Original paper

IMMUNOHISTOCHEMICAL INVESTIGATION OF ENDOMETRIAL LEUKOCYTES IN IMPLANTATION PERIOD IN RATS WITH STREPTOZOTOSIN-INDUCED DIABETES

Emel Nacar[1], Yeşim Akaydin Bozkurt[2], Ahmet Koç[3], Ahmet Nacar[4]

[1]Division of Pathology Laboratory Techniques, Turgut Ozal University Vocational School of Health Sciences, Ostim, Ankara, Turkey
[2]Department of Histology and Embryology, Faculty of Medicine, Mevlana University, Konya, Turkey
[3]Department of Histology and Embryology, Faculty of Veterinary Medicine, Mustafa Kemal University, Hatay, Turkey
[4]Department of Histology and Embryology, Medical Faculty, Hacettepe University, Ankara, Turkey

Our first aim was to determine the total leukocyte profile in implantation. Second aim was to detect the changes in uterine leukocyte profile in diabetes, a common accompanying disease. For this purpose 4 groups are formed with Wistar albino rats weighing 250-300 g. Two of the groups were non-diabetic and two of them were diabetic. One of the diabetic and one of the non-diabetic groups were left pregnant. Then uterus tissues of pregnant animals were removed in the 5th and 7th days of pregnancy together with tissues of other two non-pregnant groups. Tissues were analyzed immunohistochemically with antibodies CD45, CD3, CD4, CD8, CD56, CD68 and CD79a. It was revealed that pregnancy increased immune staining of CD68, CD3, CD45 and CD56 in endometrium. In addition it was observed that immune staining density of CD68, CD45 and CD56 decreased in diabetes. In the histopathological examination, significant degeneration was detected in the endometrium of diabetic rats. Diabetes could decrease leukocyte proportions in decidua in early pregnancy periods. Therefore immune cell therapies could be administrated in diabetes related problems of pregnancy.

Key words: implantation, diabetes, endometrium, immunohistochemistry, rat, immune cells.

Introduction

The immunosuppression mechanism necessary for continuity of gestation is not clear yet. The fetus is an allograft since its antigenic structure comes from two individuals with different genotypes. Despite this, pregnancy does not lead to a host versus graft reaction [1].

The uterine mucosa normally contains macrophages [2, 3], dendritic cells and natural killer (NK) cells [4, 5] in addition to T lymphocytes [3, 6] and B lymphocytes [7]. Following implantation, the cellular architecture of the uterine mucosa is reorganized. The rate of leukocytes in human endometrium has been reported to be 40% in one study [8] and 50% in another study [9]. Uterine natural killer (uNK) cells have been found to constitute 45% [9] and 70% [10, 11] of all leukocytes. In rats, uNK cells which survive until delivery are degranulated just before delivery and become noticeable. Macrophages, constituting 10-20% of all leukocytes in the human endometrium, can survive until delivery [12].

uNK cells are the immune cells most abundant in the endometrium during the late secretory phase and implantation [13]. A study on pregnant rats has revealed that the amount of uNK cells which have Alpha Naftil Acetate Esterase (ANAE) increased from the second day of implantation onwards and peaked on the sixth day of implantation [14]. uNK cells are phenotypically similar to CD56+ peripheral NK cells [15]. They have been reported to have important functions such as decidualization, remodeling of vessels and regulation of maternal immune responses [16]. In a study performed to show subgroups of lymphocytes in normal pregnancy, there was a significant increase in leukocytes and a decrease in NK cells especially in the first trimester with insignificant changes in B lymphocytes [17].

