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SELÇUK TIP DERGİSİ

Gebelerin Kan Lenfositlerinde AgNOR ve MN Değeri ile ANAE ve ACP-az Pozitivitelerinin Belirlenmesi

Determination of the AgNOR Parameters, MN Frequency, ANAE and ACP-ase Positivity of PBL in Pregnants

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Özet

Bu çalışma, bayanlarda hamileliğin perifer kan lenfositlerinin alfa naftil asetat esteraz (ANAE) ve asit fosfataz (ACP-az) aktiviteleri ile perifer kan lenfosit oranları üzerindeki etkilerinin belirlenmesi amacıyla yapıldı. Ayrıca perifer kan lenfositlerinde mikronükleus (MN) sıklığı ile bazı AgNOR parametreleri de belirlendi. Bayanlar Kontrol; I. trimester; II. trimester ve III. trimester olmak üzere 4 gruba ayrıldılar (n= 10). Perifer kan örneklerinden hazırlanan kan frotilerine ANAE ve ACP-az demonstrasyonu ile modifiye May-Grünwald-Giemza ve AgNOR boyamaları uygulandı. En düşük ANAE-pozitif lenfosit oranı II. trimesterde tespit edilirken (%58,2) gruplar arasındaki farklar istatistiksel açıdan önemli bulundu (p<0,05). I. (57,9%) ve III. trimesterde (%57,8) ACP-az pozitif lenfosit oranlarında istatistiksel olarak önemli düşüşler gözlendi. Hamilelik süresince perifer kan lenfosit oranlarında belirgin düşüşler dikkati çekerken (p<0,05) en yüksek null hücre oranı (%11) I. trimesterde tespit edildi. MN sıklığında hamilelik süresince belirgin artışlar gözlendi (p<0,05). AgNOR sayılarında gruplar arasında fark tespit edilmezken; AgNOR alanı/Çekirdek alanı oranının hamilelikle birlikte artarak en yüksek değerine (%10,22) III. trimesterde ulaştığı görüldü (p<0,05). Trimesterler arasında bazı farklar olsa da hamileliğin ANAE- ve ACP-az pozitif perifer kan lenfosit oranları ve perifer kan lenfosit oranı ile MN sıklığı ve bazı AgNOR parametrelerini etkilediği sonucuna varıldı.

Anahtar kelimeler: ACP-az, AgNOR, ANAE, Gebelik, Mikronükleus.

Abstract

This study was performed to determine the effects of pregnancy on the positivity rates of alpha- naphthyl acetate esterase (ANAE) and acid phosphatase (ACP-ase) of the peripheral blood lymphocytes (PBL) and PBL percentages in pregnant women. Micronucleus frequency (MN) and some AgNOR parameters of PBL were also estimated. Women divided into four groups as Control; Trimester I (TRI); Trimester II (TRII) and Trimester III (TRIII) (n= 10 for each group). ANAE and ACP-ase demonstrations, modified May-Grünwald Giemsa and AgNOR staining were performed on blood smears prepared from peripheral blood samples. The lowest T-lymphocytes percentage (58,2%) was determined in TRII and the differences between the groups were statistically important (p<0,05). There was a statistically significant decrease in the proportions of the ACP-ase (+) lymphocytes in TRI (57,9%) and TRIII (57,8%). A dramatic reduction in the percentage of PBL was recorded during pregnancy (p<0,05), whereas the highest null cell rates (11%) were found in TRI. A distinct increasing was observed in MN frequency during pregnancy (p<0,05). There was no difference in mean AgNOR counts between the groups, whereas AgNORs area/nucleus area increased during pregnancy and it gained its highest value (10,22%) in TRIII. It was concluded that the ANAE- and ACP-ase positive PBL proportions, PBL percentages, MN frequency and some AgNOR parameters were affected by pregnancy although there were some differences among the TRs.

Key words: ACP-ase, AgNOR, ANAE, MN, Pregnancy.

