The Role of CD16+, CD56+, NK (CD16+/CD56+) and B CD20+ Cells in the Outcome of Pregnancy in Women with Recurrent Spontaneous Abortion

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Abstract

Objectives: Recurrent Spontaneous Abortion (RSA) is the most common complication of pregnancy. It is considered as one of the most important issues of reproduction in the world. RSA is defined as having three or more miscarriages in the first trimester of pregnancy. Increase in peripheral blood lymphocytes may be associated with abortion; therefore, the study was aimed to investigate and compare the peripheral blood CD16+, CD56+, NK(CD16+/CD56+) and B CD20+ cells populations in diagnosis and on pregnancy outcome in women with abortion.

Materials and Methods: In this case-control study, 25 non-pregnant women with at least 3 abortions without obvious reason and 25 non-pregnant women with a living child without history of previous abortion participated. Using monoclonal antibodies anti (CD16, CD56 and CD20) and flow cytometry method, the percentage of cells with these markers was determined. Data analysis was performed by with SPSS 15 software and T-test.

Results: CD16+, CD56+ and NK (CD16+/CD56+) cells significantly increased in women with RSA compared with control group (P≤0.05) but there were no significant differences in the percentage of B CD20+ cells between the experimental and control groups (P>0.05).

Conclusion: According to the results of the present study, increased percentage of NK cells may be considered as a risk factor for RSA but involvement and the role of B CD20+ lymphocytes in RSA cannot be confirmed; however, in regard to important role of B and NK cell in the management of the immune responses, more studies are required to understand the behavior of these cells in the different stages of pregnancy more efficiently.

Keywords: Recurrent Spontaneous Abortion, B Cells, CD20, NK Cells, Flow Cytometry

Introduction

Recurrent Spontaneous Abortion (RSA) is the most common complication of pregnancy and considered as one of the most important reproductive problems in the world. RSA is defined as having three or more miscarriages in the first trimester of sequent pregnancy. It has been reported at 1-2% of women in the reproductive age (1). Numerous factors play a role in abortion. Some of these include viral and bacterial infections, chromosomal abnormalities, genetic, endocrine, anatomical factors, thrombophilia disorders and environmental factors; however, the most important factor for fetal loss in recurrent spontaneous abortion is immunological disorders (2, 3). Immunological factors such as anti-phospholipid antibodies are believed to be a main cause in RSA. It has been reported that 50% of women with recurrent miscarriage have these antibodies (4, 5). Fetal toleration by the mother which is toxic semi-allograft transplantation is the result of the balance between defense mechanisms of the mother and the invasive trophoblast cells. During pregnancy decidual lymphocytes are in direct contact with trophoblast cells. Immune cells such as lymphocytes, including Natural Killer cells (NK), T cells and B cells are present in the lower uterine endometrial tissue. NK cells are a main component of innate immunity and play an important role in the early defenses against viral infections. Also, they efficiently kill various leukemia and cancerous cells and are thought to play a role in immuno surveillance against tumors (6). Numerous investigations have shown that women with RSA have high NK cell percentage and activity both in the peripheral blood (7-10) and endometrium (11) and that the levels of these indices predict subsequent pregnancy outcome (10, 12). NK cells represent the first cellular immune defense
mechanism and are in close contact with the conceptus and placenta. NK cells comprise 5-12% of all lymphocytes and are classified into CD16+CD56dim NK cells and CD16+CD56bright NK cells based on their surface CD markers (8, 13). The predominant population of NK cells in the peripheral blood consists of CD16+CD56dim NK cells, whereas CD16−CD56bright NK cells are the main population in the endometrium (14). During implantation NK cells constitute 70-90% of the lymphocytes in the uterine endometrium (15). Increase in peripheral blood lymphocytes may be associated with miscarriage (16). The dual function of B lymphocyte is such that it plays a role in maintaining pregnancy by secreting protective antibodies, while by producing autoantibodies it can lead to pregnancy complications (17).

Evaluation of lymphocyte sub-populations in peripheral blood and endometrial tissue in diagnosis and pregnancy outcome in women with abortion is still controversial; thus, due to the availability of monoclonal antibodies and flow cytometry technology in diagnosis of immune cells, this study was aimed to investigate and compare the CD16+, CD56−, NK (CD16+/CD56−) and B CD20+ peripheral blood cells of non-pregnant women with the history of recurrent spontaneous abortion (who recently had their latest abortion) and non-pregnant women with history of normal pregnancy in order to clarify the role of these cells in the reproductive period.

