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CHARACTERIZATION OF UTERINE NK CELLS IN WOMEN WITH INFERTILITY OR RECURRENT PREGNANCY LOSS AND ASSOCIATED ENDOMETRIOSIS

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Abstract

Problem—Uterine natural killer cells (uNK) have been thought to play a key role in endometriosis and infertility. We investigated the expression of CD56, CD16 and NKp46 in endometrial tissues from 61 women with unexplained recurrent pregnancy loss (uRPL) or infertility (UI), and correlated this with the presence or absence of endometriosis. The results from the patients with sub-fertility were compared to those from 10 fertile patients.

Method of study—Mid-secretory phase endometrial biopsies were obtained and the endometrial expression of CD56, CD16 or NKp46 was identified by immunohistochemistry and quantified (ImageJ Software).

Results—The percentage of $CD16^+$ cells was higher in women with uRPL (7.9±3.2) and UI (9.0±5.5), even when these conditions were associated with endometriosis (8.9±5.3), compared to fertile patients (5.6±2.4, $p<0.05$). Likewise, the ratio of $NKp46^+$:$CD56^+$ cells was higher in women with uRPL (0.28±0.25) and UI (0.21±0.2), even when these conditions were associated with endometriosis (0.19±0.14), compared to fertile patients (0.1±0.1, $p<0.05$). No differences were observed when comparing CD56.

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Disclosure

The Authors declare that there are no conflicts of interest.

Conclusions—Women, with or without endometriosis, who have larger populations of cytotoxic $CD16^+$ uNK cells and/or higher populations of $NKp46^+CD56^+$ cells may be at greater risk for infertility disorders resulting from an inflammtory environment occuring during implantation or later during decidualization.

Keywords

endometriosis; infertility; natural killer cells; recurrent pregnancy loss

Introduction

Uterine natural killer cells (uNK) are the predominant leukocyte population in normal human endometrium [1.] Their content varies throughout the normal menstrual cycle, likely due to recruitment of peripheral NK cells (pNK) and/or *in utero* proliferation/differentiation of stem uNK cells. They represent 40% of the total leukocyte population during the proliferative phase which increases to 60% by mid-secretory phase and up to 75% in early pregnancy [2].

Approximately 70–80% of uNK cells are characterized as $CD56^{bright}CD16^{-}$ [3]. Activated uNK cells can produce angiogenic factors (VEGF, ANG2) that promote spiral artery remodeling, secrete cytokines (GM-CSF, CSF-1, TNFα, INFγ, TGFβ, LIF, IL2, CXCL10, CXL12) that direct the migration and invasion of the trophoblast, and interact directly with trophoblast antigens by expressing surface receptors such as killer immunoglobulin-like receptor (KIR) and immunoglobulin-like transcript-2 (ILT2) [4,5,6]. The activity of uNK cells is controlled by inhibitory receptors such as NKG2a and activating receptors such as NKp30 and NKp46 [7]. On the other hand, another minor subpopulation of uNK cells, characterized as $CD56^{dim}CD16^{+}$, displays cytotoxic activity towards the extravillous trophoblast and autologous endometrial cells and may create a hostile environment for implantation [8].

Dysregulation of uNK number and/or cell function (cytotoxicity, receptor expression, cytokine secretion or gene expression) has been associated with reproductive disorders such as unexplained infertility (UI), unexplained recurrent pregnancy loss (uRPL), and pre-eclampsia; however studies have been limited to relatively small populations of women and some findings have been apparently contradictory. For example, while some previous case-control studies report a higher concentration of uNK cells in women with uRPL compared to fertile women [9,10,11,12,13], other studies did not find this association [5,14]. Similarly, evidence exists for both an association and lack of association between uNK cell number/function and infertility [15,16,17,18]. Only few studies have examined changes of uNK cell numbers in endometriosis, demonstrating a lower percentage of $CD56^+$ NK cells and a defect in NK activity in the eutopic endometrium of women with endometriosis [19, 20, 21, 22]. However these studies did not focus on endometriosis patients with concomitant infertility disorders.

