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Endometrial CD4+ And CD8+ in Women with Failed Implantation Following Embryo Transfer

El-Shahawy Y. (MD)
Department of Obstetrics and Gynecology – Ain Shams University
Email: manofegypt28@hotmail.com

Abstract

To quantify and compare endometrial CD4+ and CD8+ cells in women with failed implantation following embryo transfer and infertile women using immunohistochemistry. It was a case control study that was done at Al-Amin Maternity Hospital, Saudi Arabia. Endometrial biopsies were collected from women who failed to achieve pregnancy after repeat IVF treatment from start of January 2013 till December 2014 in IVF centers, endometrial biopsies were collected in mid luteal phase of cycle. A second group of matched fertile women, and came to outpatient clinic for reasons other than infertility, were included as a control group. Using pippele catheter from all women collected Endometrial small biopsies at the 21st day of the cycle and immunohistochemical study for all biopsies was done. Higher CD4 and CD4/CD8 but lower CD8 levels were noticed in cases with implantation failure in comparison to controls with highly significant difference between the two groups as regards the three parameters. CD8 is more sensitive with higher predictive value (PPV) but less specific and less negative predictive value (NPV) than CD4. The best cut off value of CD4 was 4.1 while CD8 was 5.6. The sensitivity and specificity were higher when combining the two tests together with better accuracy. The prognostic value of measuring endometrial CD4 and CD8 cell parameters seems to be valuable. More studies are needed to confirm or refute the role of CD4 and CD8 assessments as a predictive test for screening in women with implantation failures who may benefit from immunotherapy.

Keywords

CD4 – CD8 – endometrium – implantation failure – embryo transfer.

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I. Introduction

CD4 (clusters of differentiation 4) is glycoproteins found on the surface of immune cells such as T helper cells, monocytes, macrophages, and dendritic cells. It was discovered in the late 1970s and was originally known as leu-3 and T4 (after the OKT4 monoclonal antibody that reacted with it) before being named CD4 in 1984. In humans, the CD4 protein is encoded by the CD4 gene [1-2]. CD8 (cluster of differentiation 8) is a transmembrane glycoprotein that serves as a co-receptor for the T cell receptor (TCR). Like the TCR, CD8 binds to a major histocompatibility complex (MHC) molecule, but is specific for the class I MHC protein [2]. There are two isoforms of the protein, alpha and beta, each encoded by a different gene. In humans, both genes are located on chromosome 2 in position 2p12 [3].

During implantation, maternal immunoactivation and tolerance are not only limited to the decidua but are also observed in the periphery, predominantly affecting the innate immune system. Since unexplained female infertility, as well as recurrent pregnancy loss and implantation failure, are thought to be associated with pathological maternal immunotolerance mechanisms. Although uterine NK cells are in close proximity to important reproductive events, the function of these cells is completely unknown[4]. Trials have been done to compare the number of NK cells in the non-pregnant uterine lining of women with recurrent pregnancy loss or infertility with that of women with normal fertility. Since the number of uNK cells changes rapidly after ovulation, accurate timing is important to avoid miscalculation of the results. In other words, comparing uNK cell numbers in a woman 5 days after ovulation may give much different results than a woman who is 6 days after ovulation. Uterine NK cells must be obtained by performing a biopsy of the uterine lining. However, since the density of NK cells throughout the uterine lining varies, a "deep" biopsy will give different results than a "shallow" biopsy. It is impossible to measure the depth of the biopsy in a living uterus. So, it is nearly impossible to determine whether a given patient with implantation failure has an "elevated number" of uNK cells in compared to a woman without these problems. Based on the assumed similarities between peripheral and uNK cells, it has become increasingly common to measure NK cells using blood tests in women with infertility and recurrent miscarriage which are based on the assumption that these women also have abnormalities in uterine NK cell function [5].
The purpose of this study is to measure the percentage of endometrial NK cells expressing CD4+, CD 8+ and compare the results in women with failed implantation following embryo transfer and in fertile women using immunohistochemistry.

II. Patients and Methods

This was a case-control study that was done at Al-Amin Maternity Hospital, Saudi Arabia. Endometrial biopsies were collected from 40 women who failed to achieve pregnancy after repeat IVF treatment from start of January 2013 till December 2014 in IVF centers, endometrial biopsies were collected in mid

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luteal phase of cycle. A second group of 40 matched fertile women, and came to outpatient clinic for reasons other than infertility, were included as a control group. Using pippele catheter from all women collected Endometrial small biopsies, at the 21st day of the cycle in women with regular 28-day cycles, and immunohistochemical study for all biopsies was done.

