The Relationship between Peripheral Natural Killer Cells Represented by CD56 and Repeated Spontaneous Miscarriages

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Abstract: Background: Spontaneous miscarriage is defined as the involuntary termination of pregnancy before 20 weeks of gestation or spontaneous expulsion of a fetus below fetal weight of 500 gram. NK cells are found in both peripheral blood and the uterine mucosa. There are, however, important phenotypic and functional differences between NK cells present at the two sites. Recurrent spontaneous miscarriage is usually defined as three or more spontaneous pregnancy losses (not necessarily consecutive). **Objective:** The aim of the study is to investigate the role of natural killer cells, (CD56) in cases of repeated spontaneous miscarriges. **Subjects and Methods:** This current study was conducted through the period from March 2016 to March 2017. Forty women were included in the group. They were collected from a private infertility center. **Results:** There were significant positive correlations between CD56 and Total pregnancies, Total miscarriages, early miscarriages, Miscarriages 6.0–12.0 weeks, Miscarriages 13.0–20.0 weeks, Missed abortion and previous medical treatment of miscarriage. The strongest correlation was with early miscarriages. Also Cases with positive family history of DM, thyroid diseases had significantly higher CD65%. **Conclusion:** Endometrial NK cells measured by flowcytometrey are increased in cases of repeated miscarriges. There were significant positive correlations between CD56 and Total pregnancies, Total miscarriages, early miscarriages, Miscarriages 6.0–12.0 weeks, Miscarriages 13.0–20.0 weeks, Missed abortion and previous medical treatment of miscarriage. The strongest correlation was with early miscarriages.
[Mervat Mohammed Ibrahim, Hanan Abd Elmoneam Mohammed and Sara Maustafa Mohammed Hassanein. **The Relationship between Peripheral Natural Killer Cells Represented by CD56 and Repeated Spontaneous Miscarriages.** *Nat Sci* 2018;16(8):84-89]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 11. doi:10.7537/marsnsj160818.11.

Keywords: Dendritic cells - Follicular stimulating hormone - Haematopoietic progenitor cells

1. Introduction

Embryo implantation is a complex process involving maternal hormonal changes, immune responses and maturational events in the embryo. A pregnancy could fail when these events are not synchronized. It is speculated that in women, an elevation of natural killer (NK) cells may have an effect on reproductive performance, and Natural Killer cell levels in blood are currently being used as a diagnostic test to guide the initiation of therapies in patients with infertility [(1)].

Recurrent pregnancy losses (RPL) are defined as three or more consecutive spontaneous miscarriages, which affect approximately 2% of women in reproductive age [(2)].

Multiple etiologies have been reported to cause RPL, such as uterine anomalies, coagulation disorders, autoimmune diseases, cellular immune abnormalities, endocrine disorders, and endometrial defects [(3)].

In women with RPL of unknown etiologies, various immunological abnormalities have been reported. Multiple studies have shown that women with RPL have increased numbers of CD56+ peripheral natural killer (pNK) cells either prior to or during pregnancy [(4)].

Women with elevated pNK cells have a significantly increased risk of pregnancy losses or implantation failures [(5)].

Natural killer cells have been thought to be associated with implantation failures, recurrent miscarriage (RM) or infertility due to either NK cell cytotoxicity or receptor/gene expression. Natural killer (NK) cells are known as a key component of innate immunity. NK cells are mainly found in peripheral blood; however, they are also present in several lymphoid and non-lymphoid organs, such as the spleen, tonsils, lymph nodes, liver, lungs, and intestine. In uterine endometrium, the most abundant lymphocytes are NK cells. Natural Killer cells are a type of large granular lymphocyte that belong to the innate immune system. They are derived from haematopoietic progenitor cells (HPCs) in the bone marrow and express the surface marker CD 56. During the late luteal phase of the early pregnancy, uterine stromal cells go through a decidualization under the influence of various sex steroids and cytokines [(6)].

Aim of the Work

The aim of this study is to evaluate the relationship between peripheral natural killer cells that express CD56 and repeated spontaneous miscarriage.

2. Subjects and Methods

Study design:

This case control study was conducted at a private infertility center through the period from March 2016 to March 2017. Forty women were included in case group and another forty in control group.