INTRODUCTION

Pregnancy is needed privilege immunologic status because the embryo is genetically derived from both mother and father. However, the fetus avoids immune rejection. This process is described as maternal immune tolerance by the reproductive immunologists. Actually, some interactions must occur between the embryo and endometrium for the recognition of conceptus and maintnance of the pregnancy. Finally, this period coincides with a series of some changes of the proportion and distribution of maternal immune system cells, especially T-lmphocytes and natural killer (NK) cells in peripheral blood and endometrial tissue (1, 2). Mahmoud et al. (3) have reported that a significant decrease in the total lymphocyte numbers, especially affecting B lymphocytes and NK cells in healthy pregnant women. Medina et al. (4) have observed that the B lmphopoiesis was depressed during pregnancy in mice.

Some researchers have reported that there were close relationship between immune maladaptation and some certain pregnancy disorders such as recurrent spontaneous abortion (RSA) or pre-eclampsia. T-cell subset and/or natural killer (NK) cell profiles of peripheral blood lmphocytes were found different in women with RSA and pre-eclampsia compared with healthy pregnant women. Bardeguez et al. (5) found significantly lower proportion of Th cells in women who later developed pre-eclampsia whereas Shakhar et al. (6) reported that the proportion and activity of the NK cells increased in women with RSA. Because having the predictive value of these cells for some disorders during

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pregnancy, to determine the reference data of these cells in healthy pregnant and non-pregnant women is very important (7, 8). Alpha-naphthyl acetate esterase (ANAE) is a lysosomal enzyme, which has been demonstrated in mature, immunocompetent T-lymphocytes of many mammalian species including humans (9). The enzyme is regarded as to be responsible for the cytotoxic effects of T-lymphocytes and the phagocytic activity of monocytes (10). However, it was assumed that the dot-like positivity pattern was specific for T-lymphocytes (9) whereas fine granular positivity pattern was specific for null cells (11). ACP-ase is also one of the lysosomal enzyme in lymphocytes. Some investigators demonstrated the ACP-ase reactivity in human peripheral blood T-lymphocytes (12).

Micronuclei (MN) are accepted choromosomes, chromatids or acentric chromosomal fragments which occur as a result of chromosomal damage. MN are visible after anaphase. They are small, round-shaped particles in the cytoplasm and they stain similarly to the cell nucleus. MN sizes are almost one third of the main cell nucleus. Because the demonstrating of MN in peripheral blood lymphocytes is practical, reliable and reproducible biomarker of some chromosomal damages, MN frequency test is used the demonstrate the possible genotoxic effects of several chemical substances or drugs especially used during pregnancy (13-16). Nucleolus organizing regions (NORS) are the loops of DNA containing ribosomal RNA genes. These regions are demonstrated as silver-stained black dots (AgNORS) in the cell nucleus with colloidal silver. The size and number of these structures reflect transcriptional, nucleolar and proliferative activity in relation to RNA synthesis (17).

In this study, we carried out to determine the percentages of the T-lymphocytes, null cells, ACP-ase positive peripheral blod lymphocytes, and the proportion of peripheral blood lymphocytes in healthy pregnant women. Furthermore, the MN frequency and AgNOR activity of the peripheral blood lymphocytes were evaluated to establishment of baseline values for future studies in this and/or related research because of the importance of the immune system status during pregnancy.

MATERIALS AND METHODS

Materials

This study was approved by Ethical Committee of University of Selcuk, Meram Faculty of Medicine (2008/112). Peripheral blood samples were collected from 10 healthy non-pregnant women (control group) and 30 healthy pregnant women during three different gestational stages (first, second and third trimester-TRI, TRII, TRIII, respectively, n=10) which determined by gynecologist. Peripheral blood samples were taken in heparinized tubes.

Methods

From each blood samples, seven blood smears were prepared. AgNOR staining was performed one of these smears whereas two smears were stained for each ANAE and ACP-ase demonstrations and MN frequency test.