The aim of this study was to compare the expression of CD56, CD16, and NKp46 in the eutopic endometrium from women with uRPL or UI to fertile patients, and correlate this with the presence or absence of endometriosis.

Materials and Methods

Subjects

Sixty-one women were enrolled in the study. Twenty-one women had uRPL, 30 women had UI, and 10 women had no history of infertility, recurrent pregnancy loss, or endometriosis (controls). Among women with sub-fertility disorders, 13 women with uRPL and 23 with UI had concomitant endometriosis diagnosed by laparoscopic procedure performed in selected cases when clinical signs suggested the presence of the disease, either prior or post the endometrial biopsy obtained for this study.

Sub-fertile subjects included women with UI and with 2 or more consecutive pregnancy losses (uRPL) who were evaluated in the Division of Reproductive Endocrinology at Greenville Hospital System in Greenville SC. Patients in the sub-fertility groups had regular, ovulatory cycles, at least one patent fallopian tube (without hydrosalpinges) and were 40 years of age or younger. Exclusion criteria included known uterine fibroids or septum, a history of pelvic inflammatory disease, PCOS or a partner with an abnormal semen analysis (by the World Health Organization (WHO) criteria). While a prior history of minimal or mild endometriosis was not an exclusion criterion, moderate or severe endometriosis was. Unexplained RPL were excluded if they had a known thrombophilia, genetic or immunologic abnormalities, or Müllerian defects. The fertile control group consisted of subjects who were patients in the Department of Obstetrics and Gynecology at University of North Carolina, Chapel Hill. All women who participated in the study signed an informed consent that was approved by the Institution Review Board (IRB) of Greenville Hospital System or University of North Carolina, Chapel Hill.

Tissue collection

All the participants underwent a standard endometrial biopsy by pipelle suction between days 21 to 24 of the menstrual cycle based on urinary LH surge detection (LH plus 7 to 10 days). Each patient was provided with a urinary LH detection kit (Sepal Inc., Boston, MA) and instructed to call the clinic with a positive LH surge. Tissue collected was submitted for paraffin embedding for histologic determination, dating and immunohistochemistry analysis. Women with maturational delay of endometrium (type I defect) were excluded from the study [23], whereas a type 2 endometrial receptivity defect, based on the results of the αvβ3 integrin analysis, was found in 10 uRPL and 8 UI patients [24].

Immunohistochemistry

The expression of CD56, CD16, or NKp46 in all endometrial samples was determined by immunohistochemistry. Endometrial samples were placed in 10% buffered formalin for 24 hours, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin blocks. Briefly, the tissues in paraffin blocks were cut into 5 μm thick sections using the Leica RM 2135 microtome and transferred onto glass slides. Tissues were dewaxed in xylene and rehydrated through descending concentrated alcohols. Unmasking was performed in a 110V pressure cooker in a preheated 1:100 antigen unmasking solution (Vector Laboratories, Burlingame, CA) for 4 minutes. After washing, tissues were quenched in 3% hydrogen peroxide diluted in methanol for 10 minutes. Slides were then washed in 1% PBS and

blocked in 10% NGS blocking buffer (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Tissue sections were incubated with either monoclonal mouse anti-human CD56 (NCAM) antibody at 1:50 dilution (Novocastra Leica Biosystem, Buffalo Grove, IL, USA), monoclonal mouse anti-human CD16 antibody at a 1:20 dilution (AbD Serotec, Raleigh, NC, USA), or monoclonal goat anti-human NKp46/NCR1 antibody at 1:25 dilution (R&D Systems, Minneapolis, MN, USA) at +4°C overnight in a humidified chamber. Sections were washed extensively in 1% PBS and first incubated with the specific secondary antibody for 1 hour at room temperature followed by an incubation with a 1:1000 streptavidin HRP substrate for 30 minutes at room temperature. The positive cells were then visualized by incubation with DAB peroxidase solution (3.3′diaminobenzidene terahydrochloride; Vector Laboratories, Burlingame, CA). Slides were counterstained with haematoxylin (Biocare Medical), dehydrated in ascending concentrated alcohols, cleared in xylene and mounted in Permount (Fisher Scientific). Replacing CD56, CD16, NKp46 or LIF antibodies with an equivalent amount of control mouse IgG (Serotec) resulted in absence of immunoreactivity. Decidual tissue and liver tissues from baboons were used as positive controls at the same dilutions.

