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Role of High Endometrial Natural Killer Cell Concentration in Patients with Recurrent Miscarriage

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ABSTRACT

Purpose: Lymphocyte subpopulation distribution and activation at the time of the window of implantation are likely to play a critical role in pregnancy loss. This study was planned to evaluate the prevalence of natural killer (NK) cells in the mid-secretory endometrium of women with recurrent miscarriage (RM) versus fertile controls.

Methods: The study group comprised 35 women with a history of two or more spontaneous abortions and 12 healthy fertile women as a control group. Mid-secretory endometrial tissue samples were obtained with a pipeline catheter, and endometrial NK cell phenotypes were determined by flow cytometry.

Results: While other endometrial lymphocyte populations remained constant, uterine NK cells in women with RM increased in the secretory phase. CD16+NK cell expression levels in women with RM were significantly higher than that of the fertile controls (0.57 vs. 0.08; $p<0.005$, respectively). However, decreased expression of CD4+ and CD4+3 cells and reduced ratio of CD4+/CD8+ were observed in women with RM.

Conclusion: Significantly increased levels of CD16+ in the endometrium of women with RM suggest that NK cells may have a significant role in the etiology of RM.

Keywords: Natural killer, recurrent miscarriage, abortion

INTRODUCTION

The American Society for Reproductive Medicine defines recurrent miscarriage (RM) as more than two consecutive pregnancy losses, while the World Health Organization (WHO) defines it as ≥ three miscarriages.[1-3] RM affects approximately 2.5% of women.[4] Although there is no underlying cause can be determined, many of the couples do not give term birth. A growing body of evidence points toward an immunological component of implantation failure. Pregnancy constitutes an immunological paradox since it implies that a fetus antigenically distinct from the mother is accepted by her immune system from embryo implantation to delivery. Immune balance between the mother and fetus is essential for the survival of an allogeneic fetus in the uterus. Natural killer (NK) cells are the primary immune cells that support a healthy pregnancy and have been linked to successful reproduction as a safety consideration.[5] NK cells are derived from hematopoietic progenitor cells that express the surface marker CD56,[6] which induces lymphangiogenesis, spiral artery remodeling, and trophoblast invasion.[7,8] In peripheral blood, there are two major types of NK cells: 90% are $CD56^{dim}$ CD16+ NK cells, and 10% are $CD56^{bright}$ CD16- NK cells.[9,10] However, the phenotype of uterine NK (uNK) cells, primarily $CD56^{bright}$ CD16- cells, is prevalent in the endometrium during the luteal phase and the early stages of pregnancy. Recent data indicate the presence of a subset of uNK cells, termed endometrial NK cells (eNK), with a yet-to-be-determined role.[11] This subset of cells might form a precursor of the uNK cells, given their similarity to classical uNK cell phenotype.[12] uNK cells are one of the most dominant leukocyte populations in the endometrium and account for 30% of cells during the window of implantation.[13,14]

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They play a role in immune response modulation, tissue repair promotion and vascular remodeling regulation. NK cells interact with trophoblast cells to have a role in initiating and maintaining pregnancy.[15-17]

One type of lymphocyte essential to adaptive immunity is the T cell. T cells play a role in the endometrium's local immune responses, specifically in controlling inflammation and immunological tolerance.[18] A balance between Th1 and Th2 is essential for fetal survival in the maternal uterus. For example, maternal tolerance to the semi-allogeneic fetus is thought to be predominantly related to the anti-inflammatory Th2 phenotype.[19,20] However, proinflammatory Th1 immune response is crucial for trophoblast invasion and parturition.[21,22] Additional data indicates that uNK is predisposed to release proinflammatory cytokines similar to Th1-type cytokines while reducing the anti-inflammatory Th2-type cytokines required to keep a pregnancy healthy.[23]

In women with RM, high numbers of uNK cells in endometrial biopsies taken in the late secretory phase correlated positively with the formation of blood and lymphatic vessels, spiral arteriole smooth muscle differentiation, and extent of endometrial edema. It is postulated that this exposes implanting blastocysts to excessive NK cell cytotoxicity and oxidative stress, leading to embryonic loss.[24] King et al.[25] have demonstrated that the NK percentage is >18%, which is highly specific for women with RM. Elevated NK cells play a pivotal role in RMs.[26,27] Alteration in the numbers or activity of uNK cells can, therefore, lead to reproductive failure and pregnancy complications. Owing to the wide variety of NK cell tests available to women with RM, we planned to determine whether NK cell concentrations in the late-secretory endometrium of RM women differ from those of fertile women.

