VOLUME - 12, ISSUE - 08, AUGUST - 2023 • PRINT ISSN No. 2277 - 8160 • DOI : 10.36106/gjra

Original Research Paper

Pathology

STUDY OF UTERINE NATURAL KILLER CELLS IN ENDOMETRIUM OF PATIENTS WITH RECURRENT MISCARRIAGE, DEPARTMENT OF PATHOLOGY, TERTIARY CARE CENTRE.

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ABSTRACT

Aims And Objectives: This study was done to evaluate the concentration of uterine natural killer cells by morphometry in endometrium of patients with recurrent miscarriage and to compare the number of uNK cells in women with recurrent miscarriage and fertile women without any miscarriage. **Materials And Methods:** Present study was conducted on 30 patients with history of recurrent miscarriage as study group and 30 fertile women were taken as control group over a period of two years from july 2017 to june 2019 in Department of pathology at Tertiary care centre.Endometrial biopsies were taken in the midluteal phase.Routine processing and haematoxylin and eosin staining of the endometrial biopsies were done followed by immunohistochemical analysis with anti CD56 antibody for uNK cells. **Results:** Majority of women in case group were in age group of 36-40 years. In our study, 53.3% women in case group had history of 3 previous abortions. The mean CD56 positive uNK cells in cases were 56.16/10hpf and mean CD56 positive uNK cells in controls were 25.20/10hpf. **Conclusions:** We have found that The mean NK cell concentration was significantly higher in women with recurrent miscarriages compared to fertile women in control group. NK cell concentration would help in selection of patients for immunomodulation therapy and also may throw some insight into the pathogenesis of recurrent miscarriage

KEYWORDS : Recurrent miscarriage,uterine natural killer cells,CD56,endometrial biopsy.

INTRODUCTION

Recurrent miscarriage is a common presentation in obstetrics these days. The incidence is 1-3% during reproductive years. Recurrent miscarriage (RM), historically defined as the loss of three or more consecutive pregnancies before 24 weeks gestation, occurs in about 1% of fertile women trying to conceive, and is associated with significant psychological morbidity[1].

It is heterogenous condition associated with many pathologies, but yet none are found in more than 50% of couples after numerous investigations[2]. The causes include parental and foetal chromosomal abnormalities,certain structural uterine abnormalities,antiphospholipid syndrome (APS), some thrombophilias and endocrinological disorders such as polycystic ovarian syndrome (PCOS). In cases where no known associations are diagnosed, women are termed to have idiopathic RM. It is thought to be an immune based process. So far, only screening for antiphospholipid syndrome is recommended in guidelines as a part of immunologic diagnosis.

Recently uterine natural killer (uNK) cells are considered to form a large portion of immune cells in the uterine cavity and play an important role in implantation and early pregnancy[3]. Preliminary evidence indicates that the concentration of uterine natural killer cells in women with recurrent miscarriage is increased. Hence, immunomodulation therapy can be one mode of treatment for these patients.

In the present study we shall evaluate the concentration of uterine natural killer cells in the endometrium of patients with recurrent miscarriage.

MATERIALS AND METHODS

The present study was an observational study conducted in the Department Of Pathology,at tertiary care centre for a period of 2 years i.e.; July 2017 to June 2019.

Inclusion criteria:

Properly labelled endometrial curettings and biopsy specimens in Mid-luteal phase were taken.The endometrial biopsies from 30 women in 18-40 years age group with a history of recurrent miscarriage were taken as the study group.Endometrial biopsies taken from 30 fertile women of Child bearing age without history of miscarriage were taken as control group.

Exclusion criteria:

1. Patients with anatomical disorders of uterus, thyroid dysfunctions, Anticardiolipin antibodies, lupus anticoagulant, deficiency of coagulation factors, inherited thrombophilias were excluded from our study.
2. All the patients already on immunomodulation therapy were excluded from the study.