Analysis of images

Staining was assessed by using Olympus BH2 microscope (Olympus, Melville, NY, USA) and 10 high-resolution images of each specimen were captured at 400x magnification with a Nikon DS Fi1 digital camera/NIS Elements imaging software. The ratio between positive CD16, CD56 or NKp46 cells (brown stain) and total endometrial stromal cells (blue stain) was assessed using computer assisted image analysis with color deconvolution (Image J software, NIH) for all the images. The investigators were blinded with respect to the identification of the sections.

Statistical analysis

Data are expressed as average ± standard deviation of the mean (SD). Mann-whitney test was used to compare data from women with uRPL or UI with data from fertile control patients. Median values were calculated. Statistical significance was set at $P<0.05$.

Results

Clinical characteristics of study women

Sixty-one women were included in the study. Twenty-one had uRPL, 30 had UI, whereas 10 had no history of infertility, recurrent pregnancy loss, or endometriosis (controls). Endometriosis was diagnosed in 36 sub-fertile women by laparoscopic procedure, specifically in 13 uRPL and 23 UI women. The clinical characteristics of study women were reported in Table 1. No significant differences were found in the age or BMI of women between the groups.

Percentage of CD16, CD56, or NKp46 positive cells in the endometrium

The expression of CD16, CD56 or NKp46 in the endometrium was evaluated in women with uRPL or UI with or without concomitant endometriosis, and fertile women using immunohistochemistry. Representative staining is shown in Figures 1, 2, and 3. Following

quantification, the average percent of endometrial stroma $CD16^+$ cells was found to be significantly higher in women with uRPL (7.9±3.2, p=0.03) and women with UI (9.0±5.5, p=0.02) compared to fertile women (5.6±2.4) (Figure 1). In women with endometriosis together with either uRPL or UI, the average percent of $CD16^+$ cells was also significantly higher than in fertile women (8.9±5.3 vs 5.6±2.4, p=0.01). No significant difference was found between the average percent of $CD16^+$ cells in women with uRPL compared to women with UI.

The average percent of $CD56^+$ cells in the endometrial stroma was trending lower, but not significant, in women with uRPL (18.3±14.6), women with UI (18.4±16.4) and women with endometriosis and UI or uRPL (18.4±15.4) compared to fertile women (22.3±19.9) (Figure 2). Conversely, the average percent of $NKp46^+$ cells in the stroma was significantly higher in women with uRPL (3.4±2.6, p=0.03) compared to fertile women (1.9±1.8) (Figure 3). No significant difference was found between the average percent of $NKp46^+$ cells in women with UI (2.7±2.7) and women with endometriosis and either UI or uRPL (2.9±2.7) compared to fertile women.

The ratio of the percent $NKp46^+$ cells to the percent $CD56^+$ cells in the endometrial stroma was also calculated. In comparison to fertile women (0.1±0.1), the ratio of the percent $NKp46^+$:$CD56^+$ cells was found to be significantly higher in women with uRPL (0.28±0.25, p=0.005), women with UI (0.21±0.2, p=0.04), and women with endometriosis together with either uRPL or UI (0.19±0.14, p=0.02) (Figure 4).