METHODS

Study Subjects

Thirty-five women who were admitted to the Departments of Obstetrics and Gynecology of the Kocaeli University School of Medicine for RM treatment were included in the study. The study group comprised women with a history of two or more spontaneous abortions (n=35). They were recruited into the study at the point of being admitted for further evaluation of miscarriage etiology. Women in the RM group had normal ovulatory hormone profiles and routine pelvic ultrasound, and all semen analyses were reported as being within normal limits. Women in the control group (n=12) were previously fertile and were attending a variety of procedures, mainly intrauterine device application.

This study protocol was approved by the local ethical review board at Kocaeli University Medical Faculty and was designed and carried out by the Declaration of Helsinki. All patients entered this study only after a signed informed consent for the use of the endometrium was obtained. After a detailed questionnaire with demographic and socioeconomic data was filled, all women with RM underwent hysteroscopy (H/S) and hysterosalpingography (HSG) for uterine anomaly and fallopian tubal patency evaluation. Patients with uterine anomalies after H/S and HSG were excluded from the study. The men underwent at least one semen analysis according to the WHO criteria. The women underwent day-3 hormonal assessment to evaluate their ovulatory cycles, thyroid function, prolactin, and androgen levels. The ovarian reserve was detected by measuring serum follicle stimulation hormone level and antral follicle count on the third day of the menstrual cycle. Resumption of ovulation was defined by measuring mid-luteal progesterone. The couples in whom one of the partners presented an anomaly in one or more of the above tests and a history of diabetes mellitus, thrombophilia, hyperhomocysteinemia, and abnormally high HbA1C levels were excluded from this study.

Endometrial Sampling

All patients, independent of the group, were selected for the present study based on consistent histological findings, menstrual history, and serum progesterone levels. Endometrial samples were obtained with a pipeline catheter (Vel-Med) from all the participants in the mid-luteal phase of the cycle during the implantation window (cycle days 20-24). The endometrial tissue was divided into two sections; one was fixed in 10% formalin and embedded in a paraffin block. The other was washed three times with a sterile saline solution to remove blood and stored at -80 °C for future analysis. The mid-luteal phase was calculated as the 7 to 9 days after the ultrasonographic confirmation of ovulation and was confirmed by endometrial histological dating and serum progesterone levels. All menstrual cycles studied in the current study were ovulatory according to mid-luteal serum progesterone >10 ng/mL. Endometrial dating was performed by an independent pathologist experienced in gynecological pathology. Paraffin-embedded sections of 4 mm were stained with hematoxylin and eosin and periodic acid-Schiff stain. Then, these specimens were evaluated according to the histopathological criteria of Noyes et al.[28] An out-of-date biopsy was defined as a lag of ≥3 days between the chronological and the histological day.[29]

Flow Cytometry

We measured NK cell concentrations in endometrial biopsy specimens using flow cytometry. Findings derived from the peripheral NK cells in infertile women may not represent what happens at the feto-maternal interface.[30] We, therefore, did not measure blood NK cells. There has yet to be a consensus in the published literature about the timing for NK cell testing. Although uNK cells were mostly measured in the late luteal phase in the studies, there is cycle-to-cycle variation in the number of uNK cells.[31] Therefore, endometrial biopsy was collected from women during the implantation window (cycle days 20-24) in the present study.

Human endometrium tissue was obtained from 35 women with RM and 12 fertile women. Collected endometrial samples were centrifuged at 4,000 rpm for 10 min at four °C following a heat inactivation at 56 °C. After removing the transport medium from endometrium tissues, they were washed three times with Hanks balanced salt solution (HBSS) containing 5% penicillin/streptomycin on a Petri dish and minced into small pieces. Endometrial stromal and glandular cells were

isolated by digesting with 0.5% collagenase type II in Ca^{2+}, Mg^{2+} free HBSS at 37 °C for 5 min. Following gentle pipetting, the suspension was left to form the sediment for 5 min, and the upper part of the suspension was transferred into a separate tube for centrifugation at 400 g for 5 min. The supernatant was removed. The pellet was resuspended in RPMI medium, including 10% patient serum and 0.5% penicillin/streptomycin (stroma 1). This process was repeated four times (stroma 2-4) by adding collagenase solution to the primary sediment for sequential digestions. The contents of all stroma tubes were collected together. RPMI medium, including 10% serum from the patient and 0.5% penicillin/streptomycin, was added, and after centrifugation for 5 min at 400 g, the supernatant was discarded. The pellet of stromal cells was seeded into 25 cm^2 culture flasks. After stroma 4, the suspension was left to settle for 15 seconds. The upper part was collected and resuspended in RPMI with 10% patient serum and 0.5% penicillin/streptomycin. After brief centrifugation, the pellet containing glands was seeded into 25 cm^2 culture flasks. Cells were harvested and seeded into four healthy petri dishes, about $5x10^5$ cells per well.

