



Establishment of Cut-off Values for Uterine and Peripheral Blood Natural Killer Cells During the Peri-implantation Period in Fertile Controls and Women with Unexplained Recurrent Implantation Failure

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Abstract

Background: The purpose of the study was to determine the cut-off values for peripheral and uterine natural killer (pNK, uNK) cells in fertile controls and in women with recurrent implantation failure (RIF).

Methods: In this study, 50 women with RIF and 50 fertile controls were enrolled. Midluteal endometrial biopsy samples from both cases and controls were obtained for CD 56+ cell immunohistochemistry labeling to identify uNK cells. Peripheral venous blood was also taken during the biopsy to detect pNK cells in peripheral blood mononuclear cells using flow cytometry. Cut-off values were obtained from fertile controls. Using a non-parametric Mann-Whitney U-test, the medians of the data sets were compared.

Results: The median values for uNK and pNK cell levels in the control group were 7% and 11.6%, respectively. The median value for uNK cells in RIF patients was 9%, which was higher than the one in controls but not statistically significant (p-value of 0.689). The median pNK levels (11.6% vs. 12.4%) were comparable between the RIF group and the controls. Moreover, it was found that 68% of individuals had uNK cell counts below the reference value, while 32% had excessive levels exceeding 7%. Additionally, only 51.4% of the RIF group had increased pNK cells.

Conclusion: The pNK cell cut-off values need to be used with caution because there was no difference between fertile controls and RIF women. If immunotherapy is recommended for RIF women, uNK cell testing should be used as the preferred approach.

Keywords: CD56 antigen, Endometrium, Immunohistochemistry, Natural killer cells, Recurrent miscarriage.

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Introduction

Recurrent implantation failure (RIF) is a clinical condition characterized by repetitive unsuccessful implantation that cannot be detected at a stage discernible by pelvic ultrasonography. The most recent systematic review has defined RIF as the failure to achieve implantation despite two consecutive cycles of in vitro fertiliza-

tion (IVF), intracytoplasmic sperm injection (ICSI), or frozen embryo replacement. In these cycles, a minimum of four cleavage-stage embryos and at least two blastocysts, all of good quality and at the appropriate developmental stage, are transferred (1). It is an emotionally and physically demanding experience for both clinicians and

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couples alike. The prevalence of RIF was estimated to affect about 10% of couples undergoing assisted reproductive technology (2). Before initiating effective treatment, a comprehensive evaluation of the couple must be conducted to identify the underlying causes. In women having RIF despite producing sufficient good-quality embryos, it seems logical to assume that an underlying endometrial factor is contributing to their infertility. The increased numbers of uterine natural killer (uNK) cells in peri-implantation endometrium have been reported in women with recurrent miscarriage (RM) (3) and RIF after following IVF (4). However, the reports are contradictory when it comes to the correlation of increased numbers of uNK cells with pregnancy outcomes (4, 5).

Although clinical evidence is currently lacking, there exists the biological plausibility for the involvement of uterine natural killer cells in cases of reproductive failure. This has led to a debate about whether uNK cell measurement should be included in the evaluation of women with RM or RIF. One of the biggest hurdles is the lack of standard protocol for uNK cell count reporting and particularly the cut-off values for high cell count; one group of researchers has used a 5% cut-off (6), while another has used a 12.9% cut-off value (4). There is also a lack of consensus over what constitutes a "normal" range of uNK cell counts. Conducting NK cell testing in cases of reproductive failure could be valuable if pre-pregnancy levels of uNK cells could help guide the decision for immunotherapy, potentially leading to improved outcomes in subsequent pregnancies. A recent systematic review has also revealed that there have been no reports on the treatment of women with higher uNK cells and their consequent impact on IVF treatment outcome. All relevant studies employed pNK cell testing as the recommended diagnostic method for analyzing the levels of NK cells (7).

The purpose of the present study was therefore to establish reference cut-off values for uNK and pNK cells in fertile women and to determine the number of subjects with two or more failed IVF/ICSI cycles showing elevated uNK cells and pNK cells above the reference cut-off values.

