Introduction

Endometrial receptivity is one of the critical biological processes attributed to successful pregnancy and embryo transfer (1). Abnormality in endometrial function can lead to various reproductive issues such as infertility, a tendency towards recurrent miscarriage (RM), and repeated failure of implantation. The etiology of spontaneous abortion remains unknown in approximately 50% of all subjects with RM and recurrent in vitro fertilization (IVF) failure. However, changes in the uterine environment are considered to be associated with these idiopathic disorders (2, 3). A variety of risk drivers, including endocrine disorders, anatomical and chromosomal abnormalities, and hemostatic imbalance, were attributed to spontaneous abortion. More importantly, the immune system and immunological mediators were demonstrated to be involved in this condition. However, although there is accumulating evidence demonstrating the important role of immunological mediators in more than 50% of RM subjects, physicians only use the antiphospholipid syndrome screening test (4). IVF is a commonly-used procedure of assisted-reproductive technology. However, despite breakthroughs in assisted-reproductive technology, there is still one successful pregnancy out of three IVF cycles (5). Repeated implantation failure (RIF) is a clinical phenomenon characterized by a lack of implantation after the transfer of several embryos into the uterus. On the other hand, labor resembles an inflammatory response that includes the secretion of numerous cytokines/chemokines from the resident and infiltrating immune cells into the maternal/fetal...
Inclusion and Exclusion Criteria

Spontaneous abortions before the 20th week of pregnancy. Unexplained pregnancy loss is defined as two or more recurrent abortions confirmed by vaginal ultrasound and hysteroscopy), and 5 cases (with ≥ three IVF failures). Recurrent abortions confirmed by vaginal ultrasound and hysteroscopy), and 5 cases (with ≥ three IVF failures). Participants in the control group should have had no history of miscarriage and a history of at least two miscarriages or three unsuccessful cycles of IVFs. Participants in the control group should have had no history of miscarriage and a minimum of one successful pregnancy. Patients with septic miscarriage, recorded endocrinopathies such as thyroid or prolactin abnormalities, diabetes, uterine anomalies, cancer, and a history of hormonal contraception use during the last 6 months before the last pregnancy were excluded from the study.

Procedure
After obtaining written informed consent, eligible patients were included in the study. Endometrial biopsies were performed using a pipelle suction curette during the mid-luteal phase (on days 21 to 24 of the menstrual cycle).

Immunohistochemistry
Immunohistochemistry (IHC) was used to assess the expression profile of CD56 and NKp46. In brief, endometrial biopsy specimens were immersed in 10% neutral-buffered formalin to fix for nearly 24 hours, embedded in paraffin wax, and cut into 3-4 µm thick sections with a microtome (Leica RM2235; Leica, Wetzlar, Germany). All paraffin-embedded sections were mounted onto 3-Triethoxyysilane-propylamin slides (Sigma Chemical Co.; Poole, UK), dewaxed in xylene, and rehydrated in descending concentrations of alcohol. Antigen retrieval was performed using a microwave (800W) for 25 minutes (10). After washing, tissue sections were quenched in 3% hydrogen peroxide diluted in methanol for 7 minutes to inhibit endogenous peroxidase activity. Slides were washed in PBS and incubated with protein blocker (Biopharmadx, Germany) (10 minutes for CD56 and 1 minute for NKp46). Afterwards, the primary antibodies were added to CD56 monoclonal mouse anti-human CD56 antibody (diagnostic bio-systems, Pleasanton, CA, USA) for 90 minutes at 37°C and to NKp46/NCR1 monoclonal goat anti-human NKp46 antibody (R&D Systems, Minneapolis, MN, USA) at 3 µg/mL, and incubated overnight at +4 °C in a humidified chamber, respectively. Samples were incubated for 1 hour with the HRP-labeled secondary antibody (rabbit anti-IgG mouse for CD56 and donkey anti-IgG goat antibody) at 37 °C. The peroxidase reaction was achieved with chromogen DAB (3.3′ diaminobenzidine (DAB)).

Materials and Methods

Study Design
This is a case-control study that was carried out between January and June 2019 to determine the number of uNK cells and cells expressing NKp46 in the endometrium of women with a history of RM or IVF failure considering the significant role of NKp46 in uNK cell cytotoxicity.

Participants
A total of 48 participants, aged 20 to 51 years, participated in this study. The participants included 16 healthy controls (normal pregnancy with ≥ one previous successful pregnancy and no history of abortion or infertility), 27 cases (with ≥ two previous unexplained recurrent abortions confirmed by vaginal ultrasound and hysteroscopy), and 5 cases (with ≥ three IVF failures). Unexplained pregnancy loss is defined as two or more spontaneous abortions before the 20th week of pregnancy.

