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ORIGINAL ARTICLE

Relation between Uterine Natural Killer Cells and Unexplained Recurrent Miscarriage

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ABSTRACT

Objective: To evaluate the relation between uK (uterine Killer) cells and unexplained repeated miscarriage (RM).

Patients and Methods: Eighty (80) women with unexplained repeated miscarriage and missed miscarriage of current pregnancy were studied. Fetal viability and gestational age of current pregnancy were confirmed by ultrasound, followed by suction evacuation to collect abortus specimens and uterine wall curettage to collect decidua specimens. Abortus specimens were collected for long-term monolayer cell culture and subsequent chromosome analysis using conventional G-banding technique. Decidua specimens were subjected to IHC (Immunohistochemical) staining using monoclonal antibodies specific to $CD56^+$ and $CD16^+$ expressed by uK cells.

Results: $CD56^+$ $CD16^+$ uK cells was found in 85% (68/80) of studied decidua specimens of women with unexplained repeated miscarriage, 88.5% (54/61) had normal abortus karyotyping and 73.7% (14/19) had abnormal abortus karyotyping. 73.75% (59/80) of studied women with past history of early miscarriage had $CD56^+$ $CD16^+$ uK cells in their decidua specimens and 66.25% (53/80) of studied women with past history of late miscarriage had $CD56^+$ $CD16^+$ uK cells in their decidua specimens, the association between early and late miscarriage and $CD56_+$ $CD16^+$ uK cells in deciduas specimen was significant.

Conclusion: $CD56^+$ $CD16^+$ uK cells were predominant in decidua specimens of studied women with repeated miscarriage. Significant association was found between presence of $CD56^+$ $CD16^+$ uK cells in studied decidua specimens and unexplained repeated miscarriage.

INTRODUCTION

Repeated miscarriage (RM) defined as two or more failed pregnancies (confirmed by ultrasound or histopathological examination) and is known to affect approximately 0.5–1 % of couples.`

One miscarriage increases risk of miscarriage in future pregnancy to 24%, this risk increases to 26% with previous 2 miscarriages and reaches 32% with previous 3 miscarriages, thus women with two or more consecutive miscarriages deserve meticulous study to detect definite cause and possible treatment.[2-4]

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Various factors are implicated in the pathophysiology of repeated miscarriage. Fetal causes as single gene or genomic imprinting defects account for 3.5–5% of the causes of repeated miscarriage, other fetal defects include fetal infections and developmental abnormalities.[5] Maternal causes of repeated miscarriage include; immunological causes accounting for 30% of the cases, with anti-phospholipid antibody syndrome being the most common autoimmune cause.[6,7]

Endocrine dysfunction accounts for 48.71% of the causes, while other maternal factors including anatomical detects and sub-clinical endometrial infection account for a minimal number of cases.[8,9]

Approximately 50% of repeated miscarriages are unexplained with no definitive etiology. Several authors suggest the cause to be alloimmune rejection of the fetus.[10]

NK (natural killer) cells are part of immune system lymphocytes.[11,12] uK (uterine killer) cells are short-lived,

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lymphocytes found in uterine deciduas.[13] Early in pregnancies uK cells produce angiogenic factors and are now considered important for implantation and development.[13,14]

uK cells have been linked to human reproductive disorders, including repeated miscarriage, implantation failure, fetal growth restriction and preeclampsia.[15,16]

uK cells secrete cytokines and angiogenic factors, important for placental development and establishment of pregnancy.[16]

It has been found that 37.3% of patients presented with repeated miscarriage had mild to moderate increase in NK cells and 14.7% of women with repeated miscarriage had elevated $CD56^+$ NK cells in peripheral blood.[17,18]

Other authors concluded that NK cells cytotoxicity are not related to number of peripheral NK cell and it can be estimated by NK cells markers such as KIRs (killer inhibitory receptors) or $CD16^+$ receptor expression.[19]

Because, more research work are needed to establish relation between uK cells and human reproductive disorders.[20,21]

This study designed to evaluate the relation between uK cells and unexplained repeated miscarriage.

PATIENTS AND METHODS

Eighty (80) women with unexplained repeated miscarriage and missed miscarriage of current pregnancy were included for evacuation and curettage because of current missed miscarriage (> than 8 weeks diagnosed by ultrasound). Women were studied after proper counseling, consent and approval of the ethical committee. Unexplained repeated miscarriages was defined as ≥2 previous miscarriages <20 weeks` gestation. Thorough history and examination were done for all studied women, followed by trans-vaginal ultrasound to confirm fetal viability and gestational age of current pregnancy by sonographer who was blinded to patients' data. Peripheral venous samples were collected from studied women for oral glucose tolerance test, thyroid stimulating hormone assay, prolactin, serum anticardiolipin, lupus anticoagulant assays, prothrombin gene mutations, anti-thrombin III deficiencies and thrombophilia screen. Women with septic miscarriage, documented endocrinopathies (diabetes, thyroid disorders, hyperprolactinaemia), uterine anomalies, polycystic ovary syndrome, anti-phospholipid antibody syndrome, thrombophilia, abnormal karyotype in one or both parents using leucocyte culture, autoimmune disorders, history of hormonal contraception, history of intrauterine contraceptive device application within last 3 months preceding current pregnancy were excluded from this study. Evacuation and curettage was done for all women included in this study under general anesthesia, using suction evacuation to collect abortus specimens after cervical dilatation, followed by uterine wall curettage to collect decidua specimens. Abortus specimens were collected on a special medium for long term monolayer cell culture and subsequent chromosome analysis using conventional G-banding technique. Decidua specimens were subjected to IHC (Immunohistochemical) staining using monoclonal antibodies specific to uK cells $CD56^+$ and $CD16^+$