However, there have not been any studies performed to evaluate the leukocyte profile of the uterus during implantation. Therefore, the primary aim of this study was to evaluate NK cells, T and B lymphocytes, macrophages and the complete leukocyte profile in the uterus with immunohistochemical methods. The secondary aim of this study was to determine how the cellular profile of the uterus changed in some common systemic diseases. The best model to be selected to determine this profile was diabetes since it was reported that there was an increase in spontaneous abortions in cases of uncontrolled diabetes [18]. Diabetes, which has negative effects on the whole process from implantation to delivery, has a wide variety of mechanisms of actions. Biochemical evaluations generally remain insufficient to elucidate cellular changes in the uterus during implantation. Therefore, histological evaluation of the immune cell profile in the uterus in diabetic women during early pregnancy period was aimed.

Material and methods

Animals and diabetes induction

Our study was conducted at Mustafa Kemal University Experimental Medicine Research and Application Centre, with the approval of the research ethics committee. 32 adult female Wistar albino rats were divided into 4 groups: control (group 1), diabetic (group 2), control early pregnant (group 3), diabetic early pregnant (group 4). For environmental adaptation, 4 to 5 rats were housed in one cage for 7 days under standart conditions of climate and nutrition (light period 7.00 A.M. to 7.00 P.M., 21 ±1°C, rat chow and tap water freely available). After acclimatization, rats were mated overnight and pregnancy was ascertained by the light microscopic observation of vaginal smears for the presence of spermatozoa daily. The day the smear was positive for sperm was designated as gestation day (GD) 1.

Rats in groups 2 and 4 were rendered diabetic by a single intraperitoneal injection of buffered solution (0.1 M citrate, pH 4.5) of STZ (45 mg/kg of body weight) on GD 4. After 48 hours, rats with blood glucose levels above 200 mg/dl were considered as diabetic. Rats in the other groups were administered citrate buffer (vehicle). Rats were given 5% glucose water for the first 48 h to prevent hypoglycemic shock.

General anesthesia with ketamine (50 mg/kg) and xylazine (10 mg/kg) was administered subcutaneously to the pregnant rats at GD 6 and non pregnant rats at similar days according to their cycle. Rats were opened at the midline, uterus was excised and sacrificed with decapitation.

Histological method

Tissues were fixed in 10% neutral formalin. Then fixed tissues were hydrated, dehydrated with graded alcohol series and embedded in paraffin after clearing with xylol. Five micrometer thick sections were cut and sections were stained with Crossman's modified trichrome stain to see the general structure of uterus tissues.

Immunohistochemistry

All biopsies were fixed in 10% buffered formaldehyde and paraffin-embedded by routine methods. The slides were incubated overnight at 37°C. Tissue sections of 5 μm thick were mounted on polylysine-coated slides, deparaffinized, rehydrated, and then washed with phosphate buffer saline (PBS, pH = 7.4) 3 times. Later sections were heated in citrate buffer (pH 6) for antigen retrieval. Then tissues were circumscribed with a pap pen (Super PAP Pen, PN IM3580, Becman Coulter Company, France). After washing with distilled water and with PBS, endogenous enzymes were blocked using 3% hydrogen peroxide for 10 minutes. Following a PBS wash, slides were treated with ready to use Ultra block (Lab Vision Corporation, CA, USA) for 10 minutes to prevent non specific bindings. Then primary antibodies (Table I) were added to the slides and incubated at 4°C overnight in a humidified chamber. After washing 3 times with PBS, tissue sections were incubated for 10 min. with biotinylated antibody (Lab Vision Corporation, CA, USA). Subsequently, slides were washed with PBS and were exposed to streptavidin peroxidase (Lab Vision Corporation, CA, USA) for 10 minutes. Then AEC (3-Amino-9-EthylCarbazole, AEC Substrate System, Thermo Fisher Scientific Anatomical Pathology, CA, USA) was used as chromogen. Afterwards, the slides were counterstained with Mayer's haematoxylin. Slides were examined and photographed with an Olympus DP20 camera attached Olympus CX41 photomicroscope (Olympus Corp., Tokyo, Japan).

