RESEARCH

An endometrial histomorphometric study of CD56+ natural killer cells in women with unexplained infertility

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Background. The number of peripheral blood and endometrial natural killer cells varies greatly during implantation and the first trimester of pregnancy and is thought to play a role in the maintenance of a healthy pregnancy. However, the role of endometrial CD56+ natural killer (NK) cells as an immunological mechanism in unexplained infertility is yet unknown.
Objectives. The study aimed to enumerate the concentrations of CD56+ NK cells in endometrial samples, and to statistically compare these numbers between fertile and infertile women.
Methods. A histomorphometric analysis was conducted using haematoxylin and eosin staining and an immunohistochemical approach was used for quantifying cell numbers.
Results. Fifty samples were collected in equal parts between a study group of infertile female subjects (mean (standard deviation) age 35 (4), range 26 - 42 years) and a control group of multiparous fertile individuals (mean (SD) age 43.4 (6.3), range 30 - 55). The mean number of CD56+ NK cells present at different depths for both the study and control groups did not differ significantly. Age and group (study or control) were not significantly related to the mean number of CD56+ NK cells. However, for the late secretory phase the mean number of CD56+ NK cells was significantly higher than for the early phase.
Conclusion. Our findings could not identify a statistically significant correlation between the number of CD56+ NK cells and infertility.

The standard clinical definition for infertility, as defined in the International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) glossary (2009), is 'a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse'.[1] Terms often used synonymously with infertility include:

- Reproductive failure – describing the inability to conceive or maintain pregnancy, due to either recurrent miscarriages, infertility or repeated implantation failure (in vitro fertilisation).[2]
- Repeated implantation failure – described as the failure to achieve pregnancy following 2 - 6 in vitro fertilisation cycles.[3]
- Recurrent miscarriages – described as the loss of ≥3 consecutive pregnancies before gestational age (20 - 22 weeks).[4]

Hull *et al.*[5] first described infertility as a failure to conceive over a period of 3 years of unprotected intercourse, while others report non-conception after 1 year of unprotected intercourse during the fertile phase as sufficient to diagnose infertility.[6,7] More recently, infertility has been described as the failure to conceive after 6 cycles of unprotected intercourse, regardless

of age, while the National Institute for Health and Clinical Excellence has rendered a definition that failure to conceive after 2 years signifies infertility.[8] In accordance, it is reported that 92% of the general population shows successful conception after 2 years (84% after 1 year).[9]

Prognostic factors for higher cumulative pregnancy rates include: female age <30 years; previous pregnancy in the same relationship; and <2 years' infertility. The prognosis worsens when the duration of infertility exceeds 3 years and the female partner is older than 35 years. Pregnancy rates are reported to decline by 9% each year beyond the age of 30 years.[9]

According to the American Society of Reproductive Medicine, standard infertility evaluations should include semen analysis, post-coital test and assessment of ovulation, hysterosalpingogram, and sometimes laparoscopy.[10] In agreement, the Practice Committee Bulletin on Unexplained Infertility suggests that basic evaluations should provide evidence of ovulation, adequate sperm production, and patency of the Fallopian tubes; and that unexplained infertility can only be diagnosed if these results are normal.[11] Unexplained infertility is unfortunately often misdiagnosed due to the lack of dedicated testing measures, while endometriosis, mild degrees of

tubal infertility, and poor ovarian reserve are considered the most frequent reasons for the misdiagnosis of unexplained infertility.[10]

CD56+ as diagnostic investigation

During implantation and the maintenance of pregnancy, there exists a close interaction between the endocrine and immune system. Under influence of sex steroids, there is a marked increase of a unique population of endometrial natural killer (NK) cells and lymphocytes, derived predominantly from a subset of peripheral blood NK cells and subsequently recruited to the uterus. These immune cells are believed to promote placental and trophoblast growth, provide immunomodulation of maternal-fetal interaction and have a presumptive role in the maintenance of healthy pregnancy.[3] The normal, healthy endometrium contains a variety of haematopoietic cells, including granulocytes like CD56+ NK cells, and its composition varies depending on the stage in the menstrual cycle and menopausal status. CD56+ NK cells have a primary role in the innate immune responses against viruses and transformed cells, and is thought to possibly be a critical mechanism in preventing maternal rejection of the fetus. It is thought that such immunological mechanisms influence reproductive failure, as successful pregnancy involves maternal immunity adaptation to the semi-allogeneic developing embryo.[13,14]