Alpha-Naphthyl Asetate Esterase (ANAE) Demonstration

To demonstrate the ANAE positivity, blood smears were fixed in a glutaraldehyde-acetone solution at −10° for 3 min, rinsed in distilled water, and then air-dried. The working solution was prepared by mixing 20 mg of substrate, α-naphthyl-acetate (N-8505, Sigma, Steinheim, Germany) dissolved in 0,8 mL of acetone (Merck, Darmstadt, Germany), 4,8 mL of hexazotized pararosaniline [hexazotization was performed by mixing equal volumes (2,4 mL each) of 4% sodium nitrite (Merck) and 2% pararosaniline (Merck)], and 80 mL of PBS (pH 5). Final pH of the solution was adjusted to 5,8 with 1 N NaOH, and the solution was filtered. After a 2-h incubation at 37°C, the smears were rinsed 3 times in distilled water, and nuclei were stained for 20 min in 1% methyl green prepared in acetate buffer (pH 4,2) (9).

Acid Phosphatase (ACP-ase) Demonstration

Acid phosphatase was demonstrated by the method of Sur et al. (18). Briefly, the blood smears were fixed in formal-calcium at +4°C for 10 min, and the smears were rinsed 3 times in distilled water. An incubation solution was prepared by mixing 20 mg of naphthol AS-BI phosphate (N-2125, Sigma) dissolved in 2 mL of N,N-dimethyl formamide (Sigma), 25 mL of distilled water and 1,6 mLof hexazotized pararosaniline (prepared as in the ANAE incubation solution), and 71 mL of citrate buffer (pH 5). Final pH of the solution was adjusted to 5,0 with 1 N NaOH, and the solution was filtered. After a 2-h incubation at 37°C, the slides were rinsed 3 times in distilled water, and the nuclei were stained for 20 min with 1% methyl green prepared in acetate buffer (pH 4,2).

Demonstration of MN

To determine of the MN frequency in peripheral blood lymphocytes and the proportion of the peripheral blood lymphocytes, the blood smears were stained with modified May-Grünwald-Giemsa solution (19).

AgNOR Staining

The smears were stained with a solution containing one volume of 2% gelatine in 1% aqueous formic acid and two volumes of 50% silver nitrate (Merck). The staining was performed at 37oC in the dark for 20-30 minutes (18).

Evaluation of The Stained Specimens

ANAE and ACP-ase Histochemistry

In the blood smears which were performed ANAE, two different positivity patterns were observed. In T-lymphocytes, there were one to four dot-like reddish brown granules (Fig. 1) whereas the null cells had five or much more granules (Fig. 2). Otherwise, in the smears demonstrated ACP-ase, PBL containing one to three pinkish-red cytoplasmic granules were considered ACP-positive (Fig. 3). In each of the specimens demonstrating ANAE and ACP-ase, 200 lymphocytes were counted and positivity rates were expressed as the percentage of counted cells.

MN Frequency

MN were observed small, round-shaped particles near the lymhocyte nucleus (Fig. 4). MN frequency was determined by analyzing

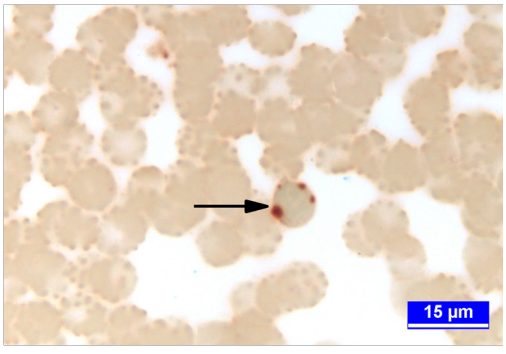

Figure 1. *A peripheral blood T-lymphocyte in a pregnant woman from TRI group. ANAE demonstration. Arrow: Peripheral blood T-lymphocyte. Bar: 15 µm.*