Inclusion and Exclusion Criteria

Association of NKp46+ uNK cells with RM and IVF failure
tetrahydrochloride; Biopharmadx, Germany) and discontinued by adding water after 5 minutes. Finally, the slides were counterstained with hematoxylin (DAKO Corporation, Carpinteria, CA), dehydrated in ascending concentrations of alcohol, cleared in xylene, mounted with a non-aqueous mounting medium (Entellan, Merck, German), and verified by optical microscopy. Positive specimens (lymphoma tissue) were used for every set to assess the validity of the immunostaining method. In addition, the phosphate-buffered saline (PBS) buffer, instead of primary antibodies, was used as the negative control. Two experienced pathologists independently analyzed all samples using microscopes (Optika, Italy). Endometrial glands and stroma were evaluated for confirmation of the day of the menstrual cycle according to Noyes et al (14). All stained cells of 10 views of each specimen were counted under a microscope at 400x magnification. The ratio between positive, CD56 or NKp46 cells (brown stain) and total endometrial stromal cells (blue stain) were calculated.

**Statistical Analysis**

The data collected in this study were analyzed using Prism version 7. Data were represented as mean ± standard deviation or median (IQR) for non-normal distribution. Mann-Whitney test was used to compare data obtained from women with RM or RIF with those obtained from the control group. P values less than 0.05 were considered to be statistically significant.

**Results**

**Demographic Characteristics**

A total of 48 participants, including patient and control groups, with a history of RM or RIF were enrolled in this study. Table 1 indicates the demographic characteristics of the women with reproductive failure (the patient group) and fertile women (the control group). No significant differences were found in the age or BMI of the two groups (P>0.05).

**The Percentage of CD56-Positive Cells in the Patient and Control Groups**

Table 1. Demographic Data of Patient and Control Groups

<table>
<thead>
<tr>
<th></th>
<th>Case (n = 32)</th>
<th>Control (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>30.9 ± 6.0</td>
<td>35.6 ± 4.4</td>
</tr>
<tr>
<td>Number of gravities</td>
<td>2.5 (2-3.75)</td>
<td>3 (3-4.75)</td>
</tr>
<tr>
<td>Number of deliveries</td>
<td>0 (0-1)</td>
<td>3 (3-4.75)</td>
</tr>
<tr>
<td>Number of miscarriages</td>
<td>2 (2-3)</td>
<td>0</td>
</tr>
<tr>
<td>Gestational age of miscarriages (wk)</td>
<td>8 (6-10)</td>
<td></td>
</tr>
<tr>
<td>Time after last miscarriage (mon)</td>
<td>11 (5-15)</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.5 ± 4.1</td>
<td>29.0 ± 2.7</td>
</tr>
</tbody>
</table>

BMI, body mass index.

Note: All data are shown as mean ± standard deviation or median (IQR).

Discussion

Uterine natural killer cells produce angiogenic factors during the first trimester of gestation, presumably playing a significant role in the successful implantation. In addition, uNK cells were found to be associated with human reproductive disorders, especially repeated miscarriage, recurrent implantation failure, preeclampsia, and fetal growth restriction (15). This study aimed to examine the number of uNK cells and the expression profile of NKp46 in the endometrium of women with a history of RM or IVF failure. The CD56 marker is classified as an isomorph of the neural cell adhesion molecule, which is expressed on neural-originated cells, conventional cytotoxic T lymphocytes, and NK cells. Both peripheral blood and endometrium are sources of natural killer cells that express the surface marker CD56 (16,17).

Studies revealed that about 80% of the uNK cells express the CD56 bright CD16- phenotype. CD56-positive cells were demonstrated to have a regulatory action, although the NCR1 or NKp46-positive cells display a cytotoxic activity (18, 19). Studies demonstrated that increased cytotoxicity level of peripheral blood NK cells in human beings is associated with an increased risk of spontaneous abortion (20). Patients aged less than 35 years with unexplained recurrent abortion demonstrated a lower risk of spontaneous abortion as compared with those aged over 35 years (21). The BMI of patients participated...
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in this study was calculated to be $27.58 \pm 4.19$ kg/m$^2$. This could be related to the standards used in this study, where we excluded a number of risk factors that render the patients susceptible to RM or IVF failure, especially diabetes mellitus, obesity, and thyroid abnormalities (22).