Reagents and materials used include;

1. Primary antibodies; which is liquid MoAb (monoclonal mouse antibody) against $CD56^+$ and $CD16^+$ expressed on NK cells.
2. Universal Kits; supersensitive immunodetection system (Biogenex Laboratories, San Francisco, USA), contains the following; (a) Negative control antibody (b) Biotinylated anti-immunoglobulin for mouse antibody (c) Label: streptavidine peroxidase complex (d) Chromogen: 2,3 DAB (Diamenobenzidine chromogen) solution, ready to use substrate buffer and H_2O_2 substrate for use with liquid DAB chromogen and substrate buffer (e) Blocking reagent to block endogenous peroxidase activity
3. Lyophilized pepsin powder, PBS (Phosphate buffer saline), counter stain (Mayer's hematoxylin), distilled water and mounting media (Canada balsam).
4. Staining jars, microscopic positive charged slides, cover slips for slides and immune-stainer.
5. Light microscope: with 100x and 400x Magnification.

IHC procedure; Decidua specimens fixed in buffered formalin (not more than 24 hours), embedded in paraffin wax, 3-micometer sections mounted with 3-aminopropyltriethoxysilane (Sigma Chemical Co, UK).

Serial sections were then stained for uK cells ($CD56^+$ and $CD16^+$) using antibodies antigen-retrieval methods.[22,23]

Primary antibodies incubated one hour (60 minutes) for $CD56^+$ and for 120 minutes for $CD16^+$ at room temperature, the staining intensity of brown reaction developed with 2,3 DAB containing 0.0I% H_2O_2 was noted, haematoxylin was used for counterstaining of sections.

Sections were dehydrated and mounted with DPX (Distrene, Plasticiser, Xylene) standard resin (Lamb Ltd, London, UK), then examined by ordinary light microscopy. Appropriate positive controls (neuroblastoma for $CD56^+$ and tonsils for $CD16^+$) were used in each run to judge the effectiveness of staining technique and mouse Ig-G [Immunoglobulin-G] were used instead of primary antibodies as negative controls.

SAMPLE SIZE AND STATISTICAL ANALYSIS

Sample size of 80 women was required in this study and calculated using G* Power software for sample size calculation (*Heinrich Heine Universität; Düsseldorf; Germany). Mean and + SD (standard deviation) were used to represents numerical values, while, number (n) and percentage (%) were used to represents categorical values. Comparison between variables was done using Chi-square (x^2) test was. A difference with a p value <0.05 considered significant.

RESULTS

Mean age of studied women was 29.6 ± 6.39 years and mean BMI (body mass index) was 26.9 ± 4.5 kg/m^2. Karyotyping study of abortus specimens showed; normal karyotyping in 76.25% (61/80) of studied specimens and abnormal karyotyping in 23.75% (19/80) of studied specimens (Table 1).

CD56$^+$ CD16$^+$ uK cells was found in 85% (68/80) of studied decidua specimens of women with unexplained repeated miscarriage, 88.5% (54/61) had normal abortus karyotyping and 73.7% (14/19) had abnormal abortus karyotyping (Table 2).

73.75% (59/80) of studied women with past history of early miscarriage had CD56$^+$ CD16$^+$ uK cells in their decidua specimens and 66.25% (53/80) of studied women with past history of late miscarriage had CD56$^+$ CD16$^+$ uK cells in their decidua specimens, the association between early and late miscarriage and CD56$^+$ CD16$^+$ uK cells in deciduas specimen was significant Table 3.

Table 1: Karyotyping analysis of the studied specimens

Variable	Number (n)	Percentage
Normal female karyotype	58	72.5
Normal male karyotype	3	3.75
Abnormal karyotype	19	23.75
Triploidy	5	6.25
Tetraploidy	10	12.5
Aneuploidy	4	5.0
Total	80	100

Table 2: Relation between karyotyping of abortus specimens and Immunohistochemical results of decidua specimens

Variable	CD56$^+$CD16$^+$uterine killer cells
	Number (percentage)
Normal karyotype (61 cases)	54 (88.5)
Abnormal karyotype (19 cases)	14 (73.7)
Total	68 (85)

Table 3: Relation between CD56+CD16+uterine killer cells and miscarriage (early and late)

Variables	CD56$^+$CD16$^+$uterine killer cells
	Number (percentage)
Number of early miscarriage	
1	13 (16.25)
2	25 (31.25)
3	12 (15)
>3	9 (11.25)
Total (80 cases)	59 (73.75)
Number of late miscarriage	
0	4 (5)
1-2	53 (66.25)
Total (80 cases)	57 (71.25)

DISCUSSION

Early in pregnancies uK cells produce angiogenic factors and are now considered important for implantation and development. uK cells have been linked to human reproductive disorders, including repeated miscarriage, repeated implantation failure, fetal growth restriction and preeclampsia,[15,16] this study designed to evaluate the relation between uK cells and unexplained repeated miscarriage.