Biopsies were fixed in 10% buffered formalin followed by processing in automated tissue processor and embedding in paraffin wax.3-4 microns tissue sections were cut and stained with H&E. Sections of 4-5 micron thickness were prepared from the corresponding paraffin blocks on poly-L-lysine coated slide for immunohistochemical staining. The Primary antibody (CD56- mouse IgG1 monoclonal antibody-prediluted Clone:123C3, PathnSitu) and Secondary antibody (Pathnsitu 43 Real En-vision rabbit and mouse) of Pathnsitu Company were used and stained according to their protocol. Evaluation of IHC was done which is characterised by dark brown cell membrane staining in uterine NK cells. The slides were scanned and CD56 positive cells were counted in ten high power fields(40x) and mean cell count was taken. The mean CD56 positive uNK cells in all the cases were compared with that of controls.

Statistical analysis of the results was done by performing Chi square test and P value at level of significance(<0.05).

OBSERVATIONS AND RESULTS

Majority of women in case group were in age group of 36-40 years and the least were in age group of 21-25 years. Majority of women in control group were in age group of 31-35 years and least were in 26-30 years.The demographic characteristics of the groups are presented in Table 1.

Table 1: Age Distribution Of Cases And Control Group

AGE GROUP	CASE GROUP	CONTROL GROUP
21-25	2	3

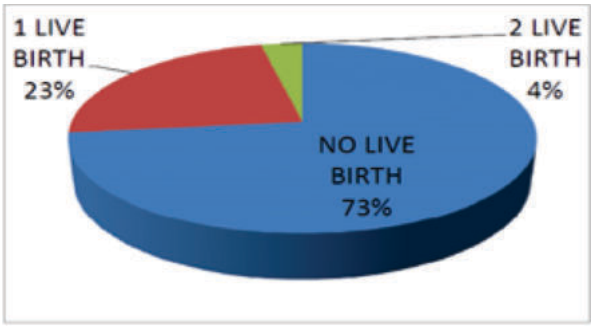
26-30	8	2
31-35	6	13
36-40	14	12

Majority (53.3%) of women had history of 3 previous abortions.Table 2 shows the number of abortions in case group.

Table 2: Number Of Abortions In Case Group

NO OF ABORTIONS	TOTAL NO.OF CASES
A3	16(53.3%)
A4	8(26.7%)
A5	6(20%)

22(73.3%) women with unexplained infertility were primarily infertile , and 8 (26.7%) were secondarily infertile.73% cases had no live births and 3.3% cases had 2 live births.Graph 1 shows the correlation of case group with number of live births



Graph 1: Correlation With Number Of Live Births In Cases

The mean NK cell concentration was high in cases compared to control group with maximum mean CD56+ cells/hpf of 58.3 in the age group of 21-25(**TABLE 3&4**).

Table 3: Comparison Of Mean Nk Cell Concentration With Age In Control Group And Cases

AGE GROUP	TOTAL NO.OF CONTROLS	MEAN CD56+ CELLS/10HPF
21-25	3	21.43
26-30	2	25.05
31-35	13	24.61
36-40	12	26.81

Table 4: Comparison Of Mean Nk Cell Concentration With Age In Control Group And Cases

AGE GROUP	TOTAL.NO.OF CASES	MEAN CD56+ CELL/10HPF
21-25	2	58.3
26-30	8	56.95
31-35	6	56.15
36-40	14	55.42

There is no significant correlation between CD56+ uNK cells and number of previous live births(**TABLE 5)**.

Table 5: Comparison Of Mean Nk Cell Concentration With Previous Live Birth In Cases

NUMBER OF LIVE BIRTHS	TOTAL NUMBER OF CASES	MEAN CD56+ NK CELLS/10HPF
0	22(73.3%)	55.74
1	3(23.3%)	57.11
2	1(3.3%)	58.9

In our study, 16 women in case group had history of 3 previous abortions with mean uNK cell concentration of 57.36 cells/10Hpf. 8 women had history of 4 previous abortions with mean uNK cell concentration of 53.37cells/10Hpf. 6 women had history of 5 previous abortions with mean uNK cell concentration of 56.7cells/10Hpf (**TABLE 6)**.

We have found that there was no significant correlation between uNK cell concentration and number of previous abortions.

