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# Natural Killer Cells in Peripheral Blood and Pregnancy Outcome in Women with Miscarriage

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**Abstract:** ***Objective: To compare the levels of Natural killer cells in women with history of miscarriage/implantation failure with controls and to observe its association with pregnancy outcome. METHODS- For Peripheral blood Natural Killer Cell analysis three colour immunofluorescence of Lymphocyte markers was performed in 1millilitres of peripheral venous whole blood collected in heparinised and EDTA tubes. The results were calculated as numbers and percentage of total circulating lymphocytes, expressed as a percentage of positive events in relation to all the events acquired by the gating.(NK cell %CD16,56=5.47-36.60; Absolute CD16+56 = 116.0-612.0/µL). Results: Mean age of pregnant cases was 27±5.2 and of pregnant controls was 26±5.22. Mean age of nonpregnant cases was 30.1±5.59 and non pregnant control was 28.3±7.7. Absolute number and percentage of NK cells were elevated in cases with history of miscarriage. The mean NK cell percentage in pregnant women with history of miscarriage was 18.66±11.99 whereas of pregnant control group percentage was 6.58%±3.72. The difference was statistically significant (P value =0.004). Mean NK cell percentage in non-pregnant patient with history of miscarriage was 17.1±10.49%. while of non pregnant control was 7.43±4.67. The difference between the two group was statistically significant (P=0.01) Conclusion: Miscarriage is a heterogeneous condition. Elevation of natural killer cells has an negative effect on reproductive performance, and NK cells levels in blood are currently being used as a diagnostic test to guide the initiation of therapies in patients with history of miscarriage.***

**Keywords:** Miscarriage, Natural killer cells, Immunofluorescence analysis, Pregnancy Outcome

## 1. Introduction

Immunological mechanisms play a role in reproductive problems such as miscarriage, infertility and implantation failure. A successful pregnancy involves maternal adaptation of the immune response to the semi-allogenic developing embryo. Implantation and growth of fetal allograft is an immunological paradox influenced by local and systemic immune responses involving immunoglobulins, cytokines, hormonal and endometrial factors and synergism between them is critical for successful implantation and subsequent conception. Natural killer cell is a part of the innate immune system, found in both peripheral blood and endometrium, derived from haematopoietic progenitor cells (HPCs) in the bone marrow and comprises 10-15% of human peripheral blood lymphocytes(PBL) . They are defined phenotypically by expression of CD56 and lack of expression of CD3 on the cell surface. They mediate nonmajor histocompatibility complex-restricted cytotoxicity of target cells. Peripheral NK cell activity and their percentage decline significantly from the first trimester of normal pregnancy. NK cell activity capable of placental damage is also down-regulated during pregnancy. An abnormal increase in the peripheral blood natural killer (pbNK) cell parameters(either in Natural Killer cells absolute value or in percentage (%)prior to conception or during early pregnancy)is associated with etiopathogenesis of Recurrent Miscarriage, implantation failure and infertility may be due to NK cell cytotoxicity or receptor/gene expression. Therefore, NK cells have been implicated to play a role in female reproductive performance. However, the exact pathogenic mechanism behind the role of NK cells in human reproduction is still unclear.

## 2. Materials and Methods

The present Observational study was conducted at Department of Obstetrics & Gynaecology, Moti Lal Nehru Medical College Allahabad over a period of one year from September 2017 to August 2018.

*Sampling Method*- Incidental Sampling

*Inclusion Criteria*-

- Woman who has definite history of spontaneous pregnancy losses, non-pregnant and pregnant.
- Normal non-pregnant and pregnant women

*Exclusion criteria*-

- Woman who received hormone treatment/ hormonal contraception/ history of intrauterine contraceptive device during last three months before study.
- Woman with history of induced abortion/ septic miscarriage.
- Woman with chronic disease or with chronic ongoing treatment/ thrombophilia/ documented endocrinopathies (diabetes, thyroid disorder, hyperprolactinemia).
- Woman with documented uterine anomalies, abnormal karyotype, polycystic ovary syndrome.

*Sampling size*- Total of 67 women of reproductive age.