Immunophenotype analysis by FACS

Endometrial cells were subjected to flow cytometry analyses. Cells were harvested and resuspended in a culture medium at 10^6 cells/mL concentration. Flow cytometry was performed using the flow cytometry instrument FACS Calibur (BD Biosciences, San Jose, CA, USA). The data were analyzed with Cell Quest software (BD Biosciences), and the forward and side scatter profiles were gated out of debris and dead cells. Immunophenotyping of endometrial cells was performed with antibodies against the following human antigens: CD45, CD14, CD45+14, CD3, CD4, CD8, CD8+3, CD16+56, CD3+16+56, CD5, CD10, CD19, CD103, CD22, CD20, CD57, cCD68, CD8, CD4+3, CD4. All of the antibodies were obtained from BD Biosciences.

Proportions of uNK cells and other lymphocyte subpopulations were determined as number (n) and percentage (%) of lymphocytes in each sample. The distribution of categorical variables between the two groups was tested with a chi-squared test. If continuous variables show a normal distribution, they are presented as mean and SD; otherwise, all results are expressed as median (range). The t-test or Mann-Whitney U test assessed statistical differences between groups. $P<0.05$ was considered significant.

Statistical Analysis

The data was analyzed by using the Statistical Package for Social Sciences software 13.0 for Windows package software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Each group's demographic and laboratory characteristics were presented in Tables 1 and 2. The average age and body mass index of the group of women with RM was not significantly different from that of the fertile controls. Karyotype analysis of women with RM was normal. The mean gestational age occurring miscarriage was 7.17 weeks. The average number of miscarriages was 3.65. One patient with RM underwent elective cerclage at 12th gestational weeks. Of the endometrial samples used for endometrial dating analysis, all fertile endometrium samples were in the secretory phase, while 31 infertile endometrium samples were in the secretory phase, three was in the proliferative phase, and one was diagnosed with simple endometrial hyperplasia without atypia.

While other endometrial lymphocyte populations remained constant, uNK cells in women with RM increased in the secretory phase. CD16+NK cell levels in RM women were significantly higher than that of the fertile controls (0.57 vs. 0.08; p=0.005 respectively). However, decreased expression of CD4, CD4+3 cells, and decreased ratio of CD4/CD8 were observed in women with RM. The two groups had no significant differences concerning human leukocyte antigen G (HLA-G) levels (1.34 vs. 1.41; p=0.57, respectively). Day-3 hormone levels of groups were similar except for estradiol (E2) levels. Women with RM had significantly higher E2 levels than fertile controls (p=0.003). Blood folic acid and homocysteine levels of women diagnosed with RM were normal. The thrombophilia panels of RM women were heterogeneous and presented in Table 3. Because the thrombophilia panel is heterogeneous

Table 1. Baseline characteristics of the patients for each group [values are n, mean ± (standard deviation)]

Characteristic	RM (n=35)	Control (n=12)	p-value
Age (y)	31.87±5.5	32.5±4.4	0.7
BMI (kg/m^2)	25.2±5.6	25.7±2.9	0.79
Smoking (mean)	8.6 (3%)	8.3 (1%)	>0.05
Gravida [mean (min.-max.)]	3.4 (2-10)	1.5 (2-6)	0.004
Parity [mean (min.-max.)]	0.3 (0-3)	1 (2-4)	0.08
Abortus [mean (min.-max.)]	3.08 (2-8)	0-0	0.09
Alive [mean (min.-max.)]	0.28 (0-2)	2.08 (2-4)	0.01

RM: Recurrent miscarriage, BMI: Body mass index, min.-max.: Minimum-maximum

Table 2. Evaluation of the expression of immune parameters and comprasion between the RM and control group