Table I. Antibodies

ANTIBODY	CELL TYPE	DILUTION	SOURCE
CD8 [CC63] 0.25 mg Ab22378 Rat Monoclonal	Cytotoxic T lymhocyte	1 : 100	Ab Abcam
Mouse anti bovine CD4 [CC30] 2 ml Ab51312, Mouse Monoclonal	Helper T lymphocyte	1 : 100	Abcam
CD3 antibody [SP7] 500μl Ab5690, Rabbit Policlonal	Mature T lymphocyte	1 : 100	Abcam
Macrophage antibody [MAC387] 100.0μg Ab31630 Mouse Monoclonal	Macrophage	1 : 100	Abcam
CD79a antibody[HM57] 100.0μg Ab3121 Mouse monoclonal	B lymphocyte	1 : 100	Abcam
CD45 antibody 1 ml Ab33923 Mouse monoclonal	Common leucocyte antigen	1 : 50	Abcam
CD56 antibody1 ml Ab9272 Mouse monoclonal	Natural killer	1 : 50	Abcam

Statistical analysis

Group 1 is compared to group 2 in order to see the effects of diabetes on normal endometrium. Group 1 and 2 is compared to group 3 and 4 to see the effects of pregnancy on endometrial leucocyte distribution. Finally group 3 was compared to group 4 to see the effect of diabetes on pregnant endometrium.

Different quantitative evaluations were made to analyze the findings of trichrome and immunohistochemical stainings. With trichrome staining tissue edema, bleeding, necrosis and epithelium degeneration were investigated. One slide for each animal and totally 8 slides for each group were examined to make quantitative analysis as Davies *et al.* described [19]. Scores were given as follows: 0, no injury; 1, mild injury with limited edema and bleeding; 2, moderate injury with limited necrosis; 3, severe injury with diffuse bleeding, edema, necrosis and cell loss.

When evaluating immunhistochemical findings, the extensity of involvement was quantified. Since leucocyte – stromal cell ration was reported as 40% and over 40% [4, 30], the extensity of the staining was evaluated as follows: 0 – no staining, 1 – 1-10% involvement, 2 – 11-25% involvement, 3 – 26-50% involvement, 4 – 50% and more involvement.

Comparisons among groups were made with χ^2 test. $p < 0.05$ was accepted significant.

Results

In histological examination, control group revealed normal endometrium structure (Fig. 1). In group 2, edema, detoriation in glandular structure and presence of inflammatory cells were seen (Fig. 2). In group 3, normal pregnant endometrium with embryo and desidual cells was seen (Fig. 3). In group 4, epithelial degeneration and necrosis in desidual cells were observed (Fig. 4).

In statistical analysis of histological results, diabetes caused significant degeneration both in normal and pregnant endometrium. Most significant differences were found between group 1 with group 4 and group 3 with group 4.

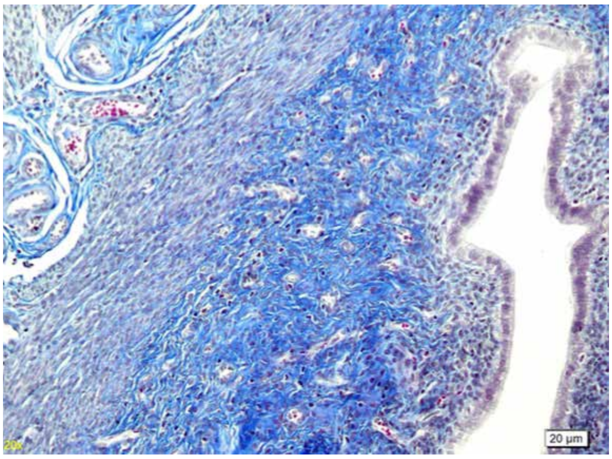

Fig. 1. Normal endometrium in control group (Trichrome stain, magnification 200×)