Methods

The present study was carried out at the ART Centre of all India Institute of Medical Sciences, New Delhi, India. The study duration was from January 2019 to May 2021. Ethical clearance was

obtained from Institute's Ethics Committee before the initiation of the study (IEC-286/03.05.2019, RP-46/2019). In this study, a total of 100 women were recruited and evenly divided into a study group and a control group.

The study group comprised of infertile women with two or more prior unsuccessful IVF/ICSI cycles undertaking fresh embryo transfer (ET) or frozen embryo transfer (FET). The control group, included fertile women who visited a gynecology outpatient department for sterilization purposes.

Based on the inclusion criteria, the study group comprised of individuals who met the following criteria: aged between 20-35 years, BMI of 19-30 kg/m^2 , a history of two or more unsuccessful IVF/ICSI cycles, normal menstrual cycles, normal ovarian reserve (AMH $>2 ng/ml$, FSH $<9 mIU/ml$, AFC >8), and a normal uterine cavity as assessed by hysteroscopy or 4D ultrasonography. Furthermore, the control group comprised of individuals who met the following criteria: age below 35 years, regular menstrual cycles, no history of infertility, and voluntary participation in the study. Based on the exclusion criteria, the following individuals were excluded from the study group: those with poor endometrium thickness ($<7 mm$), polycystic ovarian syndrome, moderate to severe endometriosis, and abnormal parental karyotype. Additionally, individuals with a history of known immunological and medical disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis, diabetes, and congenital or acquired blood dyscrasias were excluded from the study. Women whose spouses did not undergo the standard semen evaluation, including DNA fragmentation index and reactive oxygen species analysis, were also excluded from the study.

In the control group, women who had undergone any hormonal treatment within the preceding three months were excluded from the study.

Methodology: The study was initiated following ethical approval, and all eligible patients who provided consent were enrolled. Eligibility for the study subjects and controls was assessed after evaluating the inclusion/exclusion criteria. For the study group, a comprehensive history was obtained, including details of previous IVF cycles, response to stimulation, and outcomes. Clinical examinations were performed, which included measurements of weight, height, and BMI. Pre-treatment baseline hormone assays (day 2/3) were conducted in all patients encompassing serum measurements of FSH, LH, and estradiol. Serum

AMH was evaluated in the same sample using Gen II ELISA (Beckman Coulter Diagnostics, USA). Pre-treatment investigations were done in all women including evaluation of serum TSH and serum prolactin. A baseline transvaginal ultrasound (day 2/3) was done to evaluate antral follicle count (AFC) and ovarian volume. Pre-IVF assessment of uterine cavity (3D imaging, hysteroscopy/4D ultrasound, and mock embryo transfer) and fallopian tubes (to rule out hydrosalpinx) was done besides endometrial thickness measurement (days 14-18).

Sample collection: Endometrial biopsies were obtained from outpatients in the Department of Obstetrics and Gynecology at All India Institute of Medical Sciences in New Delhi, India. The biopsies were performed using a disposable Karman Cannula size 4 on day 21±2 of the menstrual cycle. The females whose dates fell outside the range of days 19-23 were not incorporated into the study. The sample was fixed in formalin and sent to the pathology laboratory for histopathological examination and immunohistochemical (IHC) staining.

During the biopsy, the peripheral venous blood was simultaneously taken from the study and control groups to measure serum progesterone and for flow cytometry.

Sample processing: Immunohistochemical staining of CD56+ cells was done using Quanto Detection Kit (Thermo Fisher Scientific, USA). The slides were prepared from paraffin-embedded tissue blocks. The applied primary antibody was CD 56 and the secondary antibody was HRP-labeled polyvalent antibody provided in the IHC detection kit. The slides were cleaned with distilled water before being counterstained with hematoxylin followed by mounting with DPX (distrene, plasticizer, xylene) as mounting media. Every effort was made to keep tissue slices moist during the immunostaining process. The incubators were placed inside a humid environment. Staining procedures for each batch included the use of validation controls.

The stromal cell count was performed on slides with a field size of 10x40, which equalled a minimum of 3800 cells. A tally counter was used for both uNK cells (CD 56 positive stained) and stromal cells (CD 56 negative stained). The percentage of uNK cells relative to the total number of stromal cells was determined for each image. The total cell count was calculated as the aver-

age of all 10 fields (8).