	RM n=35 Mean (min.-max.)	Control n=12 Mean (min.-max.)	p-value
CD45	30.6 (82.76-0.52)	32.8 (65.27-8.37)	0.68
CD14	0.56 (6.8-0)	0.27 (0.93-0)	0.91
CD45+14	1.13 (5.12-0)	0.62 (1.28-0.12)	0.09
sCD3	15.10 (40.8-0.41)	16.10 (36.7-4.58)	0.55
CD4	5.19 (15.88-0)	6.19 (13.95-1.12)	0.28
CD8	13.55 (61.8-0)	8.25 (22.35-1.39)	0.55
CD8+3	7.08 (21-0)	6.26 (21.74-0.81)	0.9
CD4+3	1.83 (10.76-0)	6.73 (12.54-3.40)	0.012
CD4 count	85.5 (274-0)	140.9 (341-7)	0.05
CD8 count	157.6 (730-0)	195.6 (449-9)	0.25
CD16+CD56	4.95 (22.32-0)	4.77 (12.16-0.32)	0.63
CD3+16+56	0.39 (2.07-0)	0.21 (0.63-0)	0.22
CD5	13.71 (30.48-0.22)	17.8 (44.07-5.43)	0.37
CD10	21.2 (76.19-0)	28.9 (69.1-4.04)	0.17
CD19	1.9 (36.41-0)	1.4 (6.1-0)	0.3
CD103	6.06 (16.72-0.07)	7.6 (15.46-2.8)	0.17
CD22	2.03 (35.16-0)	1.3 (5.11-0)	0.39
CD20	1.9 (30.74-0)	0.55 (4.41-0.14)	0.30
CD57	3.31 (16.27-0.07)	1.6642 (5.09-0)	0.24
CD16	0.57 (4.01-0)	0.08 (0.68-0)	0.005*
cCD68	2.06 (13.81-0)	6.2 (62.11-0.02)	0.69
CD4	18.32 (55.53-0)	29.38 (39.19-8.61)	0.01*
CD8	30.50 (69.23-0)	41.73 (59.11-9.01)	0.17
CD4/CD8	0.7475 (6.7-0)	0.7950 (1.14-0.13)	0.03
HLA-G	1.34 (9.62-0.12)	1.41 (6.28-0.13)	0.57

*: Statistically significant, $p<0.05$, independent samples t-test.
RM: Recurrent miscarriage, HLA-G: Human leukocyte antigen G

Table 3. Thrombophilia mutation in recurrent spontaneous group

Thrombophilia mutation	n (%)	Homozygous	Heterozygous
Factor V leiden	24 (68.6%)	0	4 (31.4%)
Factor V	26 (74.3%)	0	2 (5.7%)
MTHFRc	11 (31.4%)	6 (17.1%)	15 (42.9%)
MTHFR 1298C	9 (25.7%)	4 (11.4%)	15 (42.9%)
Prothrombin	26 (74.3%)	0	2 (5.7%)
Factor 13	18 (51.4%)	2 (5.7%)	8 (22.9%)
Fibrinogen	12 (34.3%)	5 (14.3%)	11 (31.4%)
HPA	0	5 (14.3%)	23 (65.7%)

MTHFR: Methylenetetrahydrofolate reductase, HPA: Human platelet antigen

in our study population, it cannot be the primary underlying mechanism of RM.

DISCUSSION

To test the hypothesis that local uNK cells acting in a dysregulated way could lead to miscarriage, we compared NK cell expression by the eNK cell population in RM women and fertile controls. We have shown that CD16+ expression on endometrial samples isolated from RM women during the secretory phase was significantly higher than in fertile women. Fukui et al.[26] reported that uNK cells play an essential role in implantation and that an increase in cytotoxic peripheral and uNK cells can affect reproductive performance. A study has shown that women with RM have a significantly higher NK percentage than fertile controls.[25] They also demonstrated that an NK percentage of 18% was highly specific for women with RM and defined 12.5% of women with RM as having an elevated NK cell percentage.

In humans, uNK cells are associated with the synthesis of immunoregulatory cytokines, which promote physiological angiogenesis and placental growth.[32,33] These cells accumulate around uterine spiral arteries, indicating their potential role in modulating trophoblast invasion and vascular remodeling. Therefore, higher expression levels of CD16+NK cells in unexplained infertile women may exert an unfavorable influence on embryo attachment by overproduction of cytokine and growth factor secretion, which affects placental development and vascular growth.[34] Moreover, high numbers of CD16+uNK cells in endometrial samples may cause defective spiral arteriole formation and trophoblast invasion, which inhibits embryo implantation and may cause early embryonic demişe.[25] CD16 levels were higher in the RM group. In addition, HLA-G expression was similar in the two groups.[35] In 1996, Lachapelle et al.[36] compared uNK cells in RM with fertile controls by flow cytometry analysis that described no significant difference in the overall number of uNK cells; however, there was a noticeably increased percentage of CD56+ cells that also expressed CD16 in RM patients, indicating a critical function for NK subsets in the pathophysiology of miscarriage. Kuon et al.[37] evaluated 130 women compared idiopathic RM patients and showed a higher prevalence of >300 uNK cells/mm^2 than controls (34.5% vs. 5.9%, p=0.02). In 88% of controls and 62% of RM patients, uNK cells were detected within the range of 40-300/mm^2. In a study by Zargar et al.,[38] peripheral NK cells (CD16+ and CD56+) were higher in the RM group than the control group.

