approved by the Ethics Committee of Clinical Centre "Kragujevac" and all patients had given an informal consent. The subjects were selected from pregnant women who attended Obstetrics and Gynecology Department of Clinical Centre "Kragujevac", from June 2014 to June 2015. The indications for amniocentesis were the subject inclusion criterion. Three groups of pregnant women, gestational age from 16 to 18 weeks, were recruited to the study: (i) 32 healthy pregnant women (control group) who recommended age (> 35 years) for amniocentesis, (ii) 23 pregnant women with inherited thrombophilia and (iii) 23 pregnant women with hypothyroidism. The maternal age, thrombophilia and hypothyroidism in pregnancy provide justification for amniocentesis, since in these conditions; there is an increase of the incidence of perinatal morbidity. The inherited thrombophilia was defined by following mutations: FV Leiden (G1691A), FII (G20210A), MTHFR (C677T) and PAI-1 mutation. All mutations were confirmed by molecular diagnostic methods. Pregnant women with inherited thrombophilia received proper doses of Fraxiparine or Clexane, depending on the type of mutation, and it was present in a homozygous or heterozygous form. Hypothyroidism was defined by serum concentrations of thyrotropin (TSH) and free thyroxine (FT4). Pregnant women with hypothyroidism received different doses of Eutyrox, depending on the serum hormone concentrations. The study included pregnant women who were non-smokers and free from cardiovascular, hepatic and renal disorders. Red blood cells and platelet count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, hematocrit value, prothrombin time, concentrations of C-reactive protein, hemoglobin and chorionic gonadotropin were determined in hospital laboratories. The clinical and hematological characteristics of pregnant women are shown in Table 1.

Isolation and cultivation of fetal cells from amniotic fluid

The samples, in the amount about 15 to 20 ml, were obtained by amniocentesis from amniotic fluid from pregnant women at gestation weeks from 16 to 18. The samples were centrifuged at 1800 rpm for 20 minute and supernatant removed. After centrifugation, the cell pellet was washed and resuspended in growth medium AmnioMax (Gibco, Life Technologies, USA) containing fetal bovine serum and transferred to the cell culture flask. The cells were incubated at 37 ^{0}C in 5% CO_2 atmosphere to achieving confluence.

Micronucleus in vitro assay in fetal cells

For *in vitro* micronucleus assay, it was used stock of Cytochalasin-B disolved in DMSO and diluted in medium before use. After achieving needed confluence of cell culture, 4μg/ml Cytochalasin-B was added to cell culture flask to block cytokinesis and incubated for 16 h at 37 ^{0}C in 5% CO_2 atmosphere. Wen incubation was finished and the cells were detached from the flask by 0.1% trypsin, which was inactivated

Table 1. Clinical and hematological characteristics of healthy pregnant women and pregnant women with thrombophilia and hypothyroidism.

Characteristic	Control	Thrombophilia	Hypothyroidism
Maternal age (years)	37.57 ± 0.34	37.72 ± 0.50	37.50 ± 0.84
Gestational age (weeks)	17.53 ± 0.07	17.41 ± 0.09	17.44 ± 0.60
Number of deliveries	0.94 ± 0.13	0.44 ± 0.14*	0.80 ± 0.20
Number of spontaneous abortion	0.42 ± 0.15	0.88 ± 0.19*	0.30 ± 0.10
C-reactive protein (CRP) (nmol/L)	3.65 ± 0.52	4.65 ± 0.80	4.80 ± 0.71
Red blood cells count (x 10^{12}/L)	4.11 ± 0.45	3.98 ± 0.90	3.91 ± 0.06*
Hematocrit (%)	0.36 ± 0.004	0.36 ± 0.03	0.35 ± 0.06
Hemoglobin (g/L)	121.74 ± 1.27	120.77 ± 1.67	117.10 ± 1.99*
Mean corpuscular volume (MCV) (μm^3)	87.18 ± 0.78	91.25 ± 0.84*	89.43 ± 1.00
Mean corpuscular hemoglobin (MCH) (pg/cell)	36.63 ± 6.95	45.48 ± 14.92	30.17 ± 0.37
Mean corpuscular hemoglobin concentration (MCHC) (g/L)	339.77 ± 2.55	315.07 ± 18.06	337.79 ± 3.28
Platelet count (x 10^9/L)	230.57 ± 9.41	230.39 ± 13.41	258.70 ± 14.49
Prothrombin time (PT) (s)	0.96 ± 0.013	0.98 ± 0.017	0.96 ± 0.01
Human chorionic gonadotropin (β-hCG) (U/L)	1.29 ± 0.80	2.13 ± 0.93*	1.60 ± 0.37