*Interventions*

Peripheral blood Natural killer cells –number and percentage analysis was done in women with history of spontaneous abortions either non-pregnant or in early pregnancy (5-12 weeks of gestation) and also in normal controls. For Peripheral

blood Natural killer cells as numbers and percentage of total circulating lymphocytes,1millilitres of peripheral venous whole blood was collected in heparinised and EDTA tubes. The sample was kept at room temperature (18-25'C) and not shaken. The sample was homogenized by gentle agitation prior to taking the test sample. The sample was analyzed within 24 hours of venipuncture. Immunofluorescence analysis was performed on a Coulter Flow Cytometer F C 500.

The Flow cytometer measures light diffusion and the fluorescence of cells. It makes possible the delimitation of the population of interest within the electronic window defined on a histogram, which correlates with orthoglonal diffusion of light and (Side Scatter or SS) and the diffusion of narrow – angle light(ForwardScatter or FS ).The flowcytometry is based on ability of specific monoclonal antibodies to bind to antigenic determinants expressed by leucocytes. The fluorescence of the so delimited cells is analyzed in order to distinguish the positively stained events from the unstained ones(Figure 1).

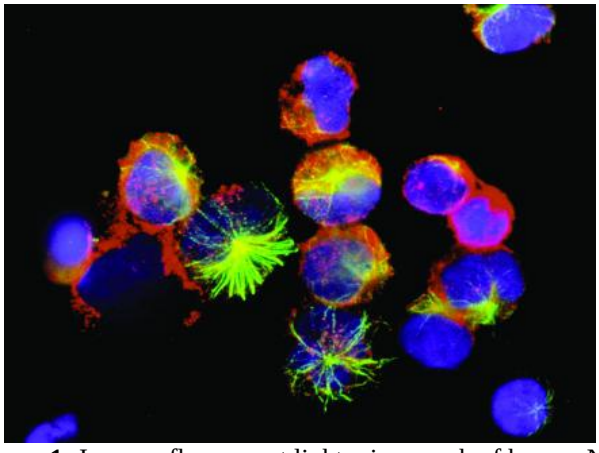

**Figure 1:** Immunofluorescent light micrograph of human NK cells

The results were expressed as a percentage of positive events in relation to all the events acquired by the gating. (NK cell %CD16,56=5.47-36.60% ; Absolute CD16+56 = 116.0-612.0/µL)

The outcome of the pregnancy was evaluated in one year period.

*Statistical Analysis*- All statistical analysis $p<0.05$ was considered as significant.

## 3. Results

A total 67 women were included in the study and 30 were non-pregnant and 37 were pregnant. Among 30 non-pregnant women, 20 women had history of miscarriage, while 10 were taken as control. Out of 37 pregnant women 27 women had history of previous miscarriage and 10 women were control. Mean age of pregnant cases was 27±5.2 and of pregnant controls was 26±5.22. Mean age of nonpregnant cases was 30.1±5.59 and non pregnant control was 28.3±7.7 (Table-1)

**Table 1:** Age distribution of cases

| S.No | | Pregnant Cases | Pregnant Controls | Non Pregnant Cases | Non Pregnant Controls |
|---|---|---|---|---|---|
| | | N. no(%) | N.no(%) | N.no(%) | N.no(%) |
| 1. | <20 Years | 1(3.7%) | 1(10%) | - | 1(10%) |
| 2. | 20-30 Years | 21(77.77%) | 7(70%) | 12(60%) | 5(50%) |
| 3. | >30 years | 5(18.5%) | 2(20%) | 8(40%) | 4(40%) |
| 4. | N no. | 27 | 10 | 20 | 10 |

Out of 27 pregnant patients with history of miscarriage 4 cases had absolute NK cell value of <116/ µL. 20 cases had NK cell value between 116-612/µL. 2 cases had NK cell value >612/µL.

The mean NK cell absolute value of pregnant women with history of miscarriage was 367.88±184.11.For the NK cell percentage 4 cases had NK cell percentage of <5.47% in peripheral blood. 20 cases had NK cell percentage between 5.47-36.6% and 3 cases had NK cell >36.6%.