CD4 and CD4+3 expression and CD4/CD8 ratio in endometrial cells were significantly lower in RM women compared with fertile women in the secretory phase of the menstrual cycle. The mechanisms responsible for the decline in CD4 and CD4+3 leukocyte numbers and CD4+/CD8+ ratio in the endometrium of RM women are unclear. The human uterus is an immune-modulated site that keeps apart the implanted semi-allogenic embryo from the harmful maternal immune response. A well-regulated cytokine network is crucial for normal immune reactions. Pro and anti-inflammatory immune responses are both postulated to be required for gestation.[39] Therefore, a decline in the lymphocyte subpopulation may disturb the balance between Th1 and Th2, which is essential for fetal survival. Studies showed no significant difference between the two groups in the T-cell count.[40]

The normal value of NK cell levels favoring or "permitting" implantation and the employed testing methodology vary between studies.[41] Our results are consistent with the results of some studies in literature but incompatible with others. Different assessment methods of NK cell numbers or percentages across the studies can be a significant source of this difference. Such contradictory results may be due to genetic and phenotypic differences in populations from different regions of the world or differences in measurement methods.

Literature evaluation revealed 13 studies comparing NK cell levels in women with RM versus controls. Six of the 13 studies evaluated peripheral NK cells, and seven evaluated uNK cells. Meta-analysis of the six studies by Seshadri and Sunkara that evaluated uNK cells expressed as a percentage of the endometrial cells in women with RM versus controls showed no significant difference between the two groups.[42] In contrast, another study that expressed uNK cells as numbers reported significantly higher levels in women with RM compared with controls.[43] Interestingly, a meta-analysis of the four studies that evaluated peripheral NK cell levels expressed as percentages showed a significant difference between women with RM versus controls.[42] The systematic review contained sixty articles comparing the CD56+ uNK level in women with RM to controls, which revealed that, in a subgroup analysis of endometrial samples, women with RM had substantially higher levels.[44] Another possibility may be statistical heterogeneity across the studies, and there is no consensus about the elevated level of NK cells. Other factors such as diurnal variation of NK cells, maternal stress, hormonal effect, exercise, time of day, parity of women, and expression of NK cells as numbers or percentages may explain the difference among the studies.[43,45] Inconsistency among the study results may vary depending on laboratory techniques, sampling methods, and the study population's selection.

Many questions remain regarding the origins, functions, and regulation mechanisms of human lymphocyte subpopulation in the etiology of women with RM because detailed, gestational time-course studies are not feasible, and endometrial sampling occurs after pathology is recognized.[46] uNK cells are transient cells endowed with angiogenic, lymphogenic properties and secretory activities that participate in the early optimization of maternal care of the fetus before birth. However, it is essential to understand that NK is not the only cell that reflects specific immune responses to pregnancy. It has been shown that T and B lymphocytes, macrophages, and NK cells are recruited into the endometrium during the mid-secretory phase of the cycle in preparation for the onset of implantation. Whatever the results, it must be remembered that RM is a heterogeneous problem. Not all women with RM will have an NK cell-related problem, and of those who do, a variety of NK cell-related problems are possible, as defined in our study. Due to the

complexity of the innate immune system, one variable and one measure cannot predict reproductive outcomes. Further studies are needed to explore the lymphocyte population's underlying role and mechanisms of action in women with RM. Targeted immunotherapy may be guided by the results of well-designed functional studies in the future, which might provide insight into the direction of uNK's effect on women experiencing reproductive issues.

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Ethics

Ethics Committee Approval: This study protocol was approved by the local ethical review board at Kocaeli University Medical Faculty and was designed and carried out by the Declaration of Helsinki.

Informed Consent: All patients entered this study only after a signed informed consent for the use of the endometrium was obtained.

Authorship Contributions

Surgical and Medical Practices: N.Y., Ş.Y.K., A.Ç., E.Ç., Concept: N.Y., E.Ç., Design: N.Y., E.Ç., Data Collection or Processing: Ş.Y.K., N.Y., E.Ç., Analysis or Interpretation: G.G., E.Ç., Literature Search: E.Ç., Writing: E.Ç.

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