Table 6: Comparison Of Mean Unk Cell Concentration With Total No.of Abortions In Case Group

NO.OF ABORTIONS	TOTAL NO.OF CASES(%) women	MEAN CD56 POSITIVE CELLS/ 10 Hpf
A3	16(53.3%)	57.36
A4	8(26.7%)	53.37
A5	6(20%)	56.7

Microscopic Pictures

Cases

H&E :Endometrium in early proliferative phase,IHC:CD56+ mean 58.3cells/hpf

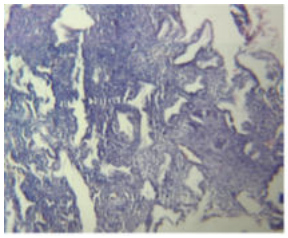


Fig 1:H&E,4X

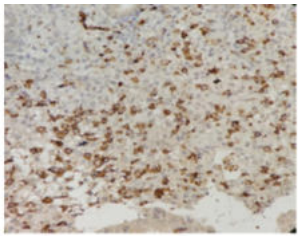


Fig 2:CD56+,4X

H&E :Endometrium in early proliferative phase,IHC:CD56+ mean 58.3cells/hpf

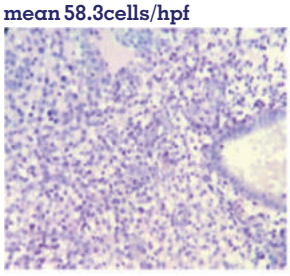


Fig 3:H&E,40X

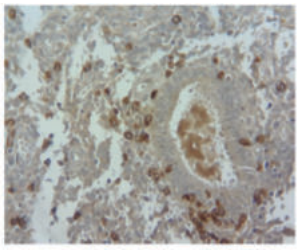


Fig 4:CD56+,40X

Control

H&E :Endometrium in proliferative phase,IHC:CD56+ mean 21.4cells/hpf

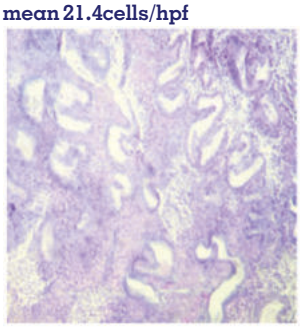


Fig 5:H&E,4X

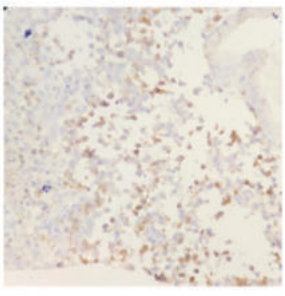


Fig 6:CD56+,4X

H&E :Endometrium in proliferative phase,IHC:CD56+ mean 21.4cells/hpf

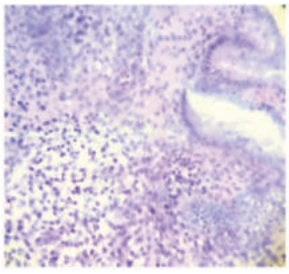


Fig 7:H&E,4

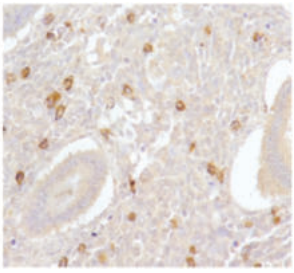


Fig 8:CD56+,40X

Comparison Of Mean Cd56+ Unk Cells In Cases And Controls

The mean NK cell concentration in cases was 56.16 and mean NK cell concentration in control group was 25.20. The mean NK cell concentration was significantly higher in women with recurrent miscarriages compared to fertile women in control group (Table: 7).

Table 7: Mean Cd56+ Unk Cells In Cases And Controls

STUDY GROUP	MEAN NO.OF CD56 POSITIVE CELLS/10HPF
CASES	56.16
CONTROL	25.20
P VALUE	0.0000153(<0.05)

DISCUSSION

Recurrent Miscarriage is the most common complication in human pregnancy in present scenario. It is clinically heterogenous condition associated with numerous conditions pertaining to the mother and foetus.