Inclusion criteria:

Maternal age less than 35 years old. History of recurrent spontaneous early miscarriage (three or more miscarriages less than 20 weeks gestation). Normal 3D pelvic ultrasound examination, no uterine anomalies and normal ovulation No past history of hypertension, diabetes or thyroid disease. Normal gynecological, hormonal, anatomical profile. Regular menstrual cycles and spontaneous ovulation.

Exclusion criteria:

Female is excluded if any of the following is present: History of hyperprolactinemia or proved to have elevated serum prolactin. History of thyroid disease or proved to have abnormal serum TSH or FT4. History of diabetes or proved to have abnormal fasting blood glucose. Women with known immunological disease (anti-phospholipid antibodies, lupus anticoagulant, anticardiolipin anti- bodies and anti-thyroid antibodies). Uterine anatomical abnormalities excluded by 3D ultrasound. Thrombophilia gene mutaion. Past history of chronic diseases (hypertension, diabetes, thyroid diseases). Previous history of pelvic inflammatory disease (PID), hydrosalpinx, endometriosis, proven polycystic ovarian syndrome, endocrinological, and metabolic disease, gynecological intervention (leiomyomas, endometrial polyps, and pelvic adhesion removal).

3. Results

The results are shown in following figures (Figures 1-8).

Table (1): Comparison between CD16 & CD56 positive and negative conditions among the studied groups regarding BMI (kg/m2).

Variable	Measure	Case (N=40)	Control (N=40)
BMI (kg/m2)	**Mean ±SD**	24.9± 2.4	24.6±2.2
	Range	20.0–31.0	20.0–31.0

Table (2): Obstetric characteristics of the studied cases.

Variables	Mean±SD	Range
Total pregnancies	4.3±2.2	2.0–12.0
Total miscarriages	3.8±2.3	2.0–12.0
Early miscarriages	3.7±2.0	2.0–10.0
Late miscarriages	0.2±0.4	0.0–2.0
Miscarriages 6.0–12.0 weeks	3.3±1.5	2.0–9.0
Miscarriages 13.0–20.0 weeks	0.3±0.6	0.0–2.0
Miscarriages >20.0 weeks	0.2±0.4	0.0–2.0
Missed abortion	2.5±2.2	0.0–8.0
Inevitable abortion	1.4±1.1	0.0–4.0
previous medical treatment of miscarriage	2.0±1.7	0.0–7.0
Previous surgical treatment of miscarriage	1.8±1.3	0.0–6.0
Preterm	0.1±0.2	0.0–1.0
Full term	0.4±0.7	0.0–2.0

Table (3): Laboratory, US and hysteroscopy findings of the case group.

Variables	Mean±SD	Range
CD65%	19.5±6.0	9.0–30.0
Hemoglobin (gm/dL)	11.2±0.8	10.0–13.0
Fasting blood glucose (mg/dL)	87.1±10.6	70.0–110.0
Prolactin (ng/mL)	18.4±4.7	10.0–27.0
TSH (mIU/L)	2.8±4.0	0.8–18.0

Table (4): Comparison between early miscarriage (6-12 weeks), early miscarriages (13-20 weeks) and late miscarriages in case groups.

Variable	Early miscarriages (6-12) weeks	Early miscarriages (13-20) weeks	Late miscarriages	P value
No of cases	**40**	**40**	**40**	0.0001
Mean (SD)	3.3 (1.54)	0.33 (0.62)	0.18 (0.07)	

Table (5): Relatioship between Positive and negative peripheral CD56 among the case group.

Variable	CD 56 postive cases	CD 56 negative cases	Total no of cases	P value
No	**33**	**7**	**40**	0.0001
Mean (SD)	20.94 (5.18)	8.14 (0.90)		

Table (6): Relatioship between Positive and negative peripheral CD56 among the control group.

Variable	CD 56 postive	CD 56 negative	Total no of control
No	**5**	**35**	**40**
Mean (SD)	12.40 (0.89)	7.03 (1.95)	

Table (7): Comparison between CD56 % among the studied groups.