Furthermore, as compared with healthy fertile women, evidence from our study on representative RM and RIF disorders indicated immunological changes, especially inducible expression of activating NKp46 receptor on NK cells along with an increased number of corresponding CD56 bright NK cells in these high-risk subjects. In fact, it can be concluded that NK cells are necessary for embryo implantation and successful pregnancy despite controversial previous evidence. Accordingly, there were contradicting empirical results from different studies. For example, Giuliani et al (10) detected a high expression level of CD16 and NKp46 positive uNK subsets in individuals with a history of abortion but they did not find any difference in CD56, which is not consistent with the findings of this study and the study conducted by Gao and Wang (23) in which a high expression level of CD56 was detected in unexplained recurrent spontaneous abortion. Liu et al also showed no correlation between the number of uNK cells and the outcomes of pregnancy in women with a history of RM and IVF failure (24). However, in another study (25), decreased expression of NKp46 in the blood and endometrium of women with an experience of abortion was reported. The main reason behind these different findings is not clear however increased expression of NKp46 is more reliable because activation of NK results in more expression of the activator NKp46 receptors in targeted cases. This has been approved through mAb-mediated blocking NCR1/NKp46 receptor on NK cells results in defective killing tumor cells. Only a few studies reported non-significant changes in the number of peripheral natural killer cells and NKp46 which is a cell-surface receptor expressed on NK cells in women with a history of RIF versus healthy women. All these studies reported that the peripheral blood level of NK cells is not a reliable marker to define the events in the uterus beyond uterine/decidual type NK cells. Other factors for the absence of similar infallible conclusions among various studies are related to different measurement methods. Apart from being invasive, this procedure is time-consuming. Additionally, it can be difficult to use stratified sampling. We suggest that the evaluation of NK endometrial stromal cells by immunohistochemical method during the luteal phase is a good estimation of the risk of abortion in women with RM and RIF and interventional therapeutic approaches should be used based on the results. Diagnosis of immunological problems in patients with pregnancy problems leads to treatment with immunomodulatory drugs. Glucocorticoid drugs (prednisolone) (26), intravenous immunoglobulin (IVIg) (27), and paternal lymphocyte therapy (through the stimulation of the immune system by paternal antigens) have been used to treat both RM and RIF complications that lead to a decrease in cytotoxicity activity and the number of uNK cells besides positive regulation of cytokine production. In the endometrium of women with RM, uNK cells and blood vessel maturation are increased and prednisolone treatment reduces uNK cells and endometrial spiral artery development in the endometrium of these patients (28). Paternal lymphocyte therapy reduces abortion in women with recurrent abortion. One of the effects of lymphocyte therapy is the reduction of NK cells (29, 30) and their cytotoxicity (31). The effect of IVIg has also been investigated on mice in addition to
humans. In a study on mice, IVIg was found to reduce miscarriage by affecting NK (32). On the other hand, measurement of Nk cells before treatment is essential because research has shown that the success rate of IVF with IVIG therapy was high in people with elevated Th1: Th2 and/or CD56 (+) cells, and in people with normal Nk or Th1: Th2, IVIG treatment had no effect (33). New strategies for modulation of immune responses against the fetus include the use of monoclonal antibodies (anti-TNF), cytokines (granulocyte colony-stimulating factor), granulocyte macrophage colony-stimulating factor), and immunosuppressive factors (tacrolimus and cyclosporine) (34). It is necessary to investigate the effect of these treatments on Nk levels in the endometrium of patients. It is also recommended that the number of uNK cells and the expression of NKp46 should be evaluated after the use of these medications. Further, considering ever-changing face regarding the amount of uNK and pursued immunological cytotoxic effects are proposed for normal pregnancy.

**Conclusion**
The increased number of uNK cells and their elevated cytotoxic activity during implantation and early pregnancy in the patient group showed a significant association between the activity of uNK cells and a higher risk of RM and RIF. Given the role of these immune cells in pregnancy abnormalities, prescribing specific immunomodulatory drugs can decrease the risk of these diseases. Larger case-control studies are required to assess endometrial function using endometrial tissue specimens derived from healthy control subjects without RM and RIF to investigate the association between uNK cells and human reproductive disorders and develop new therapeutic approaches.

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Supervision: Mehri Ghaforian, Farideh Moramezi.
Writing-original draft: Maryam Matouri.
Writing-review & editing: Maryam Matouri, Mehri Ghaforian.

Figure 3. Immunohistochemistry Staining to Detect the NKp46 Marker in Women With Recurrent Miscarriage and IVF Failure. An illustration of cell surface localization of NKp46 in the endometrial stroma derived from (A) patients, (B) fertile women, and (C) negative control (treated with PBS buffer instead of specific primary antibody) (original magnification ×400).

Figure 4. The Differences Between NKp46-Positive Cells in the Endometrial Stroma Derived From Patients With Recurrent Miscarriage and IVF Failure, as Compared With Fertile Women. **NKp46 expression was significantly higher in the patient group compared to fertile women**

Figure 5. The Percentage Ratio of the NKp46- to CD56-Positive Cells in the Endometrial Stroma. No significant differences were found in the percentage rate of NKp46- to CD56-positive cells in the endometrial stroma derived from patients with RM and RIF, as compared with the healthy controls.
All participants signed the informed consent form after receiving explanations about the study. The study was financially supported by Jundishapur University of Medical Sciences. All participants signed the informed consent form after receiving explanations about the study.

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**Informed Consent**
All participants signed the informed consent form after receiving explanations about the study.

**References**
27. Laird S. The Role of Natural Killer Cells in Human Fertility. Royal College of Obstetricians and Gynaecologists (RCOG); 2016.