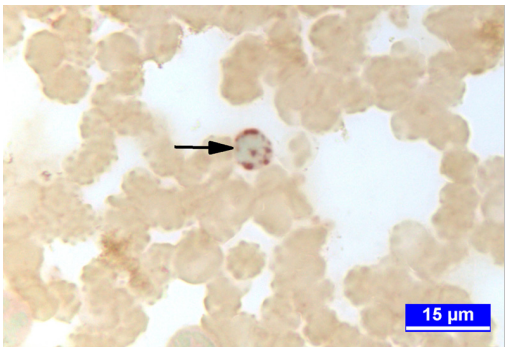

Figure 2. *A null cell in a pregnant woman from TRI group. ANAE demonstration. Arrow: Null cell. Bar: 15 µm.*

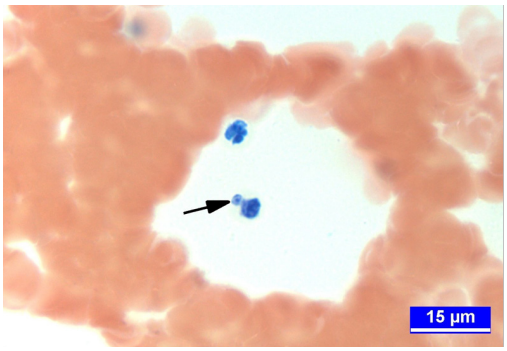

Figure 4. *A peripheral blood lymphocyte with a micronucleus (arrow) in a pregnant woman from TRIII group. Modified May Grünwald-Giemsa staining. Bar: 15 µm.*

1000 lymphocytes per smears.

AgNOR Staining

The black patches having round shapes in the lymphocyte nuclei were evaluated as AgNORs and they were measured to obtain morphological and numerical AgNOR parameters mentioned in below (Fig 5). Briefly, in each smears performed AgNOR staining, 10 lymphocytes were analyzed. The transverse diameter of lymphocytes nuclei, the nuclear area, the AgNOR area and AgNOR counts per nucleus were analysed with an image analysis programme (IM-50). Also, the percentage of the AgNOR area relative to the whole nuclear area was calculated. All blood smears were examined with a light microscope (Leica DM2500, Leica Microsystems GmbH, Wetzlar, Germany) and photographs were taken with Leica DFC 320 model digital camera.

Statistical Analyses

All statistical analyses, in particular ANOVA and DUNCAN tests, were conducted with the Statistical Package for the Social Sciences (20). The proportions of PBL, T-lymphocytes, null cells and ACP-ase positive lymphocytes were analysed with one-way ANOVA (SPSS, 10.0) following arc-sin transforming. Significance was set at $p<0.05$. The percentage of the AgNOR area relative to the whole nuclear area was analyzed with DUNCAN test (SPSS 10.0) whereas AgNOR counts and MN frequencies were analyzed using t-test.

RESULTS

Histochemistry

In ANAE demonstration, T-lymphocytes proportion of non-pregnant women was found 70% whereas the lowest T-lymphocytes percentage was determined in TRII (58,2%) and the differences between the groups were statistically important ($p<0,05$). The increasing of the null cell rates in TRI was very distinctive. A dramatic reduction in the percentage of PBL was recorded during pregnancy ($p<0,05$). There was a statistically significant decrease in the proportions of the ACP-ase (+) lymphocytes in TRI and TRIII (57,9% and 57,8% respectively). A markedly increasing in MN frequency was found during pregnancy ($p<0,05$). The highest MN frequency (14,60/1000 cells) was observed in TRIII. There was no difference in mean AgNOR counts between the groups, whereas AgNORs area/nucleus area increased during pregnancy and it gained its highest value (10,22%) in TRIII.