Among pregnant control women 50% cases had NK cell absolute number between <116/µL and NK cell percentage <5.47%. 40% cases had their NK cell absolute value between 116-612/µL and NK cell percentage less than 5.47-36.6% . 10% patients had NK cell absolute value more than >612/µL and NK cell percentage more than 36.6%. Out of 20 non-pregnant patient with history of miscarriage 1 case had absolute NK cell number of 100/L, 15 cases had between 116-612/ µL, 4 cases had >612/µL. Their mean NK cell absolute number was 442.45±184.58/µL. As far as the percentage of NK cell is concerned, 1 case had 4.6% NK cells in peripheral blood out of total lymphocytes, 15 cases each had NK cell percentage between 5.47-36.6% and 4 cases had NK cell >36.6% in their peripheral blood.

Among the non pregnant control 6(60%) cases had NK cell absolute number less than <116/µL, 4(40%) cases had NK cell absolute number between 116-612/ µL. Mean NK cell value of non pregnant control was 159.5±135.62. For the NK cell percentage, 6(60%) cases had it <5.47%, 4(40%) cases had between 5.47-36.6%. (Table-2).

**Table 2:** Natural killer cells in patients and controls

| Absolute Number of NK Cells | Pregnant Cases (n=27) | Pregnant Controls (n=10) | Non Pregnant Cases (n=20) | Non Pregnant Controls (n=10) |
|---|---|---|---|---|
| | N.no(%) | N.no(%) | N.no(%) | N.no(%) |
| <116/µL | 4(14.8%) | 5(50%) | 1(5%) | 6(60%) |
| 116-612/µL | 20(74%) | 4(40%) | 15(75%) | 4(40%) |
| >612/ µL | 3(11.11%) | 1(10%) | 4(20%) | - |
| PERCENTAGE OF NK CELLS | | | | |
| <5.47% | 4(14.8%) | 5(50%) | 1(5%) | 6(60%) |
| 5.47-36.6% | 20(74%) | 4(40%) | 15(75%) | 4(40%) |
| >36.6% | 3(11.11%) | 1(10%) | 4(20%) | - |

The mean NK cell percentage in pregnant women with history of miscarriage was 18.66±11.99 whereas of pregnant control group percentage was 6.58%±3.72. The difference between two groups is statistically significant (P value =0.004) . Their mean NK cell percentage in non-pregnant patient with history of miscarriage was 17.1±10.49%. while of non pregnant control was 7.43±4.67. The difference between the two group was statistically significant (P=0.01) (Table-3)

**Table 3:** Comparison of Mean NK cell percentage among various groups

| S, No | Group of Patients | Mean NK Cell Percentage |
|---|---|---|
| 1 | Pregnant women with history of miscarriage | 18.66±11.99 |
| 2 | Pregnant control women | 6.58±3.72 |
| 3 | Non Pregnant women with history of miscarriage | 17.1±10.4 |
| 4 | Non pregnant control women | 7.43±4.67 |

Among 27 pregnant women with history of miscarriage all 4 cases with NK cell percentage <5.47% delivered at term. and 16 cases with NK cell percentage between 5.47-36.6% also delivered at term while 4 cases aborted at 10 weeks spontaneously. 1 cases of group of >36.6% delivered at term and 2 cases aborted spontaneously before 10 weeks of gestation. Out of 10 pregnant control women 1 woman with NK cell percentage of 15% aborted while all women with NK cell <12% delivered at term.(Table-4)

**Table 4:** Association of NK cell percentage with subsequent outcome of pregnancy in pregnant female

| S.No | NK Cell percentage | Pregnancy outcome |
|---|---|---|
| 1 | <5.47% | All cases delivered at term |
| 2 | 5.47-36.6% | 16 cases delivered at term and 4 cases aborted |
| 3 | >36.6% | 1 case delivered and 2 cases aborted |

## 4. Discussion

For blood NK cell testing, apparently broad reference range for NK cell parameters(5.47%-36.6%) makes an attempt to define an abnormally high level within that range difficult, but NK cell percentage over 12 of total lymphocytes in this study was considered as high levels.