Materials and Methods
Study population
In this case-control study 25 women with RSA referred to gynecologist’s office or women’s section of Ahvaz Razi Hospital (experimental group) and 25 non-pregnant women with a history of normal pregnancy (control group) participated. All patients were in the age range of 20-35 years. Women with a history of RSA with at least three abortions in the first trimester of pregnancy without specific cause were studied. The couples then underwent complete investigations including: karyotyping to exclude the chromosomal abnormalities; blood cell counts to rule out thalassemia and anemia; check the level of TSH and T4 to rule out thyroid disorders; measuring the prolactin hormone levels to rule out hyperprolactinemia; examination of women and if necessary, hysteroscopy and Hysterosonography (HSG) to rule out anatomic abnormalities; sperm analysis and if necessary, an examination of urology to rule out male factor. Exclusion criteria included the presence of any form of disorder in any of the above tests/positive finding in the couples. Inclusion criteria included women with diagnosis of RSA with unknown cause. Control group included 25 non-pregnant women with a history of a live child and without previous history of abortion. They also were selected voluntarily among the women admitted to the laboratories of the hospital. 5 milliliters of peripheral blood was collected in heparinized tubes. All individuals provided a written informed consent before their participation in the study.

Isolation of lymphocytes
Ficol Lotion (Ficol hypac, BioSera, UK) was used in order to isolate peripheral blood lymphocytes. A ratio of 1 to 2 of Ficol and blood was poured into the test tube in a form that blood was at the top of Ficol and could not be mixed with it. It was then centrifuged in room temperature and super lymphocyte was separated based on density gradient. It was transferred by a sampler’s tip to another test tube and was washed equally with Phosphate Buffered Saline (PBS) solution. For washing, isolated lymphocytes were mixed with PBS, and centrifuged in room temperature. Supernatant was removed and the sediment was diluted again with cold PBS. The remaining steps were also performed on ice.

Flow cytometric assay
Due to the presence of antigenic markers on immune cells, monoclonal antibodies can be used to differentiate these indicators with the flow cytometry method. Isolated cells were stained with monoclonal antibodies against CD16, CD56 and CD20 which then were conjugated with fluorescein isothiocyanate (FITC) (prepared from DAKO, Denmark). Then they were analyzed with flow cytometry Becton Dikenson (BD, USA), and finally the percentage of the cells with the markers was determined.

Statistical analysis
Statistical evaluation of the data and comparison of the safety variables of experimental and control groups were done using SPSS 15 and T-test.

Results
Results show significantly increase in percentage of CD16+ (Figure 1), CD56− (Figure 2) and NK (CD16+/CD56−) (Figure 3) cells in RSA patients compared with control group (P≤0.05) but no significant difference was observed between the two group in terms of the percentage of B CD20+ lymphocytes (P>0.05) (Figure 4). However, the percentage of B CD20+ lymphocytes in the experimental group was also lower than the control group. Contrary to what was expected, a decrease was seen in the level of the B CD20+ cells which was not statistically significant (Table 1).

Discussion
The purpose of this study was to compare CD16+, CD56−, NK (CD16+/CD56−) and B CD20+ cells in women with RSA and healthy individuals. Many studies have been done to show the relationship between the levels of some immune cells with failed reproduction. In Iran and elsewhere, few studies have been performed on the relationship between B CD20+ lymphocytes with RSA. Most of the studies in the world are on B CD19 which is a marker very similar to the CD20+, and presence of each of these two markers
really confirms presence of B lymphocytes. In the present study, significant differences were observed in cells with marker CD16$^+$, CD56$^+$, NK (CD16$^+$/CD56$^+$) (P≤0.05) but B CD20$^+$ lymphocyte between two groups did not show significant difference in terms of increased B CD20$^+$ lymphocytes in women with recurrent abortion (P>0.05).

The relationship between increase in peripheral blood NK cells and reproductive failure is one of the common controversial subjects in reproductive medicine (18). NK cells are an abundant population of lymphocyte in the decidual, and are thus expected to play a physiological role during the implantation process; however, despite many similarities between the two groups of NK cells in peripheral blood and decidua, there are some differences (9). Most of peripheral blood NK cells are CD56$^{dim}$CD16$^+$, but majority of the uterine NK cells are CD56$^{bright}$CD16$^-$ (14). Karami et al. reported that the percentage of CD56$^{dim}$ cells and the level of peripheral blood NK cell cytotoxicity in RSA patients were significantly higher than healthy women which could be considered as a contributing factor for RSA. However, no significant differences were seen in percentage of CD56$^{bright}$ among women with RSA. It is possible that due to low percentage of the CD56$^{bright}$ cells population, alteration in the number of these cells between the patient and control groups may not have been accurately measured and should be reviewed in a wider population (8).