Discussion

A central regulator of the endometrial immune response is the natural killer cell. Uterine NK cell phenotype and function differ from pNK cells; an increased intensity of CD56 (bright) and a decreased expression of CD16 antigen [3] define the uNK cells, and, unlike pNK cells, uNK cells are generally less cytotoxic [4]. uNK cells are the most abundant leukocytes in the human endometrium during the window of implantation and play a key role in the maintenance of pregnancy as a result of the unique pattern of immune and angiogenic factors released and due to their interactions with the trophoblast during the early gestation.

Our study has revealed that women with larger populations of cytotoxic $CD16^+$ uNK cells, in the absence of presence of endometriosis, may be considered at increased risk for UI and uRPL. This finding is consistent with an association between uNK derangements and infertility disorders postulated by previous studies. In fact, LaChapelle et al.[10] observed a predominant contingent of $CD16^+CD56^{dim}$ cells and a significantly decreased $CD16^-CD56^{bright}$ cell subset in women with RPL compared to controls, while Quenby et al. [11] reported an increased percentage of $CD4^+$, $CD14^+$,$CD16^+$, $CD56^+$ and MHC class II^+ cells in women with RPL. In addition, Fukui et al. [15] observed an increase of the percentage of $CD16^+CD56^{dim}$ cells and decrease in the percentage of $CD16^-CD56^{bright}$ cells in women with infertility who miscarried after IVF cycles compared with women who delivered. Most recently, Junovich et al.[18] reported an increased number of $CD16^+$ endometrial NK cells in patients with unexplained infertility that correlated with a decrease in endometrial IL-6 and VEGF levels. Therefore, it can be hypothesized that an increase in “activated” $CD16^+$ uNK

cells may create an unfavorable environment for implantation. The $CD16^+$ uNK cells may produce inflammatory cytokines and secrete cytotoxic factors in response to autologous endometrial cells and fetal trophoblast cells, both of which could contribute to infertility or spontaneous miscarriages.

Activity of both $CD16^+$ and $CD16^-$ NK cells is controlled by a combination of receptors, including the natural cytotoxicity receptor, NKp46. Our study suggests that having larger populations of uterine NK cells with NKp46, in the absence or presence of endometriosis, may also increase the risk of women experiencing uRPL and UI. Similarly, Zhang et al.[25] reported higher expression of NKp46 on decidual NK cells from patients undergoing spontaneous abortions. Engagement of NKp46 on decidual NK cells was observed by Hicham et al.[26] to increase cytotoxicity of decidual NK cells, and Yokota et al.[27] recently reported that NKp46 regulates cytokine production by endometrial NK cells. Thus, simlar to CD16, the association between NKp46 and reproductive failure may relate to the increased cytotoxic activity and dysregulation of cytokine production following engagement of NKp46 on uterine NK cells. Since NKp46 is also expressed by certain populations of T cells [28], it is possible that alterations in uterine T cells may also be contributing to reproductive failure and endometriosis in our patients, but that is outside the scope of the current study.

Contrary to previous data, our study suggests that the total number of $CD56^+$ cells does not seem to play a relevant role in distinguishing women with endometriosis with UI or uRPL from fertile women. Instead, the data reported above suggests the ratio between $CD56^+$ and $CD16^+$ cells and/or the ratio between $CD56^+$ and $NKp46^+$ cells, rather than the total number of uNK cells, may be more important for maintaining a supportive environment for the establishment of pregnancy. However, further investigation on larger populations of women is needed to confirm this result.

The association between endometriosis and changes in the eutopic endometrium has been recently reviewed [29]. Although not well studied, endometriosis may be an underlying cause of sub-fertility in both UI and uRPL by inducing changes in the eutopic endometrial milieu [23, 29, 30, 31]. In our prospectively obtained patient population, endometriosis was found in the majority of women with UI and uRPL. The findings presented in this analysis of uNK cells suggest that the difference between infertility and recurrent pregnancy loss might represent a continuum of changes related to inflammatory influences of endometriosis. While women with uRPL had higher levels of cytotoxic $CD16^+$ uNK cells compared to fertile women, the levels of $CD16^+$ cells was even higher in women with UI, although the increase was not statistically significant. A similar increase in dysfunction comparing uRPL and UI was also demonstrated in a recent analysis of these samples for $\alpha v\beta 3$ integrin expression (Holoch et al. Hum Reprod, submitted 2013).