Flow cytometry: Three millilitres of peripheral blood were taken and placed in a container containing EDTA. Double centrifugation method was used to separate peripheral blood mononuclear cells (PBMCs), and all samples were cryopreserved at -80°C. For flow cytometry, 10 samples in each lot were thawed. The thawed samples were added to phosphate-buffered saline (PBS) containing falcon tubes. The samples were incubated at room temperature for five minutes after being treated with 2 ml of a paraformaldehyde solution. The samples were centrifuged twice and the pellet was resuspended in two ml of PBS and fetal bovine serum (FBS) solution. For each sample, two vials (1.5 ml) were prepared for antibody staining along with control vials. All the samples were centrifuged at 500 g for five minutes and the supernatant was discarded. Anti -CD3 BB515, anti-CD56 PE, and PerCP anti -CD16 antibodies (BD Biosciences, US) were diluted with FBS and PBS solution and were added to the samples. The samples were incubated in the dark for 45 min at room temperature. The washing step was done by centrifuging the sample in PBS at 500 g for five min. The supernatant was discarded and the washing step was repeated. The pellet was resuspended in PBS. The samples were subsequently acquired using a flow cytometer (BD Accuri™ C6 Plus personal flow cytometer; BD Biosciences, USA) and results were evaluated and expressed as the percentage of pNK cells relative to all lymphocytes. Reference cut-off value was established from fertile control women for both pNK and uNK cells.

Statistical analysis: For statistical analysis of data, SPSS vs. 25.0 (IBM, USA) was used. Kolmogorov-Smirnov test was utilized to check the normality assumptions of continuous data. Descriptive statistics including mean, standard deviation, and range were computed for normally distributed data. For skewed/non-normal data, median and interquartile range were calculated. Using a non-parametric Mann-Whitney U-test, the medians of the data sets were compared. The results of the categorization were shown as frequencies and percentages. Chi-square or Fisher's exact test was used to compare frequency of data across categories. To find out the linear association between any two study variables, bivariate correlation coefficients were computed. A two-tailed probability of p<0.05 was considered statistically signifi-

Table 1. Baseline characteristics of fertile controls and RIF subjects

Parameter	Fertile controls (n=50) Mean±SD	RIF subjects (n=50) Mean±SD	p-value
Age (years)	29.66±3.38	30.82±2.73	0.062
BMI (kg/m^2)	23.25±1.99	23.07±1.86	0.645
Cycle day of sample collection	21.22±1.26	21.48±0.90	0.241
Progesterone (P4) on the day of sample collection (ng/ml)	10.83±5.45	10.12±4.44	0.481

cant for all analyses.

Results

A total of fifty fertile controls and fifty subjects were studied to determine the cut-off values for uNK and pNK cell levels.

Table 1 shows the baseline characteristics of the control group and study population. The distribution was homogenous in terms of age, BMI, cycle day of sample collection, and progesterone level on the day of sample collection. When comparing the two groups, there was no statistically significant difference. For the fertile control group, the minimum age was 23 years and the maximum age was 35 years, while for the RIF subjects, the minimum age was 24 years and the maximum age was 35 years. The mean number of living issues in the control population was $2.64±0.92$.

The baseline hormonal profile and ovarian reserve markers in RIF subjects are depicted in table 2. In RIF subjects, the mean duration of infertility was $9.12±2.8$ years and the mean number of earlier failed cycles was $2.24±0.4$ (Table 2).

In general, 60% (30/50) of women had primary infertility and 40% had secondary infertility. Tubal factor was the prime factor of infertility in 58% (29/50) of the subjects and the remaining (42%) demonstrated unexplained infertility. The semen analysis parameters of RIF couples depicted that the mean total sperm count, progressive motility, and morphology were normal ($51.66±25.2$ million/ml, $42.12±9.3%$, and $10.34±3.2%$, respectively).