The overall mean age of pregnant group was 26.75±5.17 years while of non-pregnant group was 30.58±5.94 years (P=0.02). During the past two decades as the age of marriage is increased greater majority of women become pregnant in their late twenties (20s) and early thirties (30) and hence they very often face the problem of miscarriage.

Out of 27 pregnant women with history of miscarriage, majority (60%) of cases had NK cell absolute value between 116-612/µL. Mean NK cell absolute value was 367±184 suggesting that elevated number of NK cells in blood lead to infiltration of endometrium and complications such as miscarriages. In present study the women with previous miscarriages had higher number of NK cells in peripheral blood and this might influence the fate of present pregnancy also as NK cells play an important role in control of trophoblast growth and placental development and women with previous miscarriages who still had high NK cell levels in present pregnancy might miscarry the present pregnancy also while the patients with previous miscarriages with normal NK cell levels in present pregnancy might carry the pregnancy upto term.

17 out of 27 pregnant women with history of miscarriage had NK cell percentage>12% while only 2 out of 10 pregnant control had NK cell >12% percentage. Mean percentage of NK cells is 18% in pregnant cases compared to control who have 6.5% mean NK cells with difference of NK cells % between two groups was statistically significant (p value 0.004), suggesting that the pregnant cases with previous miscarriages who had higher NK cells in present pregnancy have higher chances of having miscarriage in present pregnancy also while women with normal levels of NK cells in present pregnancy would have a favourable outcome in present pregnancy.

All pregnant women with history of previous miscarriages who had their NK cell percentage in present pregnancy <12% had successful outcome of present pregnancy by delivering live baby in this pregnancy at term.

Majority 90% pregnant control women had NK cell percentage<12% and absolute value of NK cells less than 400/µL. Mean NK cell levels and percentage in pregnant control group was 157±107 and 6.5±3.7 respectively. The pregnant women who had no previous miscarriages and had normal levels of NK cells in early present pregnancy were likely to have a favourable outcome of present pregnancy. Majority of non-pregnant women (13/20) with previous miscarriage had NK cell absolute value >400/µL. Their absolute mean NK cell number was 442±184.58. This study revealed elevated CD56+CD16 peripheral blood NK cells in women with miscarriage. This study is not intended to establish a threshold level of CD56+CD16 peripheral blood NK cell as estimating a threshold level and testing it in same sample will create bias. We speculated in our study that women with previous miscarriage had elevated peripheral NK cells that is >400 cells/µL but still precise role of NK cells in miscarriage is not fully understood.

To our best knowledge there is no well documented study where absolute value of peripheral blood NK cells is studied in non-pregnant patients with history of miscarriage. Majority of women (13/20) with previous miscarriage had NK cell percentage >12%. Mean NK cell percentage is 17±10.49. There is no consensus in published literature as to what the definition of raised level of NK cells should be: 10, 12 or 18% but peripheral NK cell level of 12% of all lymphocytes is still regarded as the cutoff between a raised and normal level in women with miscarriages. Thus these women in present study who had previous miscarriage had high percentage (>12%) of peripheral blood NK cells.

Hosseini *et al.* (2014) also observed that in comparison to fertile group peripheral blood CO56+CD16+ subsets were significantly high in RSA patients (p value 0.01). Nearly similar to present study Shakkar*et al.*(2015) also reported NK cell percentage in primary recurrent miscarriage to be 13.17±0.93.

## 5. Conclusion

Miscarriage is a heterogeneous condition and is a challenging reproductive problem for the patient and clinician. In present study we tried to find out association between NK cell number and percentage and pregnancy outcome in patients with miscarriage as elevation of natural killer cells had a negative effect on reproductive performance. In our knowledge, this is the first trial in investigation of novel aspect of immune cause of spontaneous miscarriage. Drawing clear guidelines for the effect of NK cells on female reproductive performance and management of elevated NK cells in women with miscarriage, infertility or implantation failure is difficult due to paucity of large randomized trials and large scale studies with a biomolecular approach are needed to further substantiate our results

**Compliance with ethical standards**

- **Conflict of Interest**- Dr Meena Dayal, Dr Shadma Siddiqui, Dr Uravashi Singh declare that they have no conflict of interest.
- **Informed Consent-** Informed consent was obtained from all individual participants included in the study.

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