In conclusion, the results from our study support an association between the “activation” status of uterine NK cells and an increased risk of reproductive failure in patients with and without endometriosis. The increased CD16 and NKp46 recorded in patients with recurrent pregnancy loss and patients with unexplained infertililty suggest that alterations in the functions of uterine NK cells may occur either before implantation or during

decidualization. In agreement with the recent reports by Seshadri et al.[32] and Polanski et al.[33], the complex nature of uterine NK cells requires additional studies to determine which measurements of uterine NK cell populations and/or functions can accurately predict the risk of reproductive disorders in future patients, and also assist in determining the proper treatment for those patients.

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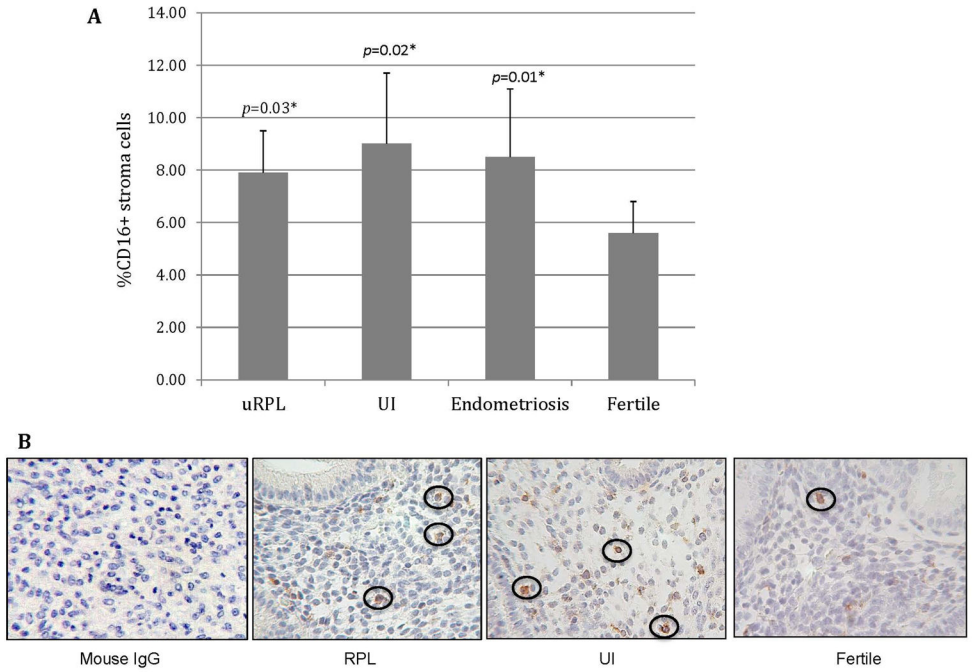

Figure 1.

(A) Percentage of $CD16^+$ cells in endometrial stroma of women with unexplained recurrent pregnancy loss (uRPL), unexplained infertility (UI) or with endometriosis associated with either UI or uRPL, and in fertile women. **(B)** CD16 expression in the endometrium of women with unexplained recurrent pregnancy loss (uRPL) or unexplained infertility (UI), and fertile women. Staining with Mouse IgG also shown for negative control. $CD16^+$ cells are circled. (400x magnification).

*CD16 expression was significantly higher in women with uRPL (p=0.03), UI (p=0.01), or endometriosis (p=0.01) compared to fertile women.