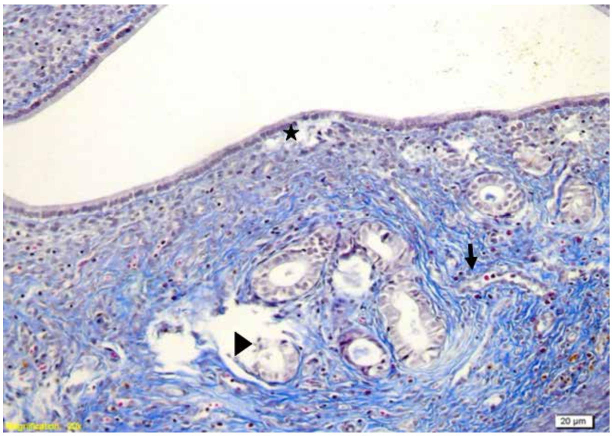

Fig. 2. Edema [*], deformation in glandular structure [arrowhead], inflammatory cells [→] in endometrium of group 2 (Trichrome stain, magnification 200×)

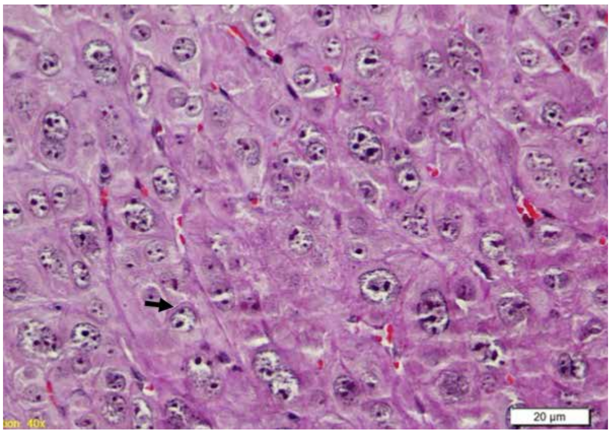

Fig. 3. Normal desidual cells in group 3 (Trichrome stain, magnification 400×

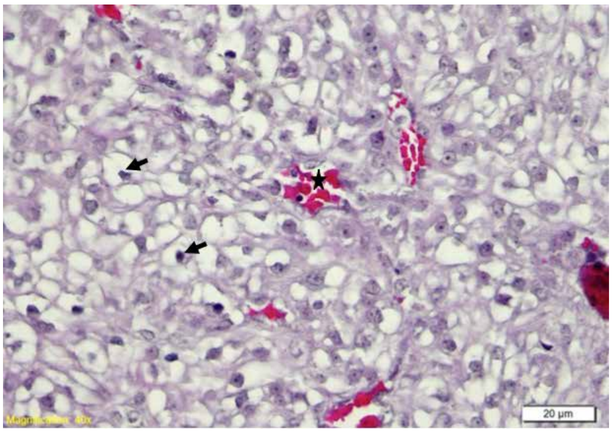

Fig. 4. Hemorrhage [*] and necrosis [→] in desidual cells (Trichrome stain, magnification 400×)

In immunohistochemical analysis with anti-CD3, CD4, CD8, CD45, CD56, CD68 and CD79a antibodies, it was found that pregnancy significantly increased CD68 (Table II), CD45 (Table III) and CD56 (Table IV) staining; and didn't affect CD79a and CD3 staining. Groups didn't show any significant involvement in terms of CD4 and CD8 staining. Diabetes caused no significant change with regard to CD68, CD56, CD3 and CD79a involvement (statistical data not shown). However significant decrease in CD45 staining was observed in diabetic groups.

Discussion

Several studies have been conducted to reveal changes in endometrial leukocytes during pregnancy [20, 21]. However, there have been few studies on effects of diabetes on endometrial leukocytes [22]. The present study reports that pregnancy significantly increased NK, macrophage and total leucocyte population in the endometrium. Additionally, diabetes decreased total leucocyte counts with no significant effect on leucocyte subtypes separately.

The number of uNK cells increase in the endometrium in early gestation [23, 24] and the rate of these cells in the endometrium increases to 70% [21, 25, 26]. They were reported to accumulate especially around trophoblasts and spiral arteries [4]. Consistent with the literature, CD56+ cells were found to increase considerably in the early gestational period in the present study.