Cases not associated with any etiological factor are termed idiopathic RM. Immune based etiology is one of proposed theories,although the exact mechanism is not fully understood.

The immune cells composition in endometrium is T cells, macrophages, uNK cells with very few neutrophils and hardly any B cells. These cells vary in proportion throughout the menstrual cycle increasing during the late secretory phase. This indicates that endometrial leucocytes play an important role in implantation and maintenance of pregnancy.

Uterine Natural Killer cells have most dramatic rise in number and become most predominant leucocyte during implantation and early pregnancy and express receptors for invading extra villous trophoblastic tissue.

T cells constitute for 40% leucocytes in endometrium during proliferative phase[4] but decreases in proportion due to rise in uNK cells in secretory phase. T cells express either CD8 or CD4 antigen.The CD4+ cells produce cytokines of Th1 and Th2 type The difference in ratio of T cell subtypes, CD8 and CD4 and the balance between Th1 and Th2 cytokines that are produced by these cells, play an important role in initiation and maintenance of pregnancy. Th1 cytokines promoting trophoblast tissue lysis and Th2 cells helping in implantation and maintenance of pregnancy. Macrophages also increase during secretory phase and early pregnancy and produce Th2 cytokines playing an important role in trophoblast invasion.

All uterine NK cells express the CD56 antigen but lack CD16 and CD57 which differentiates them from peripheral blood NK cells(pNK) which are CD16 bright positive. They are predominantly found in stratum functionalis and, start to increase in number after ovulation. In pregnancy, uNK cells are found in highest number in decidua basalis closest to invading EVT at implantation. In third trimester, uNK cell number decreases but remains in both decidua basalis and parietalis.

Sources Of Uterine Nk Cells:

The process how uNK cells arrive in large numbers into endometrium during late secretory phase is uncertain, but two main theories exists. In-utero proliferation and differentiation of stem cells or indigenous NK cells in the endometrium, or Recruitment of haematopoietic stem cells or NK cells from peripheral blood which subsequently differentiate in uterine microenvironment into uNK cells phenotype.

These uterine NK cells produce various cytokines such as GM-CSF, CSF-1, TNF-α, IFN-γ, TGF-β, LIF, MIF, IL-2 and IL-10[5]. GM-CSF stimulate DNA synthesis and CSF-1 increase production of HCG and Human placental lactogen by trophoblast. On the other hand IFN- □ inhibit EVT invasion by increasing their apoptosis. Therefore any alterations in production of cytokines could contribute to the imbalance of this unique fetal-maternal interface immune phenomena leading to abnormal implantation and the clinical presentation of a miscarriage.

Uterine NK cells also produce high levels of angiogenic growth factors like VEGF, placental growth factor, angiopoietin-1 and 2 important for vascular remodelling.

Uterine Natural Killer Cells and Recurrent Miscarriage:

uNK cells are essential for decidualisation, implantation and the maintenance of pregnancy and it may be changes in their numbers or function that results in miscarriages. The association between high Unk cell density and RM has been repeatedly reported in prepregnancy endometrium.Function of uNK cells is controlling angiogenesis, and their density was found to be positively correlated with endometrial angiogenesis and uterine artery blood flow in women with RM Embryo implantation and early pregnancy development occur in a relatively hypoxic environment (2-3% O2)[6]. Therefore, inappropriate blood flow to the intervillous space could be associated with oxidative stress damage to the developing placenta. One theory is that increased uNK cell density is associated with increased number of spiral arteries which may lead to inappropriate blood flow to the developing foetal-placental unit, causing oxidative stress and consequent miscarriage[7]

The incidence of recurrent miscarriage has been increasing not only after normal conception, but even with IVF. Hence it has been hypothesised that a local endometrial factor is contributing to it.

NK cell is one such local factor which is being studied recently and therapies targeting these cells are now in use. This is further strengthened by evidence of cell-cell interactions between uNK cells and EVT, and possible functions of uNK cells that play direct important roles in the process of implantation. Alternately, uNK cell density may serve as a marker of an underlying endometrial or immunological phenomenon that leads to miscarriage.