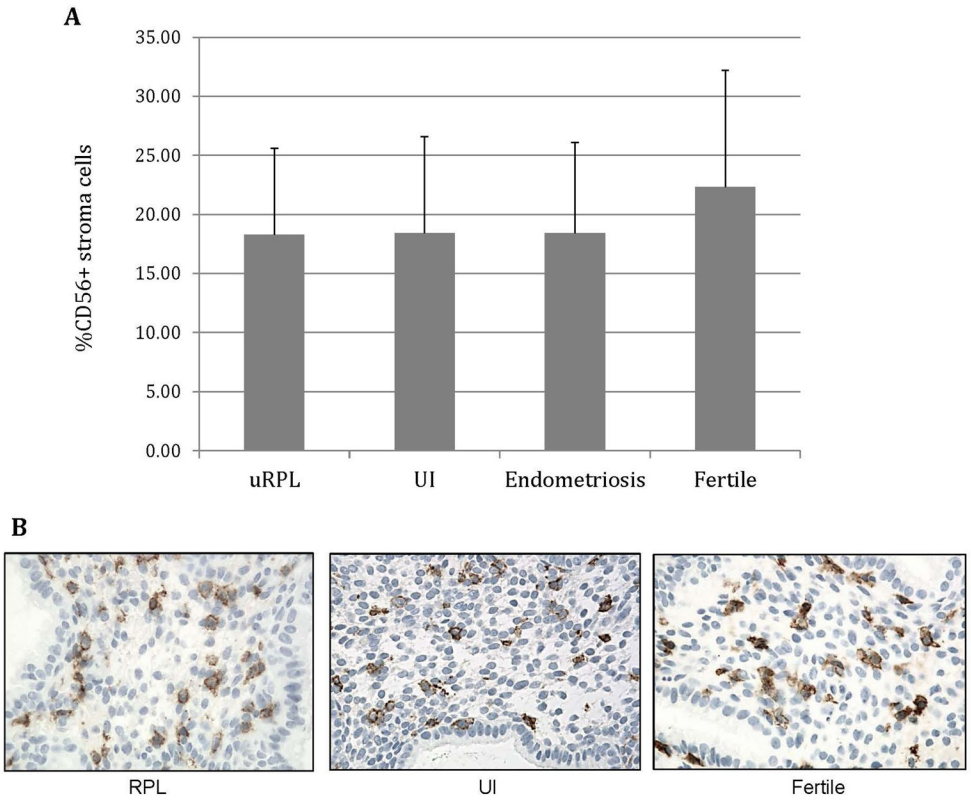

Figure 2.

(A) Percentage of $CD56^{+}$ cells in endometrial stroma of women with unexplained recurrent pregnancy loss (uRPL), unexplained infertility (UI) or with endometriosis associated with either UI or uRPL, and in fertile women. **(B)** CD56 expression in the endometrium of women with unexplained recurrent pregnancy loss (uRPL) or unexplained infertility (UI), and fertile women. (400x magnification).