It is known that peripheral blood NK cells are phenotypically and functionally different from endometrial NK cells, with less than 10% resembling endometrial NK cells and differing in surface antigen expression.[15] Endometrial NK cells have little cytotoxic activity, but are a rich source of cytokines, particularly angiogenic ones, which are possibly involved in tissue remodelling during the formation of the placenta and the regulation of trophoblast invasion and angiogenesis.[14,16,17] Granulated lymphocytes (including CD56+ NK cells) are present in large numbers in pre-decidualised endometrial stroma in the mid- and late-secretory phases.

There are two theories to explain these large numbers:

- The recruitment of CD56+ NK cells from peripheral CD56+ NK cells which differentiate in the uterine microenvironment into the endometrial phenotype.
- That endometrial CD56+ NK cells stem from the proliferation and differentiation of stem cells in the endometrium.[12]

An immunohistochemical analysis in the non-pregnant endometrium shows increases of endometrial CD56+ NK cells during the secretory phase (alleged time of implantation).[12] These numbers remain high during early pregnancy, constituting 70% of the T lymphocytes at the interface between maternal decidua and the invading trophoblast and are the most predominant leucocyte population during the time of implantation and early pregnancy.[3,4] In contrast, 30% of the endometrial T lymphocytes were CD56+, as opposed to the peripheral blood (5 - 15%). This percentage remains constant during the menstrual cycle in both the proliferative and the secretory phases.[3,4]

Successful pregnancy outcomes have been reported after intravenous immunoglobulin G (IgG) therapy in patients with recurrent pregnancy losses. Intravenous IgG therapy is shown to down-regulate elevated circulating peripheral blood CD56+ NK cells, suggesting an association between increased CD56+ NK cell numbers and unexplained infertility.[18] Research on the association of increased peripheral or endometrial CD56+ NK cells and infertility, however, remains ambiguous.

Research problem and objective

The role of endometrial CD56+ NK cells in the immunological mechanism of unexplained infertility is yet unknown. Quantification of endometrial CD56+ NK cells, or the comparative percentages of CD56+ NK cells in the circulating peripheral blood during the secretory phase of individuals with unexplained infertility have not been documented. It is also unknown whether these values differ in infertile and multiparous fertile individuals.

Determining these values could help to determine the role played by endometrial CD56+ NK cells in the immunology of unexplained infertility, and the aim of the study was therefore to enumerate and statistically compare endometrial CD56+ NK cell numbers in both infertile and control patients. The study only evaluated endometrial CD56+ NK cell numbers; peripheral blood levels were reserved for future research. The study was approved by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, and the National Health Research Ethics Council (ref. no. 8/2013).

Methods

Volunteering women with a history of infertility were recruited from the Endocrine and Reproductive units of three Gauteng-based hospitals in South Africa: Steve Biko Academic Hospital, Kloof Hospital and Kalafong Hospital. All women had normal menstrual cycles, normal ovarian and pelvic ultrasound examinations, normal hysterosalpingographies, normal mid-luteal progesterone levels, and their partners showed normal semen analysis (Table 1). The control group consisted of gynaecology outpatients with no associations with infertility. Anonymous samples were used for this histomorphometric, cross-sectional analysis and all participants signed informed consent.

Endometrial biopsies

Endometrial biopsies were performed by clinician gynaecologists as an outpatient procedure using an endometrial sampler during the secretory phase of the menstrual cycle (Z-sampler, Bioteque America Inc., USA). Specimens were placed in 10% buffered formalin and study numbers were allocated to each sample.

Haematoxylin and eosin staining

The first evaluation step was done on haematoxylin and eosin (H&E)-stained slides under light microscopy to verify the histological representation of the endometrium, and to exclude inflammation, hyperplasia, atypia or evidence of malignancy. Samples were categorised into three phases:

Early secretory phase: presence of tubular-shaped glands; subnuclear vacuoles in more than 50% of the epithelial cells, involving more than 50% of glands; presence of mitotic activity in the glandular cells (Fig. 1).