Table 2. Baseline hormonal profile and ovarian reserve markers in RIF subjects (n=50)

Parameter	Mean±SD
Duration of infertility (years)	9.12±2.86
Number of previous failed cycles	2.24±0.47
FSH (mIU/ml)	6.37±1.93
LH (mIU/ml)	4.06±1.43
AMH (ng/ml)	4.04±1.62
Antral follicle count (AFC)	10.08±2.94
TSH (mIU/L)	2.85±0.77
Baseline endometrial thickness (mm)	8.29±0.88

The values for both endometrial and peripheral blood NK cell levels were obtained from both fertile control population and RIF subjects (Table 3). Since the obtained data exhibited skewness or non-normal distribution, the median was calculated to determine the cut-off values. The median and range for uNK cells in controls and subjects are depicted in figure 1 and that of pNK cells are depicted in figure 2. The control group exhibited median uNK cell levels of 7% and pNK cell levels of 11.6%. The median uNK cell percentage in RIF participants was 9%, which, although higher than in the control group, did not reach statistical significance (p-value of 0.367). Comparable median pNK levels were also observed across the control and RIF groups (p-value of 0.633).

All RIF individuals did not have increased NK cell counts and 32% of patients exhibited in-

Table 3. Median values for pNK and uNK cell levels (%)

NK cell levels	Controls (n=50)	Subjects (n=50)	p-value
	Median (IQR)	Median (IQR)	
Median uNK cell levels (%)	7 (18.25)	9 (17.25)	0.367
Median pNK cell levels (%)	11.6 (9.53)	12.4 (11.65)	0.633

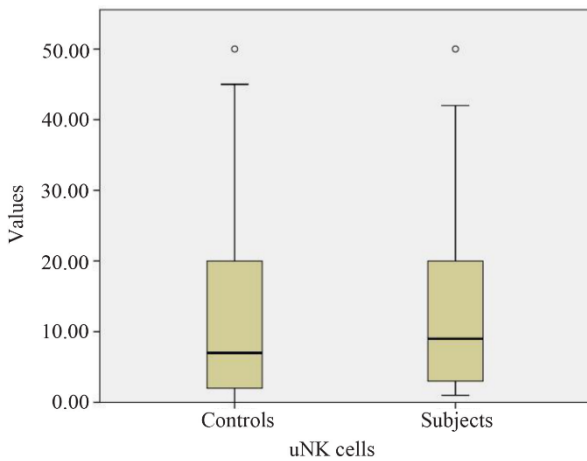


Figure 1. Box and Whisker plot depicting median and range of values for uNK cells in controls and subjects

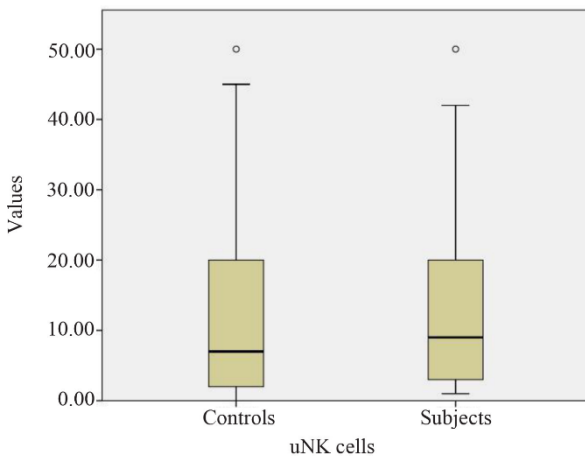


Figure 2. Box and Whisker plot depicting median and range of values for pNK cells in controls and subjects

creased uNK cell levels (more than 7%), whereas 68% had levels below the standard threshold. pNK cells were also increased in 51.4% of individuals with RIF.

A positive correlation was found between uNK and pNK cell levels in RIF subjects, although not statistically significant (p-value of 0.06). In fertile controls, there was also a nonsignificant correlation between uNK and pNK cell levels (p-value of 0.594) (Table 4).

Discussion

Both uterine mucosa and peripheral blood lymphocytes include natural killer (NK) cells, which are an essential component of our innate immune system. They are large granular lymphocytes generated from bone marrow haematopoietic progeni-

Table 4. Correlation between uNK and pNK cell levels among subjects and controls

Study population	Pearson correlation	p-value
Subjects (n=50)	0.608	0.06
Fertile controls (n=50)	0.77	0.594

tor cells that express surface antigen CD 56 (9). CD 56 dim and CD 16+ cells make up the vast majority of peripheral NK (pNK) cells (90%) (10), whereas most of uNK cells are CD 56 bright and CD 16- (11). NK cells induce apoptosis or lysis of target cells by the release of granular components (perforins, granzymes) from their cytoplasm or the production of cytokines such as interleukin -10, tumor necrosis factor-alpha, transforming growth factor-beta, and interferon-gamma. uNK cells are a strong supply of cytokines, specifically angiogenic, which are responsible for trophoblast invasion and angiogenesis, whereas pNK cells exhibit considerable cytotoxicity with anti-viral and anti-neoplastic properties (11). Two theories, however, provide explanations for the origin of uNK cells: either they differentiate from pNK cells within the uterine microenvironment, or they arise from the growth and differentiation of endogenous NK cells or stem cells within the endometrium (11,12).