Exclusion criteria:

- ***Women had a known or any definitive cause explaining the failure of IVF-ET***
    1. History of maternal endocrinopathies, hyperprolactinemia, luteal deficiency (detected by repeated low midluteal progesterone level), abnormal thyroid hormones, hyperandrogenism, polycystic ovarian syndrome.
    2. Acquired (antiphospholipid antibody syndrome) or hereditary thrombophilia.
    3. Uterine malformation had been ruled out by ultrasound and hystroscopy .
    4. Abnormal karyotyping.

All included women were subjected to revising history and examination sheets with particular emphasis on personal history: age, residence, education level and socioeconomic status, Complaint regarding infertility, obstetric history including parity and gravidity. Investigations have been collected from all women, which included mainly: glucose tolerance curve, anticardiolipin and lupus anticoagulant antibodies, TORCH serology, pelvic sonar and hysterosalpingogram and semen analysis from their husbands.

Endometrial biopsy preparation procedure:

Endometrial biopsies were collected around the expected day of implantation (day 21 of 28 day cycle) from regularly cycling women using a small pippele catheter

A. The patient is asked to lie on the table with her feet in the stirrups for a pelvic examination. A speculum was inserted into the vagina to spread the walls of the vagina apart to expose the cervix. The cervix was then cleansed with an antiseptic solution. A tenaculum held the cervix steady for the biopsy.
B. The pippele catheter was inserted into the uterine fundus and with a scraping and rotatory motion some tissue was taken. The tissues were fixed in 10% buffered formaline for preparation of paraffin block and then 5 µm thick section was cut from the paraffin on positively charged slides stained with Hematoxylin and Eosin to demonstrate T-lymphocyte subsets using CD4 (helper – T) and CD8 (suppressor T) markers by immunohistochemical study.
C. The antibodies used were:
    - CD4 marker used was CD4-Ab-8 (clone 4 B12) manufactured by NeoMrkers For Lab Vision Corporation. It is used for detection of T-helper cells. Ab used at 1:10 – 1:20 for 60 min at room temperature

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using ultravision LP systems to detect Ab.

- CD8 marker used was clone SP16 (rabbit-monoclonal antibody) manufactured by NeoMrkers For Lab Vision Corporation. It is used for detection of T-suppressor cells. Ab used at 1:50 for 30 min at room temperature using ultravision LP systems to detect Ab.
- Immunohistochemistry was performed according to Hsu and Raine 1981 [6] and applying the supersensitive ABC universal kit (Biogenix, USA)

The steps of the technique can be summarized as follows:

i. Deparaffinization of the formaline-fixed paraffin embedded tissue section in xylene and descending grades of alcohol.
ii. Antigen retrival (unmasking of the antigen) by treating the slides in citra solution in microwave for 5 minutes.
iii. Incubation of the tissue sections with the primary antibody (Anti CD4 and Anti CD8) for one hour. Incubation of the tissue sections with the secondary biotinylated antibody (link antibody) for 30 minutes.
iv. Incubation of the tissue section with the peroxidase-labeled streptavidin.
v. End result of the series of reactions was labeled by adding a substrate, chromogen (coloring agent) that leads to formation of a brownish precipitate at the antigen sites.

Ethical consideration:

Institutional review board (IRB) approval: the ethical scientific committee for approving the study discussed the protocol and informed consent was obtained before participation.

Consent procedure:

The Investigator made certain that an appropriate informed consent process was in place to ensure that potential research subjects, or their authorized representatives, were fully informed about the nature and objectives of the clinical study, the potential risks and benefits of study participation, and their rights as research subjects. The Investigator obtained the written, signed informed consent of each subject, or the subject's authorized representative, prior to performing any study-specific procedures on the subject. The Investigator retained the original signed informed consent form.

Subject Confidentiality:

All laboratory specimens, evaluation forms, reports and other records that leave the site would not include unique personal data to maintain subject confidentiality.

Sample size calculation:

The required sample size has been estimated using the Power Analysis and Sample Size software version 08.0.9 (PASS;NCSS;LLC;Kaysville, Utah). The test used for calculation is the two-sided z-test and type 1 error has been set at a two-sided value of 0.05 (confidence level, 95%).