Mid-secretory phase: presence of angulated glands; supra-nuclear vacuoles in the epithelial cells; no mitotic activity; increased glandular secretions and progressively increased stromal oedema. Presence of spiral arterioles and eosinophilic cytoplasm in the stromal cells.

Late-secretory phase: presence of closely packed serrated-shaped glands; pre-decidua changes around spiral arterioles forming a compact layer; presence of mitotic activity in the predecidual cells; apoptotic activity within the glands; fibrin thrombi in the small vessels; extravasation of erythrocytes into the stroma (Fig. 2).[12]
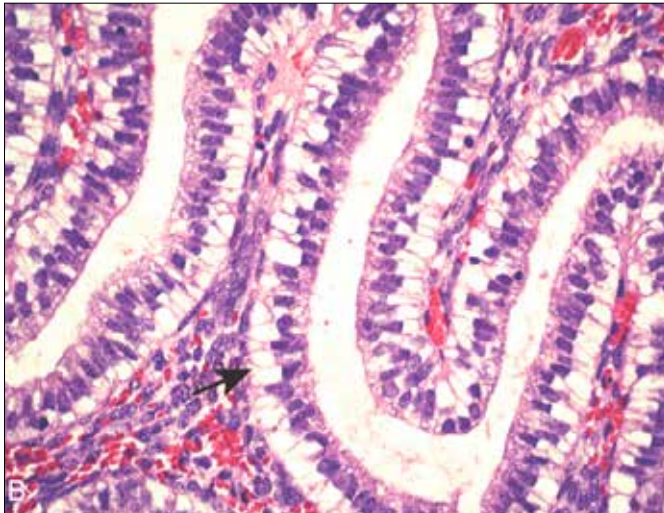
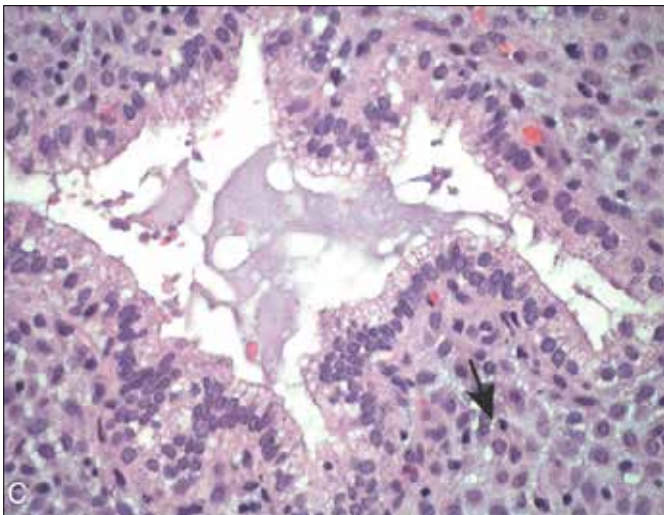*Fig. 1. Early secretory phase with subnuclear vacuoles.*

Fig. 2. Late secretory exhaustion and predecidual changes.

Immunohistochemistry

A second step of evaluation was done through the immunohistochemistry of each sample. Two sections (4 µm) were cut off all the specimens with a standard microtome and dried overnight at 37˚C. Sections were manually dewaxed in xylene twice for 5 minutes and brought to distilled water before being treated with 0.38% EDTA and incubated for 30 minutes. The sections were washed in distilled water and treated with 3% H_2O_2 solution for 10 minutes, washed again and placed in buffer wash for 5 minutes. All sections were covered with primary mouse anti-human NK cell, CD56+, monoclonal antibodies (Dako, Denmark) at a dilution of 1:100 for 30 minutes. The slides were washed in a buffer and dried, covered with FLEX detection system (Agilent Technologies Inc., USA) sera and incubated for 30 minutes at room temperature. Samples were washed again in a buffer wash solution and incubated with 3,3-diaminobenzidine tetrahydrochloride twice for 3 minutes at room temperature. After being washed again in tap water, samples were counterstained in haematoxylin (2 min) and eosin blue (5 min). The slides were dehydrated and mounted and adequate positive controls (lymphnode tissue) were placed on each slide.