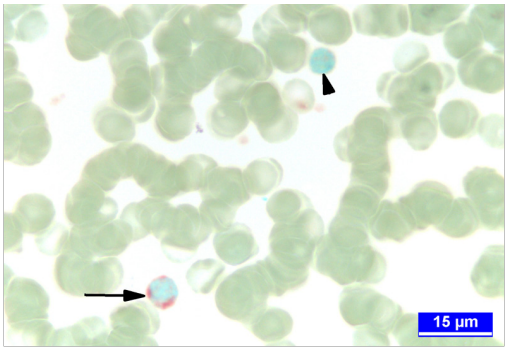

Figure 3. *An ACP-ase-positive (arrow) and negative (arrow heads) peripheral blood lymphocyte in a woman from control group. ACP-ase demonstration. Bar: 15 µm.*

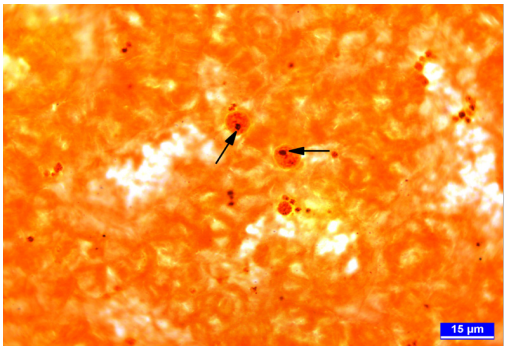

Figure 5. *Two peripheral blood lymphocytes (arrows) contained AgNOR dots in a woman from control group. Bar: 15 µm.*

Tablo 1. *MN frequency, some AgNOR parameters and the proportions of the peripheral blood lymphocytes, T-lymphocytes, null cells, and ACP-ase lymphocytes in different gestational stages.*

Groups	Proportions of peripheral blood T-lymphocytes (%)	Proportions of peripheral blood nul cells (%)	Proportions of peripheral blood I ACP-ase lymphocytes (%)	Proportions of peripheral blood lymphocytes (%)	Ratio of AgNORs area to nucleus area (%)	Mean AgNOR number per nucleus of peripheral blood lymphocytes	Frequency of MN in peripheral blood lymphocytes (MN/1000 lymphocytes)
Control	70,00±1,6^{a}	2,00±0,51^{b}	69,9±1,84^{a}	39,4±1,21^{a}	7,39±0,28^{b}	1,45±0,12^{a}	5,8±0,87^{c}
TRI	60,9±1,36^{b}	11,00±1,22^{a}	57,9±2,59^{b}	35,2±2,26^{a}	8,66±0,44^{ab}	1,57±0,08^{a}	11,7±1,06^{b}
TRII	58,2±1,55^{b}	2,2±0,57^{b}	66,4±3,4^{a}	27,3±2,19^{b}	9,55±0,7^{a}	1,56±0,15^{a}	11,8±1,35^{ab}
TRIII	62,2±1,81^{b}	2,5±0,5^{b}	57,8±1,51^{b}	24,9±1,46^{b}	10,22±0,87^{a}	1,44±0,08^{a}	14,60±0,03^{a}

a-c: Values within a column with no common superscripts are significantly (p<0.05) different, ±SE.
TRI: First Trimester
TRII: Second Trimester
TRIII: Third Trimester

DISCUSSION

During the succesfull pregnancy, embryo, fetus and placental tissues escape the maternal immune responses because these structures resemble the semi-allogenic graft. At the begining of the implantation process, the embryo causes temporary inflamation that facilitated placental deveopment. These events affect the proportions of the some certain peripheral blood cells. The major changes are observed in the lymphocyte subpopulations (1, 21, 22). Iwatani et al. (23) determined that distinct decreasing in absolute counts of T-and B-lymphocyte and T-cell subsets in the first trimester and during the pregnancy. Watanabe et al. (24) reported that significant decrease in the number of cytotoxic T-cells and hepler T-cells in early and late pregnancy, respectively. The same investigators (24) also observed the decreased numbers of T-alpha beta, CD5- B and CD5+ B cells during pregnancy. In this study, the mean proportion of the T-cell demonstrated by using ANAE histochemistry was determined as 70% in healthy non-pregnant women. However, in the first trimester, T-lymphocyte number was decreased down to 60,9% and this suppression was proceeded during pregnancy (p<0,05, Table 1). It is suggested that the early suppression of T-lymphocytes may be aimed to survive of embryo.