Values are means ± S.E.M. * $p < 0.05$, thrombophilia and hypothyroidism versus control.

after 2 to 3 minute by adding 1 ml DMEM, containing fetal bovine serum. The cell suspension was centrifuged at 1000 rpm for 10 minute, washed by 0,9% NaCl and subjected to a mild hypotonic treatment by 0.75% KCl in 1 minute. Then, the cells were centrifuged 1000 rpm for 10 minute and fixed in Carnoy's fixative (3:1, methanol : acetic acid) for 30 minutes. Finally, the cells were resuspended in a small volume of fixative (100-200 µl), dropped onto cleaned slides and dried in air. The slides stained by 4% Giemsa stain in buffer (pH 6.8), for a few seconds. The slides were covered with a coverslip and observed under a microscope. The minimum 1000 binuclear cells were scored from each slide for the presence of micronuclei. The identified and scored micronuclei were expressed as percentage micronuclei, at the 1000 binuclear cells (12).

Statistical analysis

The data is expressed as mean ± S.E.M. The statistical analysis was performed by using SPSS (version 20.0) for Windows. The independent *t*-test was used for comparison of data between the control group and the examined groups. The correlation coefficient (*r*) was determined by using Pearson Correlation. The statistical significance was accepted at the $p \leq 0.05$ for all comparisons and in all correlations. Only significant correlation coefficients are reported.

RESULTS

Clinical and hematological characteristics of healthy pregnant women and pregnant women with thrombophilia and hypothyroidism

Table 1 shows clinical characteristics of healthy pregnant women and pregnant women with thrombophilia and hypothyroidism. The maternal and gestational ages were not significantly different between the groups. Based on the results, significantly a low level of successful completion of pregnancy and significantly a higher incidence of spontaneous abortions were shown in women with thrombophilia compared to healthy pregnant women. In addition, pregnant women with thrombophilia showed significantly higher values of mean corpuscular volume (MCV) and chorionic gonadotropin (β-hCG) in their blood. Red blood cells count and concentration of hemoglobin were significantly lower in the blood of women with hypothyroidism in comparison to healthy pregnant women. Concentrations of C-reactive protein (CRP) and hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), prothrombin time (PT), platelet count and hematocrit were not significantly different between groups.

White blood cells count, the number and percentage of different types of leukocytes in the blood of healthy pregnant women and pregnant women with thrombophilia and hypothyroidism

The white blood cells count in the blood of healthy pregnant women and pregnant women with thrombophilia and hypothyroidism is presented in Figure 1. The obtained results show there is no difference in the number of white blood cells between the examined groups. Figure 2 shows results of the number of certain types of leukocytes in blood of healthy and pregnant women with thrombophilia and hypothyroidism. The results show no difference in neutrophils, lymphocytes and basophiles count between these groups. Pregnant women with thrombophilia have a significantly higher number of monocytes and basophil granulocytes in their blood

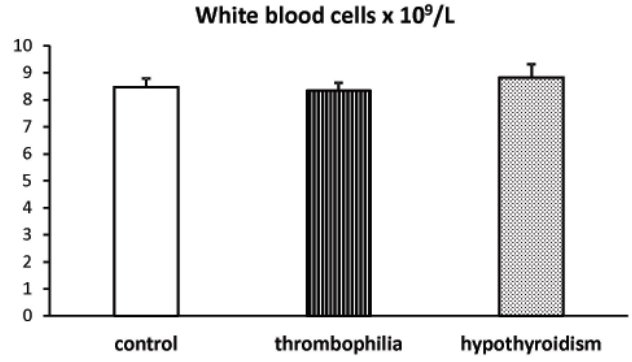

Figure 1. Total number of white blood cells in healthy pregnant women and pregnant women with thrombophilia and hypothyroidism.
Values are means ± S.E.M. * p < 0.05, thrombophilia and hypothyroidism versus control.

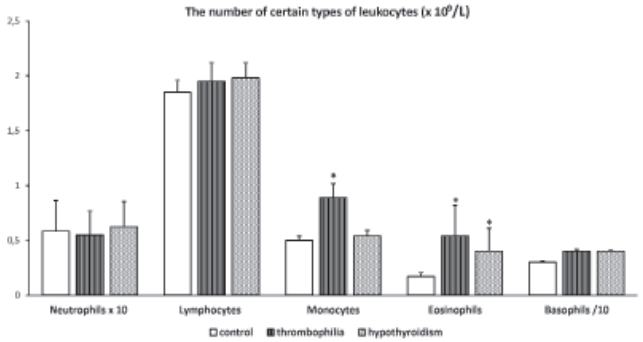

Figure 2. The number certain type of leucocytes in healthy pregnant women and pregnant women with thrombophilia and hypothyroidism.
Values are means ± S.E.M. * p < 0.05, thrombophilia and hypothyroidism versus control.