Statistical analysis: Retrieved data were recorded on an investigative report form. The data were analyzed with SPSS® for Windows®, version 15.0 (SPSS, Inc, USA). Description of quantitative (numerical) variables was performed in form of mean,

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standard deviation (SD) and range. Description of qualitative (categorical) data was performed in the form of numbers and percent. Analysis of numerical variables was performed by using student's unpaired t-test (for two groups) or ANOVA (for more than two groups). Analysis of categorical data was performed by using Fischer's exact test and Chi-squared test. Logistic regression analysis was performed to calculate association between variables and their odds ratios. Association between variables was estimated using Pearson's correlation coefficient (for parametric variables) and Spearman's correlation coefficient (for non-parametric variables). Significance level was set at 0.05.

III. Results

The current study was conducted on women recruited at Al-Amin Maternity Hospital, Saudi Arabia, during the period between January 2013 and December 2014. The study included 2 groups of women: group I (study group) [n=40]; women who failed to achieve pregnancy after repeat IVF treatment and group II (control group) [n=40]; matched fertile women, who came to outpatient clinic for reasons other than infertility.

Endometrial small biopsies at the 21st day of the cycle were collected by using pippele catheter from all women and immunohistochemical study for all biopsies was done to measure endometrial CD4+ and CD8+ cell numbers which were analysed as a percentage of total stromal leucocytes in five randomly selected fields of view in the stratum functionalis of each sample.

Table (1) shows that there was no significant statistical difference between the two groups as regards age, body mass index (BMI), but there was a significant difference between the two groups as regards parity and gravidity. CD4 level and CD4/CD8 were higher in cases in comparison to controls but CD8 level was lower in cases in comparison with controls with highly significant difference between the two groups as regards the three parameters

Table (2) shows a significant statistical inverse correlation between CD4 and parity and significant statistical positive correlation between CD8 and parity by Spearman correlation. On the other hand there was no significant correlation of CD4 or CD8 versus other variables among cases

Table (3) shows a significant statistical inverse correlation between CD4 and parity and significant statistical positive correlation between CD8 and parity by Spearman correlation. On the other hand there was no significant correlation of CD4 or CD8 versus other variables among controls

Table (4) shows that CD8 is more sensitive with higher predictive value (PPV) but less specific and less negative predictive value (NPV) than CD4. The best cut off value of CD4 was 4.1 while CD8 was 5.6. The sensitivity and specificity were higher when combining the two tests together with better accuracy.

IV. Discussion

Many studies tried to confirm a connection between levels of certain lymphocyte subpopulations and reproductive failure. In the current study which was conducted on women recruited at Al-Amin Maternity Hospital, Saudi Arabia, during the period

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between January 2013 and December 2014 and included 2 groups of women: group I (study group) [n=40]; women who failed to achieve pregnancy after repeat IVF treatment and group II (control group) [n=40]; matched fertile women, who came to outpatient clinic for reasons other than infertility. Using pippele catheter from all women collected Endometrial small biopsies at the 21st day of the cycle and immunohistochemical study for all biopsies was done to measure endometrial CD4+ and CD8+ cell numbers. CD4 level and CD4/CD8 were higher in cases in comparison to controls but CD8 level was lower in cases when compared to controls with highly significant difference between the two groups as regards the three parameters. These results agreed with a similar study [7], which reported that lymphocyte subpobulations percentage was elevated in women who suffered from recurrent miscarriage and with a chromosomally normal pregnancy. Also the results agreed with (Thum MY et al. 2013) [8], who reported an association between high numbers of pre-pregnancy uterine lymphocytes and miscarriage in a subsequent pregnancy after biopsy, but result was just significant (P =0.04), Two explanations for these discrepant results due to numbers included in that study were small; of 16 women who became pregnant, only 4 miscarried, and It is impossible to measure the depth of the biopsy in a living uterus.

In the present study, there was a significant statistical inverse correlation between CD4 and parity and significant statistical positive correlation between CD8 and parity by Spearman correlation. On the other hand there was no significant correlation of CD4 or CD8 versus other variables among cases, these result matched with another study [9],who reported Infertile women had significantly elevated circulating lymphocytes ($P < 0.001$) compared with infertile women. These results disagreed with (Gavin et al. 2012) [10], who reported that there was no significant association between simple enumerations of peripheral blood NK cells with IVF treatment outcome and pregnancy outcome, it was unclear at what stage the blood samples were obtained from the study subjects, but in our study inclusion criteria regular menstrual cycle, being in midluteal (secretary) phase

The current study shows that CD8 is more sensitive with higher predictive value (PPV) but less specific and less negative predictive value (NPV) than CD4. The best cut off value of CD4 was 4.1 while CD8 was 5.6. The sensitivity and specificity were higher when combining the two tests together with better accuracy. The results of the current study are in agreement with a similar study [11], which showed retarded endometrial development was found in 9 out of 20 women (45%) in the recurrent abortion group. Measurable differences in glandular development (i.e., numbers of glands and glandular epithelial height) were found in this subgroup. The percentage of endometrial CD8+ suppresser T-lymphocytes (+ve cluster differentiation) was significantly decreased and CD4+: CD8+ ratio was significantly increased in the recurrent aborters ($P < 0.05$). In contrast, the proportion of B-lymphocytes (CD20+) was significantly increased ($P < 0.05$). The proportion of natural kuller (NK) cells was identical in both groups CD 16+ CD56 dim NK cell subset with cytolytic activity was higher in the habitual aborters.