Variable	CD 56 % in case group	CD 56 % in control group	P value
No	40	40	0.0001
Mean (SD)	19.18 (6.63)	7.73 (2.57)	

Table (8): Comparison between CD56 positive and negative conditions among the studied groups.

	Variable	CD 56 postive in case group	CD 56 negative in control group
Case group	**No**	33	7
	Mean (SD)	20.94 (0.90)	8.14 (0.34)
Control group	**no**	5	35
	Mean (SD)	12.40 (0.89)	7.03 (1.95)

4. Discussion

Recurrent miscarriage (RM) is defined as three or more consecutive spontaneous pregnancy losses before gestational 24 weeks. About half of the cases are idiopathic, and an altered endometrial environment has been suggested as a potential contributory factor.

Successful implantation requires coordinated vascular development and maintenance in the maternal-embryo interface to provide nourished environment [(7)].

It is speculated that in women, an elevation of natural killer (NK) cells may have an effect on reproductive performance, and NK cell levels in blood are currently being used as a diagnostic test to guide the initiation of therapies in patients with infertility [(8)].

They have been thought to be associated with implantation failures, recurrent miscarriage (RM) or infertility due to either NK cell cytotoxicity or receptor/gene expression [(1)].

Normal pregnancy is an orchestrated process that requires both promotion of fetal growth and maintenance of immune tolerance. Among immune cells, uterine NK (uNK) cells are the most distinguishable lymphocytes during the first trimester of pregnancy, constituting >70% of all leukocytes in human deciduas. uNK cells exist only during early pregnancy and decrease after the placenta is formed [(9)].

Uterine natural killer (uNK) cells are the major leucocytes present in the non-pregnant endometrium and pregnant decidua. They are part of the innate immune system, and are found in both peripheral blood and endometrium. Although both peripheral NK (pNK) and uterine NK (uNK) cells express the surface antigen CD56, pNK cells are phenotypically and functionally different from uNK cells and <10% of pNK cells resemble uNK cells. They have a unique phenotype to be CD56bright CD16 – whereas in peripheral blood CD56dimCD16+ NK cells constitute the major subpopulation [(10)].

Peripheral NK cells (CD56dim) have been demonstrated to show significant cytotoxic activity with well-established antiviral and anti-neoplastic

functions, while uNK cells have little cytotoxic activity, but are a rich source of cytokines, particularly angiogenic ones, with possible roles in regulation of trophoblast invasion and angiogenesis [(11)].

uNK cells were found to be a major source of cytokines and angiogenic growth factors and to be frequently aggregated around the spiral arteries in early pregnancy, reflecting a role of uNK cells in mediating vascular changes during implantation and pregnancy [(12)].

NK cells cause cytotoxic effects by inducing lysis or apoptosis of the target cells mediated by the release of granular components within their cytoplasm (perforin, granzymes) or secretion of cytokines, such as tumour necrosis factor-alpha, interleukin (IL)-10, interferon-gamma and transforming growth factor-beta [(12)].

There are studies observed an increased proportion of mature endometrial vessels in a subset of population with increased numbers of uNK cells during the mid-luteal phase of the menstrual cycle in women with recurrent reproductive failure, which has provided an indirect evidence for the uNK cells-associated alteration of vascularization.

A high proportion of peripheral NK cells may not reflect the condition of the endometrium where implantation occurs and the mechanism of how these cells can be associated with miscarriage is unclear. In contrast large numbers of uNK cells appear in the mid-secretory phase although the mechanism is still not known. There are two theories: recruitment from pNK cells which subsequently differentiate in the uterine microenvironment into uNK cell phenotype through a series of organized processes, or uNK cells come from the *in utero*proliferation and differentiation of stem cells or endogenous NK cells in the endometrium [(13)].

The current study is retrospective study that was conducted at a private infertility center through the period from March 2016 to March 2017. Forty women were included.

The forty patients have history of repeated spontaneous miscarries (more than or equivalent to three miscarries) all are less than twenty weeks of gestation.

All patients are less than 35 years old and have no history of any endocrinal disorders or known chronic disease.

All patients have regular menstrual cycles, spontaneous ovulation, normal hormonal profile, normal 3D ultrasound examination, normal thrombophilia gene screening and no immunological disorders.