CD16 is expressed by neutrophils, activated macrophages, and natural killer cells. CD56 is expressed by natural killer cells as one of neural cell adhesion molecule.

pNK (peripheral natural killer) cells are known to be found in blood and endometrium. pNK and uNK are phenotypically and functionally different.[11] Both pNK (peripheral natural killer) and uNK (uterine natural killer) express the surface CD56.[11]

Studies have shown that 90% of pNK cells express a CD56^{dim} CD16$^+$ phenotype, while 80% of the uNK cells express a CD56^{bright} CD16$^-$ phenotype, with the CD56 cells known to have a regulatory function, while the CD16 cells have a cytotoxic function.[24-26]

In humans, it has been proved that the elevated circulating cytotoxic NK cells (not the count) increase the risk of miscarriage.[27] Women<35 years old with unexplained repeated miscarriage were studied to minimize risk of chromosomal abnormalities and miscarriages associated with advanced maternal age.[28]

BMI of women included in this study was 26.9 ± 4.5 kg/m^2, this might be due to our selection criteria as we excluded some risk factors that might predispose to RM such as obesity,[29,30] diabetes mellitus and thyroid disorders.

Eighty abortus specimens were cytogenetically analyzed using tissue culture and conventional G-banding technique,

because comparative genomic hybridization (without culture) was not introduced in our institute until recently. Cytogenetic analysis using tissue culture and conventional G-banding method has some limitations; contamination, culture failure and maternal cells growth.[31]

In this study, Karyotyping studies showed; normal karyotyping in 76.25% (61/80) of studied abortus specimens and abnormal karyotyping in 23.75% (19/80) of studied abortus specimens. A 29-57% rate of chromosomal abnormality was previously reported during analysis of miscarried tissue from women suffering RM,[32-35] and the higher of normal chromosomal study in miscarried tissue of women with RM confirms that there may be other factors other than chromosomal abnormalities associated with RM.[36]

$CD56^+$ $CD16^+$ uNK cells were found in 85% (68/80) of studied decidua specimens of women with unexplained repeated miscarriage. Quenby et al.[16] reported that women with RM had significantly more uNK than controls and Clifford et al.,[37] also showed that there were increased $CD56^+$ uK cells in women with unexplained repeated miscarriage.

Increased expression of $CD56^+$ $CD16^+$ uNK was also reported in deciduas obtained after spontaneous miscarriage in women with history of repeated miscarriage.[38]

Quenby et al.[39] used IHC to investigate leukocyte populations in mid-luteal endometrial biopsies of 22 women suffering from RM compared to 9 women without RM, they found that $CD4^+$, $CD14^+$, $CD16^+$ and $CD56^+$ uNK cells were significantly higher in RM group than controls.

Lachapelle et al.[40] compared endometrial specimens from 20 women with RM with endometrial samples collected during the secretory phase from 15 fertile controls. Lachapelle et al.[40] found that the percentage of uK was similar in the two groups although, greater percentage of $CD56^+$ $CD16^+$ uK were found in women with RM.

$CD56^+$ $CD16^+$ uNK cells was found in 85% (68/80) of studied decidua specimens of women with unexplained repeated miscarriage, 88.5% (54/61) had normal abortus karyotyping and 73.7% (14/19) had abnormal abortus karyotyping, this difference was statistically not-significant. Yamamoto et al.[41] also, reported same findings, when they studied uNK cells in decidua specimens of both chromosomally normal and abnormal missed miscarriages.

Although, in this study we found expression of $CD56^+$ $CD16^+$ uNK cells in decidua specimens of women with RM is high, Yamamoto et al.[41] did not found over expression of $CD56^+$ $CD16^+$ uK cells in deciduas of studied women with missed abortions, because their study was limited to sporadic cases of missed abortions, not RM cases.

73.75% (59/80) of studied women with past history of early miscarriage had $CD56^+$ $CD16^+$ uK cells in their decidua specimens and 66.25% (53/80) of studied women with past history of late miscarriage had $CD56^+$ $CD16^+$ uK cells in their decidua specimens, the association between early and late miscarriage and $CD56^+$ $CD16^+$ uK cells in deciduas specimen was significant. Findings of this study suggests that $CD56^+$ $CD16^+$ uK cells are predominant in decidua of women with RM. Women refused to participate in this study and use of tissue culture and conventional G-banding technique for cytogenetic analysis and karyotyping of abortus specimens were faced as limitations during this study.

CONCLUSION

Further Large case controlled studies are needed to compare decidua specimens from RM cases with decidua specimen from normal cases without RM to establish relation between uK cells and human reproductive disorders and to improve future treatment for such cases.

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