Joseph-Horne et al. assessed the luteal phase progesterone excretion in fertile and infertile population and revealed that the values for the early luteal phase were considerably higher in fertile women than in the infertile group (13). To validate the luteal phase of the menstrual cycle, the progesterone levels were tested simultaneously on the day of sample collection for histological dating as well as uNK and pNK cell assessment. As all females had normal menstrual cycles, all samples were obtained on day 21±2 of the menstrual cycle. This difference in cycle day of sample collection and progesterone levels did not correlate with uNK and pNK cell levels in the present study.

The cut-off value for uNK cell levels in the present study was 7% (median) as obtained from the control group whereas in RIF subjects, the median value was 9%. CD56 immunohistochemical analysis of the peri-implantation endometrium from fertile controls and RIF participants is shown in figure 3. Similar findings were observed by Tucker et al. who used immunocytochemistry to determine the proportion of stromal cells positive

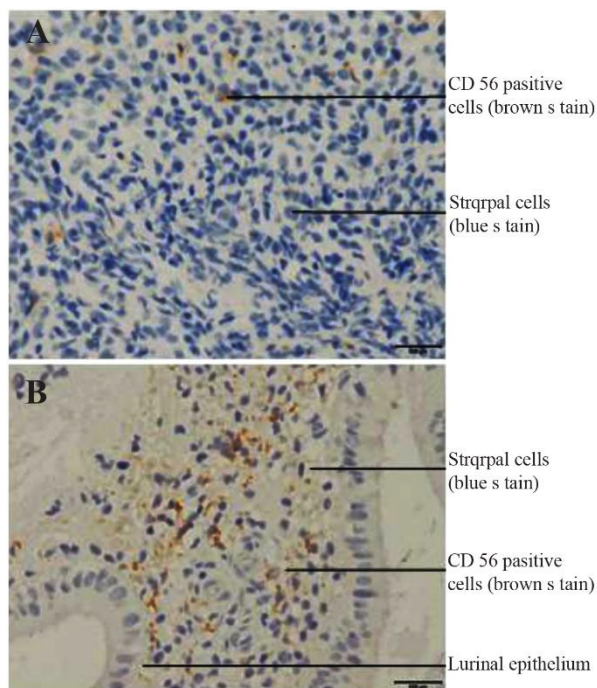


Figure 3. Photomicrograph depicting immunostaining of peri-implantation endometrium from fertile control women and women with RIF. CD 56 (brown stain) in normal endometrium (A) CD 56 (brown stain) in endometrium from RIF woman (B). IHC (200X)

for CD56, CD16, and CD69 in endometrial samples from 15 healthy controls and 40 patients with RIF (4). Endometrium from women with RIF following IVF had a substantially greater density of CD56+ cells (p-value of 0.005) compared to endometrium from control women with median (range) CD56+ cell density of 5% (2.1-19.2%). The comparison of uNK cell percentages among women with recurrent reproductive failure and fertile controls was performed in a retrospective research by Chen et al. They concluded that median percentage of uterine natural killer cells was 2.5% (range: 0.9%-5.3%) in fertile ovulating controls from China and the reference range was 1.2-4.5% (5th-95th percentile). The percentage of median uNK cell was 3.2% (range of 0.6-8.8%) and the median RIF uNK cell was 3.1% (range of 0.8-8.3%), both substantially higher than the control groups (14). These low median values for uNK cells in controls and RIF group as compared to our study might be attributed to ethnic variations, although there are no studies on the variation of uNK cell levels comparing Asian women with others. Donoghue et al. could not find any significant difference in the cell density of CD56+ or CD16+ uNK cells in women with RIF com-

pared to women with implantation success (15). The finding differed from the present study as they included cases with endometriosis, PCOS, and fibroids in their study that might have affected the uNK cell levels.