Blood samples were obtained during mid luteal phase of the menstrual cycle. The flow cytometric analysis was performed at Flow Cytomtery Laboratory of a private clinic.

In our study, the mean age (SD) of cases was 27.7±4.2.

There is less than third of cases had family history of DM and thyroid.

In our study there are significant positive correlations between CD56 and Total pregnancies, Total miscarriages, early miscarriages, Miscarriages 6.0–12.0 weeks, Miscarriages 13.0–20.0 weeks, Missed abortion and previous medical treatment of miscarriage. The strongest correlation was with early miscarriages, regarding the family history, there are no significant difference between cases with and without family history of DM, thyroid diseases regarding age and obstetric history.

Studies of peripheral NK cells

Emmer et al. [(4)] investigated both pNK cells number and activity, while ***Aoki et al.*** [(14)] only studied pNK activity. ***Emmer et al.*** [(4)]***; Aoki et al.*** [(14)] both agreed with our study in that they studied women with idiopathic RM whereas ***Yamada et al.*** [(15)], the study with the largest sample size in this group, differs from our study in that it included women with known associations of RM such as endocrine disorders, APS and thrombophilia. Although they reported that high pNK cells number and activity predicted subsequent biochemical miscarriage and miscarriage of normal karyotype, more than half of the women investigated had another possible contributing factor to their miscarriage, creating potential bias in the results. The three studies included women after only two miscarriages which does not fit the ESHRE (European Society of Human Reproduction and Embryology) definition of RM [(16)].

Studies of uterine NK cells

Testing of uNK cells involves an endometrial biopsy that can only be carried out in the pre-pregnancy period. Immunohistochemistry was the method used in most of the studies of uNK cells. This is more time consuming than flow cytometry but it reveals the location of the uNK cells [(17)]. Analysis using flow cytometry involves digesting the tissue, and thereby potentially losing cells and antigens. Furthermore, it needs a large sample of endometrium that may be difficult to obtain in some women. Against our results for significant relationship between peripheral NK cells and repeated spontaneous miscarriges, ***LaChapelle et al.*** [(18)] reported no difference in uNK cell density between women who subsequently miscarried and those who had live births. Although, ***Michimata et al.*** [(19)] included women after two miscarriages and used immunohistochemistry for analysis while ***LaChapelle et al.*** [(18)] included women with three miscarriages and analysed NK cells using flow cytometry.

Meta-analysysis of Seshadri and associates

Seshadri and associates [1] conducted a meta-analysis of the following studies [20], that evaluated peripheral NK cell levels expressed as percentages showed a significant difference between women with RM versus controls (SMD 1.36; 95% CI 0.04; 2.69; P = 0.04; Fig. 6a). The $I2$ was 95% indicating significant statistical heterogeneity across the studies. They also did a meta-analysis of ***Aoki et al.*** [14]***;*** that expressed peripheral NK cells as numbers showed significantly higher levels of peripheral NK cells in women with RM compared with controls (SMD 0.81; 95% CI 0.47; 1.16; $P < 0.00001$; Fig. 6b). The $I2$ was 0% indicating no statistical heterogeneity. These two studies agreed with our study in studying peripheral NK cells, in RM and also in their results.

Seshadri and associates [1] also conduct a meta-analysis of ***Clifford et al.*** [2]***; Lachapelle et al.*** [18]***; Tuckerman et al.*** [21] that evaluated uNK cells expressed as a percentage of the stromal cells in women with RM versus controls showed no significant difference between the two groups (SMD 0.40; 95% CI −1.24; 2.04; P= 0.0.63; Fig. 6c). The $I2$ was 96% indicating significant statistical heterogeneity across the studies.

Conclusion

Endometrial NK cells measured by flowcytometrey are increased in cases of repeated miscarriges.

There were significant positive correlations between CD56 and Total pregnancies, Total miscarriages, early miscarriages, Miscarriages 6.0–12.0 weeks, Miscarriages 13.0–20.0 weeks, Missed abortion and previous medical treatment of miscarriage. The strongest correlation was with early miscarriages.

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6/25/2018