This morphometric evaluation was performed using transmitted light microscopy and an eyepiece to a magnification of ×400. The magnification of ×400 was defined as the high-powered field (HPF) used in this study. The number of positively staining cells in ten, randomly selected, non-overlapping HPFs were counted and labelled as level 1. The same procedure was repeated from the same subject's endometrial sample in a second (4 µm deeper) section and was labelled as level 2.

A paired *t*-test was used to compare the mean number of CD56+ NK cells of the two different tissue sections (level 1 and level 2) for both study and control groups. A linear model was fitted to test for a relationship between the mean number of CD56+ NK cells and the covariates group (study or control), age and phase (early, mid or late). For covariates that were significant, where applicable, post-hoc comparisons of least-squares means of CD56+ NK were performed with a Bonferroni correction.

After omission of 3 samples deemed to fall outside the inclusion criteria, the 53 original samples were reduced to 25 samples in both the study group (mean (standard deviation (SD)) age of 35.0 (4.0), range 26 - 42 years) and control group (mean (SD) age 43.4 (6.3), range 30 - 55). None of the participants in the study group had a history of live birth and had been diagnosed as infertile. Infertility specialist teams of the respective gynaecology units were used. In the control group, the median (range) number of successful pregnancies was 3 (3 - 7).

Results
H&E results
The paired *t*-test performed showed that for both the study and control groups there was not a significant difference between

Table 1. The selection criteria for subject recruitment used by gynaecologists collecting the endometrial samples

Inclusion criteria	Exclusion criteria
Childbearing age	Advanced age (>35 years)
Normal menstrual cycles	History of previous ovarian surgery
Normal ovarian examination on ultrasound	Polycystic ovarian syndrome
	Thyroid disease
Normal transvaginal ultrasound	Hyperprolactinaemia
Normal hysterosalpingography	Hypothalamic causes of infertility secondary to abnormal weight changes
Normal sonohysterography/ diagnostic office hysteroscopy	
	Endometriosis
Normal pelvic examination on ultrasound	Reversible adhesive tubal disease
Normal mid-luteal progesterone/ Luteinising hormone levels	Uterine leiomyoma
	Uterine polyps
Normal semen analysis of partner	Asherman's syndrome (uterine synechiae)
	Congenital uterine cavity anomalies
Additional inclusion criteria for control group	Male partner infertility
Multiparous (≥2 full-term pregnancies)	

Table 2. Mean number of endometrial CD56⁺ NK cells per 10 HPFs in the three stages of the secretory phase of the total population

Stage of secretory phase	CD56⁺ cells in level 1 and 2, mean (SD)	Age of participants, mean (SE)
Early	16.17 (5.22)	38.07 (1.73)
Mid	30.88 (4.75)	39.00 (1.27)
Late	37.16 (4.91)	40.41 (1.96)

NK = natural killer; HPF = high-powered field; SD = standard deviation; SE = standard error.

Table 3. Mean numbers of endometrial CD56⁺ NK cells per HPF in both the study and control group*

Group	N Obs	Variable	N	Median	Mean (SD)	Min	Max
Control	25	l1_hpf1	25	17.00	30.16 (29.01)	1.00	120.00
		l1_hpf2	25	15.00	23.16 (21.08)	3.00	100.00
		l1_hpf3	25	20.00	27.68 (28.29)	2.00	124.00
		l1_hpf4	25	20.00	23.72 (16.72)	2.00	62.00
		l1_hpf5	25	20.00	25.08 (18.62)	6.00	78.00
		l1_hpf6	25	19.00	24.52 (17.01)	5.00	72.00
		l1_hpf7	25	20.00	25.32 (17.58)	3.00	59.00
		l1_hpf8	25	20.00	26.60 (20.87)	6.00	77.00
		l1_hpf9	25	16.00	24.36 (17.05)	2.00	64.00
		l1_hpf10	25	20.00	24.28 (17.45)	0.00	60.00
		AvgL1	25	18.70	25.49 (18.18)	4.80	72.10
Study	25	l1_hpf1	25	24.00	32.16 (28.61)	1.00	102.00
		l1_hpf2	25	15.00	29.52 (35.94)	0.00	163.00
		l1_hpf3	25	20.00	29.20 (25.68)	0.00	96.00
		l1_hpf4	25	17.00	29.48 (32.87)	0.00	110.00
		l1_hpf5	25	22.00	36.80 (39.03)	1.00	151.00
		l1_hpf6	25	20.00	32.88 (33.69)	1.00	139.00
		l1_hpf7	25	21.00	26.08 (22.18)	0.00	92.00
		l1_hpf8	25	23.00	31.76 (28.77)	2.00	115.00
		l1_hpf9	25	23.00	34.76 (29.70)	0.00	104.00
		l1_hpf10	25	22.00	27.04 (22.07)	0.00	83.00
		AvgL1	25	21.40	30.97 (26.95)	2.00	112.60