The cut-off value for pNK cell levels was found to be 11.6% (obtained from a fertile group) in the present study. Flow cytometry dot plots of the control and RIF subjects have been shown in figure 4. This is in accordance with the findings of Beer et al. who demonstrated that women with multiple IVF failures had considerably greater levels of CD56+ pBL than normal fertile controls, and those with CD56+ pBL of 12% or fewer had a much higher conception rate (16). Another study by Sacks et al. demonstrated the cut-off value of 18% (17). Santillán et al. reported pNK cell levels to be $13.4 \pm 1.2\%$ (range of 2.63–29.01) in RIF subjects and $8.4 \pm 0.7\%$ (range of 5.72–13.28) in fertile controls (18). The present study, however, revealed no difference in median pNK cell levels in RIF and fertile controls. This might be due to the small sample size of the study.

All RIF individuals did not have increased NK cell counts and 32% of patients exhibited increased uNK cell levels (more than 7%), whereas 68%

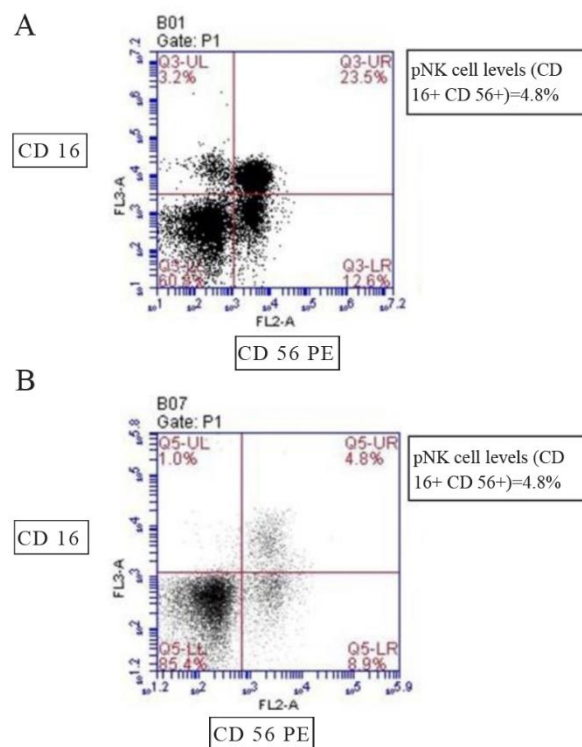


Figure 4. A) Flow cytometry dot plots showing pNK cell levels (CD 16+ CD 56+) in RIF subjects and B) control population

had levels below the standard threshold. This implies that not all women with RIF exhibit elevated NK cell levels, thus caution should be exercised when considering the use of these values for immunotherapy decisions during IVF. As there was no variation in pNK cell levels among fertile controls and women with recurrent implantation failure, the cut-off values of pNK cells should also be used with caution. In contrast, Santillán et al. revealed that uNK levels above 250 CD56 cells/hpf were observed in 53% of idiopathic RIF patients, while only 5% of healthy controls exhibited similar levels (18). Chen et al. observed that around 29% (10/34) of RIF women had an uNK cell proportion that exceeded the upper limit. However, they utilized the 5th and 95th percentiles to establish the lower and upper boundaries and determined that 47% of women with RIF had an uNK cell count that was beyond the reference range (14).

The 10th and 90th percentile cut-offs for NK cells in surrogates were identified in a study as 19.58% and 44.3%, respectively. Furthermore, 10.5% of patients with implantation failure (compared to 9.5% in surrogates) had NK cells below the cut-off of 19.58% whereas 17.5% were identified to have NK cell levels over 44.3% (compared to 9.5% in surrogates). However, there was no statistically significant difference in uNK cell density in surrogates and women who experienced failed implantation (19).

A comprehensive review and meta-analysis revealed that uNK levels were considerably higher in the endometrium of women with RM and RIF. Moreover, it was found that measuring pNK as a predictor of uNK behavior had limited clinical support. However, before utilizing this in clinical settings, a standardized reference range must be established. uNK measurement has the potential to be a more useful diagnostic tool (20). Investigations on the function of uNK cells in cases with RIF and RM have produced inconsistent findings and contradictory conclusions. These differences arise due to the diversity of the population being studied and the lack of established scientific techniques for determining the normal range and functioning of uNK cell counts (21).