In this study, an important finding was the null cell percentages which determined by using alpha naphthyl acetate esterase (ANAE) histochemistry. Null cells have been accepted as natural killer (NK) cell (25, 26) and some investigators (9, 11) claimed that these cells could be demonstrated with many ANAE positive granules in human. It is supposed that these cells increase at the begining of the pregnancy. Iwatani et al. (23) found significant increasing in the percentage of NK cells in the first trimester. The researchers (23) also pointed out that NK cells proportions gradually decreased in the second and third trimesters. Watanabe et al. (24) reported the increase of NK+3 cells in early pregnancy. However, they observed that decrease of NK+3 cells proportions in late pregnancy. Koç and Kanter (27) evaluated rat endometrial tissue on which performed ANAE demonstration and they observed the significant increase in NK cells counts of pregnant rat endometrium in early pregnancy. Similarly, Sur et al. (28) found that the null cells demonstrated by ANAE histochemistry increased in peripheral blood and endometrial tissue of mice in early pregnancy. Because of the null cells are accepted as NK cells, their specific staining pattern which observed in ANAE performed smears, and their proportion shows distinct changes during pregnancy, it is considered that they have clinical diagnostic value. Besides, Andalip et al. (29) reported the proportion of NK cells were 9,21% in healthy women whereas they found 13,48% in women with recurrent spontaneous abortion (RSA). Beer et al. (7) claimed that the elevations of CD56+ NK cells to over 18% during a pregnancy was a reliable prognostic data of impending pregnancy loss. In this study, we examined the ANAE demonstrated smears, and the null cells percentage of the non-pregnant women was found as 2% but we determined the distinct increase in the proportion of the null cells up to 11% in the first trimester (p<0,05, Table 1). In the second and third trimesters, the proportions of the null cells were decreased (2,2% and 2,5%, respectively, Table 1). It is thought that the increase of null cells in the first trimester may be response of mother's immune system to embryo. Afterwards decrase of null cells in the second and third trimesters may be related to the maternal acceptance of the embryo.

Acid phosphatase (ACP-ase) is a lysosomal enzyme which considered specific for T-lymphocytes in many mammals (12). In this study, it was observed that the proportions of the ACP-ase positive peripheral blood lymphocyte decreased in the first (57,9%) and third trimesters (57,8%), and these differences were statistically important (p<0,05, Table 1). There is no knowledge pointed out about the possible relation between the ACP-ase positive PBL proportion and pregnancy. However, because of these lymphocytes assumed T-lymphocytes in mammals, the results of ACP-ase histochemistry obtained from this study may be interpreted as ANAE results.

Iwatani et al. (23) found that the numbers of T-, B- and T cell subsets decrased with a decrease in the total lymphocyte number in the first trimester. Mahmoud et al. (3) observed the significant decrease in the mean numbers of the T-, B-, NK and T-cell subsets concomitantly the decrease of PBL number in the third trimester. In this study, we determined the similar results mentioned the previous studies. The proportion of the PBL was gradually decreased during the pregnancy, correspondingly the results obtained from ANAE and ACP-ase histochemistry (Table 1).

Different factors that may cross the placental barrier effect the fetal development. Because the fetal cells can rapidly divide, many factors may cause DNA damages. Micronucleus frequency (MN) test performed on umblical cord or peripheral blood lymphocytes has become the prefered cytogenetic test used to evaluate genotoxic effects of many enviromental or biological factors (30). Stankovic et al (31) reported the

significant increase the MN frequency of PBL in pregnant women and fetal cord blood lymphocytes in the years of 2000 and 2001 after bombing Serbia as compared to year 1995. They measured MN frequency before bombing as 9,61 and 3,74 in 1000 cells in PBL and cord lymphocyte, respectively. After bombing of Serbia, they found the MN frequency as 28,26 and 22,22 in 1000 cells in the same order.