In our study we have included women in age range 18-40 years with history of 3 recurrent miscarriage in case group and fertile women in age range 18-40 years as control group. The majority of women in case group were in age range of 36-40 years and mean age is 34.2 and the majority of women in control group were in age group 31-35 years with mean age being 35.6(Tabe:8).

Table 8: Comparison Of Patients Age In Present Study And Other Studies

STUDY	MEAN AGE IN CASES	MEAN AGE IN CONTROLS
Present study	34.2	35.6
Clifford et.al8,	32	32.9
Ruben J. Kuon et.aI9	34.7	27

In our study 22 women had no previous live births, 7 women had one previous live birth and 1 woman had two previous live births. The mean number of live birth was 0.3(Table :9).

Table 9: Comparison Of No.of Previous Live Births In Cases And Control Group In Present Study And Other Studies

STUDY	MEAN NO.OF PREVIOUS LIVE BIRTHS	RANGE
Present study	0.3	0-2
Quenby SM et.al10,	0.3	0-2

In our study 16 women had history of 3 previous abortions, 8 women had 4 previous abortions and 6 women had 5 previous abortions the mean no.of abortions in our study was 3.6.

Quenby S et.al11, in their study found the mean number of miscarriages were 4.4 with range:3-17 which was higher compared to our study. The mean no.of previous abortion in control group was 0.4 with range 0-2(Table:10).

Table:10: Comparison Of No.of Previous Abortions In Cases In Present Study And Other Studies

STUDY	MEAN NO.OF PREVIOUS ABORTIONS	RANGE
Present study	3.6	3-5
Quenby SM et.al,	4.4	3-17

In our study we have included endometrial curettings and biopsy from 30 women with history of 3 previous spontaneous pregnancy loss as case group and women of reproductive age group with normal reproductive history in control group.

The CD56 positive uNK cells analysis showed that the mean uNK cell concentration in endometrium of cases was found to be 56.16. The mean uNK cell concentration in control group was found to be 25.20. In our study, the uNK cell concentration in cases was significantly higher compared to that in control group(Table:11).

Table:11: Comparison Of Cd56+ Unk Cell Concentration In Cases And Controls In Present Study And Other Studies

STUDY	Comparison of CD56+ uNK cell concentration in cases and control Group
Present study	Increased in cases
Clifford et.al8,	Increased in cases
Quenby et.al10,	Increased in cases
Tuckerman et.al11,	Increased in cases
Ruben J Kuon et.al9,	No difference
Shimada et.al12	No difference

Summary

The present study is an observational study done at Department of Pathology,Tertiary care centre over a period of two years from July 2017 to June 2019. A total of 30 women with history of recurrent miscarriage were taken as the study group, 30 fertile women were taken as control group. Endometrial biopsies were taken in the midluteal phase.Routine processing and haematoxylin and eosin staining of the endometrial biopsies were done followed by immunohistochemical analysis with anti CD56 antibody.

A positive immunoreaction is characterized by darkbrown cell membrane staining in Uterine Natural Killer cells.The slides scanned and CD56 positive cells were counted in ten high power fields (40x) and mean cell count was taken.

The mean CD56 positive uNK cells in all the cases were compared with that of controls.The mean CD56 positive uNK cells in cases were 56.16/10hpf and mean CD56 positive uNK cells in controls were 25.20/10hpf. The mean NK cell concentration was significantly higher in women with recurrent miscarriage compared to fertile women in control group.

CONCLUSION

Uterine natural killer cells, with their ability to release cytokines have a dual role both in facilitating pregnancy,as well as causing recurrent miscarriages,when they are excess in number.

Uterine natural killer cell evaluation help in diagnosis of immunological causes of recurrent miscarriage, Hence its evaluation should be included in the screening protocol. It is also recommended that population based studies should be done to set a cutoff value for number of uNK cells which would indicate a risk of subsequent recurrent miscarriage.

Research has shown that disarray in immune mechanism can be manipulated by decreasing the concentration of Unk cells.Hence immunomodulation therapy targeting these NK cells would prevent recurrent miscarriage

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