The rate of CD45+ cells increases to 25% in the late secretory phase of the cycle [27]. In gestation, the rate of CD45+ leukocytes increases [4, 20, 28, 29]. It has been reported that the ratio of leukocytes to decidual cells in the early gestational period is 40% [30] and their involvement is the most marked around glands and the vessels [28]. The present study also revealed that pregnancy considerably increased

Table II. Statistical analysis of CD68 staining

CD68	Mean ± SD (standart deviation)
Group 1	0.75 ±0.46
Group 2	0.62 ±0.51^{a}
Group 3	2.25 ±0.46^{b,c}
Group 4	1.75 ±0.46^{d,e}

$p < 0.05$ *is accepted as significant*
$^{a}p = 0.590$ *not significant when compared to control*
$^{b}p = 0.001$ *significant when compared to control*
$^{c}p = 0.001$ *significant when compared to group 2*
$^{d}p = 0.135$ *not significant when compared to group 3*
$^{e}p = 0.006$ *significant when compared to group 2*

Table III. Statistical analysis of CD45 staining

CD45	Mean ± SD
Group 1	1.62 ±0.51
Group 2	0.75 ±0.46^{a}
Group 3	3.50 ±0.53^{b,c}
Group 4	2.12 ±0.35^{d}

$p < 0.05$ *is accepted as significant*
$^{a}p = 0.018$ *significant when compared to control*
$^{b}p = 0.001$ *significant when compared to control*
$^{c}p = 0.001$ *significant when compared to group 2*
$^{d}p = 0.002$ *significant when compared to group 3*

Table IV. Statistical analysis of CD56 staining

CD56	Mean ± SD
Group 1	1.50 ±0.53
Group 2	1.00 ±0.53^{a}
Group 3	2.62 ±0.51^{b,c}
Group 4	2.00 ±0.53^{d}

$p < 0.05$ *is accepted as significant*
$^{a}p = 0.202$ *not significant when compared to control*
$^{b}p = 0.010$ *significant when compared to control*
$^{c}p = 0.097$ *not significant when compared to group 4*
$^{d}p = 0.010$ *significant when compared to group 2*

CD45 involvement, which was marked around the vessels and the glands.

CD3 proteins are used to determine T cells in extrathymic tissues. The rate of decidual CD3 positive lymphocytes during pregnancy is controversial. Flow cytometric evaluations of human decidual tissues in the early gestation and the first trimester revealed a decrease in the number of CD3 positive cells in decidual tissues in the pregnant endometrium compared to the nonpregnant endometrium [15, 31, 32]. However, there have been studies revealing that there are not such marked changes in pregnancy as we also found [4]. The differences have been attributed to the size of decidual samples, the method of counting the cells and immunohistochemical methods used [32]. Besides the current study is the first on effects of diabetes on CD3 involvement and did not show a difference between the groups.

Macrophages constitute 10% of CD45 cells (general leukocyte antigen) in a non-pregnant endometrium and 20-30% of the cells in the deciduas in the first trimester [2, 10]. In a study on subclasses of leukocytes in the endometrium at the time of implantation and early gestation [23], the rate of CD68 positive macrophages was found to increase, which is comparable with the present study. The effect of diabetes on the rate of macrophages was reported in a few studies. In a study by Kim *et al.*, there was not a considerable difference in the rate of macrophages stained with CD14 and that of the macrophages stained with CD68 [33] consistent with our findings.

Studies on histopathological examinations of diabetes related uterine changes mostly revealed findings consistent with those obtained in the present study. One study performed in 2003 [34] revealed that there were considerable epithelial changes in diabetic rats. In another study on effects of progressive hyperglycemia on the endometrium, a thickened basal membrane, phagocytic blood elements extending into the basal membrane, differentiation in the stromal cells, vacuolization and degeneration were observed [35]. Several other studies showed that diabetes caused atrophy, lipid accumulation and involution in the uterus [36, 37].