There are many studies which performed to assess the potential genotoxic effects of some certain drugs used during pregnancy on umblical cord and/or peripheral blood lymphocytes. Grujicic et al. (16) analysed MN frequency of cord blood lymphocytes in the neonates whose mothers prescribed betamimetics in combined with verapamil used in the prevention of premature labor. The investigators concluded that this administration has elevated MN frequency. Milosevic-Dordevic et al. (15) investigated the effects of gestogens administrated in the prevention of spontaneous abortions and they determined that gestogens induced MN frequency in the peripheral blood lymphocytes of pregnant women. Yeşilada et al. (32) observed an association between increased MN frequency of peripheral blood lymphocytes and polycystic ovary syndrome (PCOS). The same investigators claimed that many factors induced genetic instability such as hyperandrogenism, hyperinsulinemia and oxidative stress might caused DNA damages and increased MN frequency. In this study, we detected MN frequency of PBL as 5,8 in 1000 cells in non-pregnant control women. However, we observed increased MN frequency during pregnancy and the highest MN frequency was determined in third trimester (14,6 in 1000 cells, $p<0,05$, Table 1). The gradually increase of MN frequency during pregnancy may be explained to increased genetic instability of the lymphocytes caused by hormonal changes.

The nucleolus organizer regions (NORs) are loops of DNA shyntesizing rRNA. NORs are accepted an indication of rDNA transcriptional activity. The proteins associated with these regions are visualized as silver-stained black dots in the cell nucleus and this staining technique so-called Ag-NOR technique. Ag-NOR technique may applied to many different cells including peripheral blood lymphocytes (18, 33). Because some AgNOR parameters, such as size, number, and area, may change according to cellular proliferative and/or secretory activity, these parameters can be evaluated in some certain disorders, including malignant transformation (34). Neudeck et al. (35) claimed that the some AgNOR parameters measured in the villous trophoblastic cells were reliable indicator in complete and partial hydatidiform mole. Suresh et al. (36) reported that the increased AgNOR counts had diagnostic value in partial hydatiform mole. Yang (37) suggested that increased AgNOR numbers of gestational trophoblastic tumors might be useful tool to detect the early malignant change of hydatidiform mole. Similarly, Oltulu et al. (38) observed that AgNOR technique was much easier and cheaper than β-hCG levels in diagnosis of gestational trophoblastic disease. As it is understood, the previous studies demonstrated NORs using AgNOR technique were generally concerned pregnancy associated trophoblastic disorders. However, in this study, some AgNOR parameters in PBL were evaluated during pregnancy. AgNOR counts per lymphocyte were found range 1,44 from 1,57, and there was no statistically difference between the groups ($p>0,05$, Table 1), but we determined that the percentage of the AgNOR area relative to the whole nuclear area was increased during the pregnancy and the differences between the groups were important ($p<0,05$, Table 1). The highest value was detected in third trimester (10,22%). The gradually increased percentage of the AgNOR area relative to the whole nuclear area reflects the increased trascriptional activity of the cells during pregnancy. It is thought that the lymphocytes may endeavour to provide increased physiological demands needed for defending of the mother and fetus because they are very sensitive to infectious agents during pregnancy.

In this study, it was concluded that the some certain changes occuring in numbers of peripheral blood lymphocytes demonstrated ANAE and ACP-ase histochemistry during pregnancy. The most distinctive findings were obtained from null cells. Also, it was concluded that the some morphologic AgNOR parameters and MN frequency might be useful tool to determine some cellular activities and possible genomic damages, respectively. Besides, the results obtained from this study may be used as reference values in the evaluation of immune status of pregnant women. On the other hand, we suggest that these techniques used in this study could serve as a laboratory aid for early prediction of some pregnancy disoders such as unclarified infertility or spontaneous abortions, because these techniques are much cheaper, less time consuming, simple and can be performed in most laboratory. However, the further studies should be planned on large population involved the women with pregnancy associated disorders to explain our understanding of the possible immunological mechanisms underlying mentioned problems.

Acknowledgements

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