In Perricone et al. study on women with RSA, increase in percentage of CD56$^+$ cells and absolute number were seen (19). Also in Hadinedoushan et al. research, RSA patients showed higher NK cytotoxicity than normal pregnant women with no history of abortion (20). Atia and Elzaher study showed that the number of CD56$^+$ decidual NK cells was increased significantly in RSA cases (21). In a survey conducted by Tuckerman et al. When endo-
metrial CD56bright NK cells were analyzed, the number of NK cells in women with a history of RSA was dramatically higher than in the control women (22). Also in another study conducted by Kwak et al. the number of NK cells with CD56+ marker in non-pregnant women with a history of RSA compared to healthy pregnant women increased dramatically. Also pregnant women with a history of RSA compared with pregnant women in the control group showed a significant increase in the number of cells CD56+, NK (CD56+ / CD16−) and B (CD19+) lymphocytes (23) which is in agreement with our study.

The results obtained by the authors are contradictory. For example, Souza et al. (24), using Cr-release method, showed that peripheral blood NK cell cytotoxicity did not differ between patients and control group. Also Emmer et al. reported no differences between RSA and normal controls prior to conception in the numbers of CD56+ and CD56−/CD16+ cells and NK cytotoxicity (12). In contrast to this study, Karami et al. (8) results showed a clear and dramatical enhancement in NK cytotoxicity among RSA women compared with normal women. These results are in consistent with Yamada et al.(10), using both methods of 51Cr release and flow cytometric analysis. One reason for the contradictory results obtained from different investigations may be due to sampling time taken from patients with RSA.

NK cells contribute to the cytokine response. This cytokine response is generally characterized either as a TH1 type (with production of IL2, IFNγ and TNF-α) or a TH2 type (with IL4, IL5, IL10, IL13). Normal pregnancy might be the result of a TH2 cytokine response. IFNγ secreted by TH1 cells increase activity and cytotoxicity of NK cells which correlate with recurrent abortion. Several studies indicated women with RSA tend to produce a predominantly TH1 type response both in the period of implanta
tion and during pregnancy (25,26).

Lachapelle et al. study showed B CD20+ lymphocytes increased in the endometrium of these patients (27). Results from Lachapelle study were not consistent with our study. Ghafourian Boroujerdina et al. study showed that the mean percentage of population of B CD22+ lymphocytes in infertile women compared to healthy women did not significantly change which is in agreement with the results of our study (28).

The results from different studies on B cells are controversial. For example in Darmochwal-Kolarz et al. study, percentage of B CD19+ lymphocytes was significantly lower in women with a history of RSA compared with normal women (29), which is partly consistent with the result of our study (due to being like marker CD19 and CD20) whereas in study of Kwak et al., B CD19+ lymphocytes levels in women with a history of RSA was higher than in normal subjects (23). Also in Jablonowska’s study, women with a history of RSA during the first trimester of pregnancy demonstrated an increase in the number of B CD19+ compared with healthy pregnant women (30). The reason for this discrepancy may be associated with several immunological theories proposed for RSA. Some of these hypotheses suggest increased cytotoxicity of decidua NK cells or peripheral blood against fetal antigens (7,31).

Some antigens are associated with HLA (32,33) and others suggest a role of autoimmune disorders in RSA (34,35). During pregnancy, the maternal immune system faces a double dilemma: tolerance against growing semi-alloge
cne fetus and protection of the mother and the progeny vs. pathogens at the same time. This requires a fine and extremely regulated balance between immune activation and tolerance (36). Yamada et al. reported that trophoblast cells in women with recurrent abortion can stimulate peripheral blood mononuclear cells, their proliferation and production of toxic factors for fetus (37). Professional antigen presenting cells, B lymphocytes and especially the B-1a cells can activate T cells and thus set or create the immune response. Various studies have shown that pregnancy disorders such as RSA and preeclampsia are associated with the prevalence of TH17 cells. B-1a lymphocytes are strong inducers for differentiation of TH17 (36,38).

Several studies suggest that women with a history of RSA have an abnormal immune response along with an increase in peripheral blood NK and B cells compared with normal control group women (23).

Conclusion
The present study showed brief information on the relation of the population of the B CD20+ lymphocytes, CD16+, CD56+ and NK (CD16+/CD56+) cells in peripheral blood of women with history of RSA and healthy women. According to the results of present study, increased percentage of NK cells may be considered as a risk factor for RSA but involvement and the role of B lymphocytes in RSA may not be confirmed. Also the role of secreted cytokines from T cells and their effect on B and NK cells should be considered further. Thus more studies are necessary to clarify these immunological mechanisms, including the performance of these cells and their behavior in different stages of pregnancy, as well as the role of cytokines in RSA.

Ethical issues
After getting approval from the ethics committee of Ahvaz Jundishapur University of Medical Sciences, blood samples were taken voluntarily from women admitted to the laboratories of the Razi Hospital. Also for the purpose of the study and taking blood, informed consent was obtained from the cases.

Conflict of interests
The authors declare no conflict of interests.

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