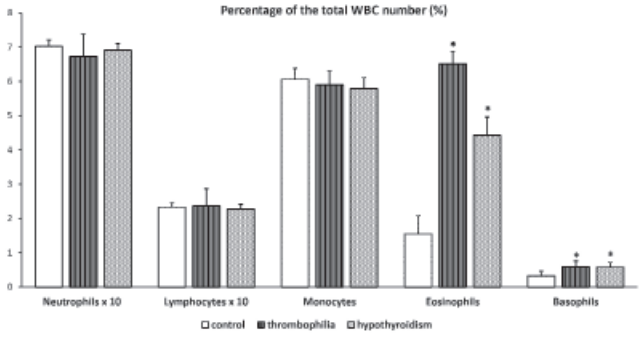

Figure 3. Percentage of distribution certain type of leucocytes of the total white blood cells number (WBC) in healthy pregnant women and pregnant women with thrombophilia and hypothyroidism.
Values are means ± S.E.M. * p < 0.05, thrombophilia and hypothyroidism versus control.

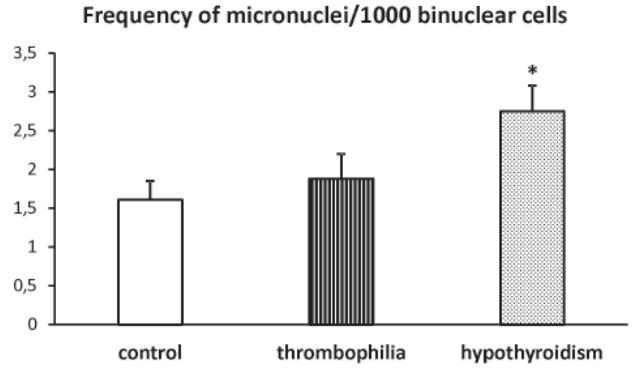

Figure 4. Frequency of fetal cells micronuclei in healthy pregnant women and pregnant women with thrombophilia and hypothyroidism.
Values are means ± S.E.M. * p < 0.05, thrombophilia and hypothyroidism versus control.

than healthy pregnant women. The number of eosinophil granulocytes is significantly higher in the blood of women with hypothyroidism in comparison to healthy pregnant women. The results of percentage different type of leucocytes are presented in Figure 3. The percentage of eosinophil and basophil granulocytes is significantly higher in the blood of women with thrombophilia and hypothyroidism compared to healthy pregnant women. The percentage of other type leukocytes showed no significant difference in blood between the examined groups.

Frequency of micronuclei in fetal cells

The frequency of micronuclei in fetal cells is presented in Figure 4. Our results show significantly higher frequencies of micronuclei in fetal cells of pregnant women with hypothyroidism than in healthy pregnant women. The difference in the frequency of micronuclei in fetal cells of healthy pregnancy and pregnant women with thrombophilia is not indicated.

Correlations

The results of this study show that the frequency of micronuclei in fetal cells has a significant positive correlation with percentage of eosinophil (r = 0.915, p < 0.011) and basophil (r = 0.767, p < 0.044) granulocytes in maternal blood in pregnant women with hypothyroidism. The positive correlations of eosinophil and basophil granulocytes percentage in maternal blood with frequency of micronuclei in fetal cells were not significant in healthy and women with thrombophilia.

DISCUSSION

Hormonal and metabolic changes during normal pregnancy predispose mother to thrombosis and higher hormone output by thyroid gland (3,14). Inherited thrombophilia in pregnancy additionally increases predispose to thrombosis and thromboembolic events (1) leading to fetal

morbidity and mortality (3). The results of this work show that pregnant women with inherited thrombophilia in comparison to healthy pregnant women have a significantly lower incidence of life birth and a significantly higher incidence of miscarriages of the total number of pregnancy. In pregnancy with thrombophilia increased clotting in the placental vasculature resulting in placental infraction and hypoxic condition (15), leading to spontaneous abortion. The increased concentration of β-hCG in maternal serum indicates ischemic condition of the placenta leading to placental infraction (16-17). According to our results, the concentration of β-hCG is significantly higher in maternal serum with thrombophilia than in healthy pregnancy. Reports in the literature are in contradiction and indicate no significant difference (18) and significantly lower (19) concentration of β-hCG in pregnancy with inherited thrombophilia compared to healthy pregnant women.