High amounts of pNK cells were seen in 51.4% of the study group, which is significantly greater than the 11% found in previous research on women with RIF (17); however, the analysis of NK cells in the study might have been significantly influenced by the samples being collected during

the IVF cycle and the analysis being conducted by an off-site laboratory. Moreover, the study included RIF women with endometriosis and autoimmune diseases which might have affected NK cell levels. In the present study, the blood samples were taken during an unstimulated cycle at a fixed time during the mid-luteal phase in all patients. The RIF subjects comprised only those with either unexplained or tubal factor infertility, without any apparent cause that could have influenced NK cell levels.

All other factors of infertility which could either impair the implantation or affect NK cell levels like anovulation, male factor, endometriosis, diminished ovarian reserve, uterine fibroids, polyps, synechiae or septum were excluded. Thus, the underlying immunological cause of infertility in all the subjects recruited in the present study could be unexplained recurrent implantation failure. None of the patients had any autoimmune disorder or thrombophilia. All patients had normal karyotypes.

All the samples were processed and analyzed by flow cytometry using consistent methodology performed by a single individual.

When the correlation between uNK and pNK cells was studied, a positive correlation was detected between uNK and pNK cell levels in RIF subjects, which was not statistically significant. In fertile controls, there was also an insignificant correlation between uNK and pNK cell levels. There is a lack of available data on the correlation between uNK and pNK cell levels specifically in women with RIF. Our finding differed from the study conducted by Santillán et al. who found a weak but positive correlation between pNK and uNK cell levels (Pearson correlation=0.38; p-value of 0.012) which further increased (Pearson correlation=0.587; p-value of <0.001) when idiopathic RIF group was considered (18).

The above contradictory findings only reflect a lack of evidence in supporting immunomodulatory therapy for RIF. Despite the extensive research, the exact role of uNK cells in pregnancy is still uncertain. It is also unclear whether the reported increase in uNK cell numbers which is associated with abnormal pregnancy pathologies like RPL, RIF, or pre-eclampsia, is directly causal or if it reflects more underlying issues with the endometrium. It is known that uNK cells and pNK cells are distinct from each other. Therefore, relying solely on measurements of pNK cells is not sufficient to comprehend the involvement of uNK cells

in reproductive failure. At present, there is no indication for routine uNK cell testing in women undergoing infertility or seeking IVF treatment. uNK cell testing may have a role in the selected group of RIF women with no other cause of implantation failure. The measurement of uNK cells must be standardized and the definition of "normal" and "high" levels should be based on established reference ranges derived from standardized methodology.

Only cases of unexplained and tubal factors of infertility were included in our study, thus making our study group a true representation of unexplained RIF. This led to the hypothesis that an underlying immunological etiology could be the cause of their implantation failures. In the present study, a fertile control group was used to establish a reference cut-off value for uNK and pNK cell levels. As these cut-off values have not been derived for the Indian population in any of the studies, the possibility of ethnic differences in NK cell levels was ruled out by not employing the reference values from different ethnic groups. Also, in this study, an attempt was made to determine the correlation between uNK and pNK cells in both fertile and RIF subjects, with the goal of exploring the feasibility of using pNK cell testing as a potential alternative to more invasive uNK cell testing. However, the most important limitation of the research was the small sample size of the study group.

Conclusion

The current study has established the threshold values for uNK and pNK cell levels to be 7% and 11.6%, respectively. Considering that not all women with RIF have abnormally high NK cell counts, these data should be utilized with caution when deciding on immunotherapy during IVF. The lack of any noticeable difference in pNK cell levels between fertile controls and women experiencing recurrent implantation failure implies that the use of cut-off values should be approached cautiously. The utilization of uNK cell testing may be deemed a favorable approach in cases where immunotherapy is recommended for this cohort of female patients. The potential promise of NK cell testing in the realm of assisted reproduction for women experiencing unexplained recurrent implantation failure necessitates further validation of our findings through larger trials.

Conflict of Interest

No conflicts of interest were declared by any of the authors.

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