NK = natural killer; HPF = high-powered field; SD = standard deviation.
*The magnification of ×400 was defined as the high-powered field (HPF) used in this study.

the mean CD56⁺ NK cells measured at level 1 and level 2 with $p=0.6636$ and $p=0.8836$, respectively. The results for the two levels were combined in further analysis. In the linear model with mean CD56⁺ NK cells as dependant variable and group, age and phase as covariates, the results indicated a significant difference ($p=0.0173$) in the mean number of endometrial CD56⁺ NK cells/10 HPFs between the phases. In the post-hoc test least-square means of the mean CD56⁺ NK cells were compared for the different phases making a Bonferroni correction. There was a significant difference in the mean CD56⁺ NK cells between early and late secretory stages, ($p=0.0165$ after the Bonferroni correction) (Table 2). Controlling for group and phase, there was no significant relationship between age and mean CD56⁺ NK cells ($p=0.2171$). There was also no significant difference in the mean CD56⁺ NK cells for the 2 groups when keeping age and

phase constant ($p=0.8476$; study and control mean (SD) values were 28.77 (4.65) and 27.36 (4.63), respectively.

Histomorphometric results

From the histomorphometric analysis, each sample was evaluated as two separate slide sections (level 1 and 2) and the number of endometrial CD56⁺ cells/HPF for each level was counted (Table 3). The results from the analysis are given above; there was no statistically significant difference in the number of CD56⁺ NK cells between levels and groups.

Discussion and conclusion

This cross-sectional histomorphometric study was conducted to establish the potential role of endometrial CD56⁺ NK cells as an immunological mechanism in unexplained infertility. The study revealed no statistically significant associations between the numbers of CD56⁺ NK cells present in the endometrium compared with age, number of successful pregnancies, mean number of cells per HPF, or between accumulated cell numbers per 10 HPFs in either of the groups.

However, the only positive statistical association in our results was the difference in number of CD56⁺ endometrial NK cells found between the early and late secretory phase (Table 2). The significance of this finding warrants further investigation, but in agreement, literature does show variation in endometrial NK cells numbers throughout the menstrual cycle, with a dramatic increase between days 6 - 7 after the luteinising hormone (LH) surge.[15] This is the putative time of implantation and the number of endometrial CD56⁺ NK cells have been shown to remain high during early pregnancy, encompassing 70% of the endometrial leucocytes in the first trimester before steadily declining and being absent at term.[15] Despite NK cells being the most predominant leukocyte population during the time of implantation and early pregnancy, the precise role of endometrial NK cells and their relative contribution of cytokine secretion or cytotoxicity in implantation and maintenance of a successful pregnancy remain unfounded.[3,4,13] Other research still shows associations with increased numbers of endometrial CD56⁺ NK cells and infertility, while women with reproductive failure are often treated with steroids, intravenous immunoglobulin, and tumour necrosis factor-alpha (TNF-α) blocking drugs, that aim to suppress CD56⁺ NK cells. However, a review of various forms of immunotherapies also did not show significant differences between treatment and control groups.[19]

In essence, the hypothesis that endometrial NK cells might play an immunological role in unexplained infertility could not be supported or denied by our findings, but more conclusive evidence for a causative role for NK cells in unexplained infertility could be yielded if the testing of secretory-phase endometrial cell numbers could be standardised.[4,20] At present there is no agreed method for assessing endometrial NK cell numbers, and currently adopted methods vary greatly in the mean numbers of endometrial NK cells present, the percentage of endometrial NK cells versus that of stromal cells, or versus CD45⁺ cells.[6,15] On our part, follow-up studies of peripheral blood v. endometrial NK cell population numbers would help to further this investigation.

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