In conclusion, the results of this study may shed light on the pathophysiology of abortions, the rates of which are increased in diabetics. Because diabetes related changes in the number and location of the immune cells, which protects the developing embryo with several mechanisms, may cause problems during implantation and subsequent developmental period. Based on this relation, immune therapies could protect developing embryo and fetus from diabetes-induced developmental complications.

The authors declare no conflict of interest.

This study was supported by Mustafa Kemal University Scientific Projects Unit with Project Number 1004 D 0105.

References

1. Bulla R, Fischetti F, Bossi F, et al. Feto-maternal immune interaction at the placental level. Lupus 2004; 13: 625-629.
2. Bonatz G, Hansmann ML, Buchholz F, et al. Macrophage- and lymphocyte-subtypes in the endometrium during different phases of the ovarian cycle. Int J Gynaecol Obstet 1992; 37: 29-36.
3. Song JY, Fraser IS. Effects of progestogens on human endometrium. Obstet Gynecol Surg 1995; 50: 385-394.
4. Bulmer JN, Longfellow M, Ritson A. Leukocytes and resident blood cells in endometrium. Ann N Y Acad Sci 1991; 622: 57-68.
5. Klentzeris LD, Bulmer JN, Warren A, et al. Endometrial lymphoid tissue in the timed endometrial biopsy: morphometric and immunohistochemical aspects. Am J Obstet Gynecol 1992; 167: 667-674.
6. Loke YW, King A, Burrows TD. Decidua in human implantation. Hum Reprod 1995; 10: 14-21.
7. Salamonsen LA, Woolley DE. Menstruation: induction by matrix metalloproteinases and inflammatory cells. J Reprod Immunol 1999; 44: 1-27.
8. Trundley A, Moffett A. Human uterine leukocytes and pregnancy. Tissue Antigens 2004; 63: 1-12.
9. Rieger L, Segerer S, Bernar T, et al. Specific subsets of immune cells in human decidua differ between normal pregnancy and preeclampsia – a prospective observational study. Reprod Biol Endocrinol 2009; 7: 132.
10. Mor G, Straszewski-Chavez S, Abrahams V. Macrophage-trophoblast interactions. Methods Mol Med 2006; 122: 149-163.
11. Bulmer JN, Lunny DP, Hagin SV. Immunohistochemical characterization of stromal leucocytes in nonpregnant human endometrium. Am J Reprod Immunol Microbiol 1988; 17: 83-90.
12. Hunt JS. Stranger in a strange land. Immunol Rev 2006; 213: 36-47.
13. Kimber SJ. Leukaemia inhibitory factor in implantation and uterine biology. Reproduction 2005; 130: 131-145.
14. Koç A, Kanter M. Sıçanlarda implantasyonda endometriyum dokusunun hücresel ve sıvısal savunma sistemi hücreleri üzerinde histokimyasal ve histometrik araştırmalar. Yüzüncü Yıl Üniversitesi Sağlık Bilimleri Dergisi 2000; 6: 122-130.
15. Starkey PM, Sargent IL, Redman CW. Cell populations in human early pregnancy decidua: characterization and isolation of large granular lymphocytes by flow cytometry. Immunology 1988; 65: 129-134.
16. Santoni A, Carlino C, Gismondi A. Uterine NK cell development, migration and function. Reprod Biomed Online 2008; 16: 202-210.
17. Kühnert M, Strohmeier R, Stegmuller M, et al. Changes in lymphocyte subsets during normal pregnancy. Eur J Obstet Gynecol Reprod Biol 1998; 76: 147-151.
18. Mills JL, Simpson JL, Driscoll SG, et al. Incidence of spontaneous abortion among normal women and insulin-dependent diabetic women whose pregnancies were identified within 21 days of conception. N Engl J Med 1988; 319: 1617-1623.
19. Davies JK, Shikes RH, Sze CI, et al. Histologic inflammation in the maternal and fetal compartments in a rabbit model of acute intra-amniotic infection. Am J Obstet Gynecol 2000; 183: 1088-1093.
20. Mincheva-Nilsson L, Baranov V, Yeung MM, et al. Immunomorphologic studies of human decidua-associated lymphoid cells in normal early pregnancy. J Immunol 1994; 152: 2020-2032.