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This in partial agreement with another study [12] which described the identification of a CD161−CD56+ CD8 T cell subset capable of negative regulatory function: cytolysis of activated CD4 T cells. Many questions remain for further exploration of this interesting population of cells. Multiple other negative regulatory CD8 T cell subsets have been described including FoxP3+ CD8 T cells. Determining the differences between various regulatory CD8 T cell subsets regarding marker expression and function should be addressed. Additionally, the CD8 T cell clones previously described by this group expressed IFN-gamma following activation. As these negative regulatory CD8 T cells also phenotypically resemble terminally differentiated effector CD8 cells, these populations should be directly functionally compared in future studies.

A prospective study would be required to account for such possible bias that is known to affect peripheral blood lymphoctes levels and activation status, including exercise and stress [13-14]. Concerns need to be administered in further study in embryo quality (primarily genetic) is almost certainly the most significant factor determining IVF success, Thus, it is important to have markers to identify IVF failures of chromosomally normal embryos. So prospective clinical trials are necessary to answer the question of the effectiveness of embryo selection. Also to improve implantation success is directed for better embryo selection and improving endometrial receptivity[15]. Selection of patients may limit conclusions originating from our study. So studies have to add for inclusion criteria minimum two deliveries to avoid recurrent pregnancy loss to confirm fertility, as a significant proportion of cases of recurrent pregnancy loss (15%) remain unexplained, so it may target suitable patients who might attempt treatment with additional immune suppressive therapy, only 40% of women experiencing implantation failures would be helped if immunotherapy were 100% effective [16].

V. Conclusion

Quantification of the percentage of expression of different cell surface markers in peripheral blood natural killer cells has a promising role to offer to be integrated with various methods to predict the outcome of embryo transfer.

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Table (1): The demographic differences between Study and Control Groups.

	Group I [Study Group] (n=40)	Group II [Control Group] (n=40)	*P*
Age (Years) Range: Mean ± SD:	24 – 40 32.37 ± 5.1	18 – 45 31.5 ± 5.6	>0.05* NS
Body mass index Mean ±SD:	35.3±5.1	35.2 ± 5.2	>0.05* NS
Gravidity Mean ± SD:	1 ± 0.3	3 ± 1.06	<0.05** S
Parity Mean ± SD:	0.7 ± 0.2	2 ± 1.5	<0.05** S
CD4 Mean ± SD:	6.68 ± 2.36	4.275 ± 1.89	<0.001 HS
CD8 Mean ± SD:	4.55 ± 1.62	6.4 ± 1.69	<0.001 HS
CD4/CD8 Mean ± SD:	1.66 ± 0.91	0.827 ± 0.57	<0.001 HS

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Table (2): Correlation between CD4 and CD8 versus different variables among cases:

	CD4		CD8	
	R	**P**	**r**	**P**
Age	0.07	> 0.05	0.03	> 0.05
BMI	0.06	> 0.05	0.05	> 0.05
Gravidity	-0.09	> 0.05	0.06	> 0.05
Parity	-0.39	<0.05 S	0.24	< 0.05 S
CD8	-0.24	> 0.05		

Table (3): Correlation between CD4 and CD8 versus different variables among controls:

	CD4		CD8	
	R	**P**	**r**	**P**
Age	0.05	> 0.05	-.23	> 0.05
BMI	0.07	> 0.05	-0.17	> 0.05
Gravidity	-0.44	> 0.05	-0.11	> 0.05
parity	-0.41	< 0.05 S	0.31	< 0.05 S
CD8	-0.11	> 0.05		

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Table (4): Validity of CD4 and CD8 in prediction of implantation failure:

	CD4	CD8	Both	CD4/CD8
AUC	0.45	0.71	0.78	0.42
Best cuff off	4.1	5.6	-	0.72
Sensitivity	61%	70%	73%	65%
Specificity	73%	56%	74%	58%
PPV	57%	61%	50%	49%
NPV	71%	44%	70%	66%
Accuracy	63%	65%	73%	50%