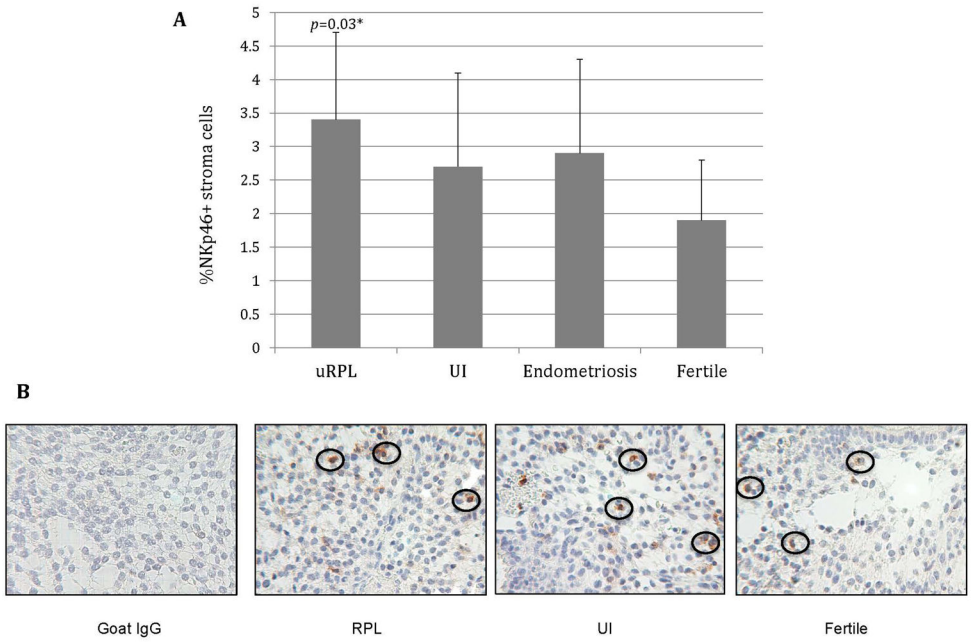

Figure 3.

(A) Percentage of NKp46^{+} cells in endometrial stroma of women with unexplained recurrent pregnancy loss (uRPL), unexplained infertility (UI) or with endometriosis associated with either UI or uRPL, and in fertile women. **(B)** NKp46 expression in the endometrium of women with unexplained recurrent pregnancy loss (uRPL) or unexplained infertility (UI), and fertile women. Staining with Goat IgG also shown for negative control. NKp46^{+} cells are circled. (400x magnification).

*NKp46 expression was significantly higher in women with uRPL (p=0.03) compared to fertile women.

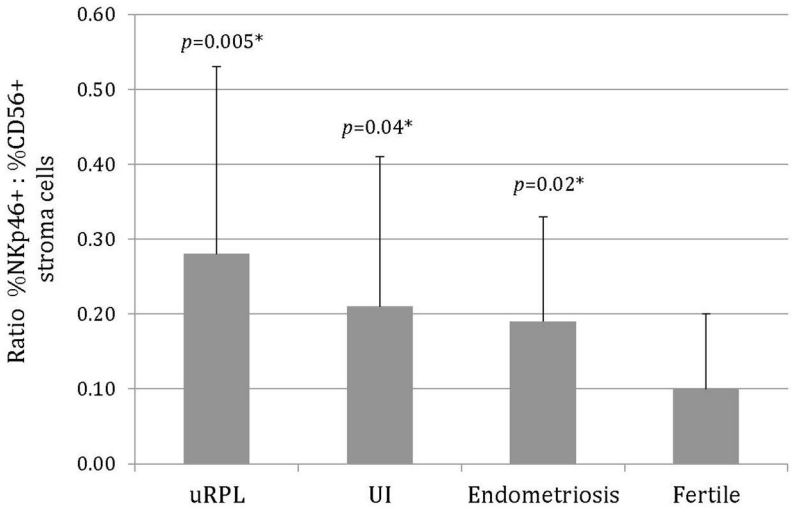

Figure 4.

Ratio of $NKp46^+$:$CD56^+$ cells in endometrial stroma of women with unexplained recurrent pregnancy loss (uRPL), unexplained infertility (UI) or with endometriosis associated with either UI or uRPL, and in fertile women.

*Ratio of $NKp46^+$:$CD56^+$ cells was significantly higher in women with uRPL (p=0.005), UI (p=0.04), or endometriosis (p=0.02) compared to fertile women.

Table 1

Clinical profile of women enrolled in the study.

	uRPL	UI	Fertile	P*
Women (n)	21	30	10	-
Women with Endometriosis (n)	13	23	-	-
Age (years)	34.2±3.0	31.9±4.3	30.4±8.0	0.07
Body mass index (Kg/m^2)	26.3±7.3	23.4±6.9	22.2±1.4	0.14
Gravidity (n)	4.8±2.0	0.2±0.4	1.2±1.7	<0.05
Miscarriages (n)	4.2±1.9	-	-	-

Data are expressed as number (n) or mean ± standard deviation.

uRPL, unexplained recurrent pregnancy loss; UI, unexplained infertility.

*T-test between study groups.