21. Ho HN, Chao KH, Chen CK, et al. Activation status of T and NK cells in the endometrium throughout menstrual cycle and normal and abnormal early pregnancy. Hum Immunol 1996; 49: 130-136.
22. Savion S, Gidon-Dabush S, Fein A, et al. Diabetes teratogenicity is accompanied by alterations in macrophages and T cell subpopulations in the uterus and lymphoid organs. Int Immunopharmacol 2004; 4: 1319-1327.
23. Slukvin II, Breburda EE, Golos TG. Dynamic changes in primate endometrial leukocyte populations: differential distribution of macrophages and natural killer cells at the rhesus monkey implantation site and in early pregnancy. Placenta 2004; 25: 297-307.
24. Shi Y, Ling B, Zhou Y, et al. Interferon-gamma expression in natural killer cells and natural killer T cells is suppressed in early pregnancy. Cell Mol Immunol 2007; 4: 389-394.
25. Moffett-King A. Natural killer cells and pregnancy. Nat Rev Immunol 2002; 2: 656-663.
26. Croy BA, van den Heuvel MJ, Borzychowski AM, et al. Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. Immunol Rev 2006; 214: 161-185.
27. Kamat BR, Isaacson PG. The immunocytochemical distribution of leukocytic subpopulations in human endometrium. Am J Pathol 1987; 127: 66-73.
28. Stewart JA, Bulmer JN, Murdoch AP. Endometrial leucocytes: expression of steroid hormone receptors. J Clin Pathol 1998; 51: 121-126.
29. Quack KC, Vassiliadou N, Pudney J, et al. Leukocyte activation in the decidua of chromosomally normal and abnormal fetuses from women with recurrent abortion. Hum Reprod 2001; 16: 949-955.
30. Ozenci CC, Korgun ET, Demir R. Immunohistochemical detection of CD45+, CD56+, and CD14+ cells in human decidua during early pregnancy. Early Pregnancy 2001; 5: 164-175.
31. Starkey PM, Clover LM, Rees MC. Variation during the menstrual cycle of immune cell populations in human endometrium. Eur J Obstet Gynecol Reprod Biol 1991; 39: 203-207.
32. Vassiliadou N, Bulmer JN. Quantitative analysis of T lymphocyte subsets in pregnant and non-pregnant human endometrium. Biol Reprod 1996; 55: 1017-1022.
33. Kim JS, Romero R, Cushenberry E, et al. Distribution of CD14+ and CD68+ macrophages in the placental bed and basal plate of women with preeclampsia and preterm labor. Placenta 2007; 28: 571-576.
34. Garris DR, Garris BL. Diabetes-induced, progressive endometrial involution characterization of periluminal epithelial lipoatrophy. Diabetes 2003; 52: 51-58.
35. Garris DR, Williams S, Smith-West C, et al. Diabetes-associated endometrial disruption in the Chinese hamster: structural changes in relation to progressive hyperglycemia. Gynecol Obstet Invest 1984; 17: 293-300.
36. Garris DR, Garris BL. Lipoatrophic diabetes-associated utero-ovarian dysfunction: influence of cellular lipid deposition on norepinephrine indices. Horm Res 2002; 58: 120-127.
37. Garris DR, West RL, Pekala PH. Ultrastructural and metabolic changes associated with reproductive tract atrophy and adiposity in diabetic female mice. Anat Rec 1986; 216: 359-366

Address for correspondence

Emel Nacar, PhD
Turgut Ozal University Vocational School of Health Sciences
Division of Pathology Laboratory Techniques
Ostim
06500 Ankara, Turkey
e-mail: emelakdeniznacar@gmail.com