In normal pregnancy increases the total number of red blood cells, but relative less than increases the plasma amount, which results in a dip in hemoglobin concentration. This dilutional anemia is physiological condition in normal pregnancy (20). Our results indicate that the total number of red blood cells and concentration of hemoglobin are significantly lower in pregnant women with hypothyroidism in comparison to healthy pregnancy. The data in the literature suggests that anemia is often the first sign of hypothyroidism (21), but based on our results, the reduction in the total number of red blood cells and concentration of hemoglobin in women with hypothyroidism are in the level of reference values for pregnancy (20). A higher level of MCV, which observed in pregnant women with thrombophilia, is within reference range (80 - 100 μm^3).

Numerous reports in the literature suggest that hemostasis is intimately linked to inflammation, where in each process propagate and intensifies the other, creating the potential for a vicious cycle of thrombosis and inflammation (22,23). The results obtained in our study show that the total number of white blood cells is not significantly different in pregnancy with thrombophilia and hypothyroidism compared to the control one. Interestingly, we found a significant higher number of monocytes and eosinophils in the whole blood of pregnant women with thrombophilia than in healthy pregnancy. In addition, we found a significant difference in leukocyte formula between thrombophilia and control, and our results show that the blood of pregnant women with thrombophilia presence of eosinophils and basophils is significantly higher. Hypercoagulable state in pregnancy with inherited thrombophilia is accompanied with increasing in platelets activity and aggregability (3). Activated platelets degranulate and express of P-selection on surface, which binds to ligands located on monocytes (24). The result of this interaction is the formation of platelet-leukocyte aggregates that produce proinflammatory, procoagulant, oxidative and mitogen substances that can cause arterial thrombosis (25). Lukanov and coworkers (26) showed that levels of platelet-leukocyte aggregates are significantly higher in pregnant women with thrombophilia. The increased number of eosinophils is associated with a high risk of thromboembolism. During degranulation, eosinophils release tissue factor, which initiates of clotting cascade, and major basic protein that inhibits potent anticoagulant thrombomodulin and activates of platelets (27). Released major basic protein from eosinophils induces activation and number increase of basophils, which have granules that contain histamine, platelet-activating factor, several cytokines, proteolytic enzymes and bioactive proteoglycans (28).

Thyroid hormone showed to influence the immune system and disturbance in thyroid hormone secretion may disturb the immune response (29). In the present study, a higher number of eosinophils were observed in pregnant women with hypothyroidism than in healthy pregnant women. Eosinophils percentage distribution of the total WBC number is also significantly higher in pregnant women with hypothyroidism. Jafarzadeh and co-workers (30) suggest that hypothyroidism is a condition characterized by an increase in eosinophils. It is known that thyroid hormones regulate human hematopoietic system and that eosinophils differentiation from bone marrow is stimulated in hypothyroidism condition (9). Thyroid hormones exert a depressive effect on the differentiation and the number of basophils. Literature data show that number of basophils is not statistically different in hypothyroidism comparison to healthy subjects (31). Our results also show that there is no difference in the number of basophils between pregnant women with hypothyroidism and healthy ones. However, on the basis of these results, the basophils percentage distribution of the total WBC number was significantly higher in pregnant women with hypothyroidism.

Eosinophils and basophils are capable for oxidative brust by production of reactive oxygen species (32,33). Reactive oxygen species produced in large amount from maternal eosinophils and basophils can cross the placenta and induce genomic instability and consequence to formation of micronuclei (10) in fetal cells. Our results indicate a significantly higher frequency of micronuclei in fetal cells in pregnant women with hypothyroidism than in healthy pregnant women. Based on the present results, the frequency of fetal cells micronuclei is significantly positive correlated with the percentage of maternal eosinophil and basophil in pregnant women with hypothyroidism. These results suggest that an increased percentage of the distribution and activities of eosinophils and basophils in hypothyroidism contribute to the formation of micronuclei in fetal cells and can affect to a normal fetus development and successful completion of pregnancy.

CONCLUSION

In summary, the results of the present study demonstrate significant hematological changes in pregnant women with thrombophilia including higher levels of β-hCG, number of monocytes and eosinophils. Hematological

changes in pregnancy with hypothyroidism were reflected in the increase of eosinophil number. Eosinophil and basophil percentage distribution were documented in thrombophilia and hypothyroidism. The increased fetal cells micronuclei frequency and their correlation with eosinophils and basophils were indicated in hypothyroidism. Future studies require investigating a concentration of products activities of maternal eosinophil and basophil in amniotic fluid and their correlation with the frequency of micronuclei in fetal cells.

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Conflict of interest

The authors have no conflicts of interest to declare.

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