ORIGINAL ARTICLE

Ovarian Stimulation Affects the Levels of Regulatory Endometrial NK Cells and Angiogenic Cytokine VEGF

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Problem
Endometrial NK cells play a critical role in uterine vascularization producing angiogenic factors. Impact of ovarian stimulation on endometrial expression of NK cells and VEGF in normal fertile oocyte donors and the effect of endometrial injury treatment on these parameters have been investigated.

Method of study
Endometrial tissue was obtained from oocyte donors during natural and stimulated cycles. NK cell subsets were measured by flow cytometry. VEGF was determined by ELISA and flow cytometry. Endometrial angiogenic parameters were determined by ultrasound Doppler. Local injury was induced by scratching endometrial tissue previous to implantation window.

Results
Ovarian stimulation decreased endometrial levels of NK cells and vascularization index but increased VEGF levels. Local injury normalized only the $CD56^{+}$ NK cell count.

Conclusion
Hormonal therapy for ovarian stimulation may be associated with poor endometrial vascularization. Local injury before the implantation window seems not to influence endometrial angiogenic parameters altered by ovarian stimulation.

Introduction

Endometrial cytokines and Natural Killer (NK) cells seem to be involved in embryo receptivity and vascularization of the endometrium during implantation window.[1,2] Based on the intensity of CD56 expression, NK cells can be divided into two subpopulations.[3,4] Most of NK cells present in the endometrium at the time of implantation are $CD56^{bright}$ and express low levels or no CD16 (regulatory NK cells). They represent the main source of NK cell-derived immunoregulatory cytokines,[5] such as vascular endothelial growth factor (VEGF), suggesting a possible role for these cells in inducing specifically localized angiogenesis.[6] Moreover, VEGF may be involved in the recruitment of peripheral NK cells to the uterus.[1]

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It is known that female sex steroids regulate the number of endometrial NK cells during menstrual cycle and pregnancy.[1] The association of falling progesterone levels with the death of endometrial NK cells strongly suggests a progesterone-induced modulation.[7] For a long time, it has been hypothesized that gonadotrophins and gonadotropin-releasing hormone (GnRH) analogs used to induce multifollicular development might also affect endometrial receptivity, either directly or indirectly.[8] Clinical studies suggest that patients with a high gonadotrophin response exhibit embryonic implantation failure associated with supraphysiological levels of estradiol (E_2).[9] Low levels of exogenous E_2 could maintain the window of receptivity for an extended period of time, whereas high doses of E_2 can rapidly initiate a refractory state. In summary, levels of E_2 seem to determine the duration of implantation window.[10]

Local injury produced by endometrial biopsy increases cytokine secretion and endometrial cell growth simultaneously with a magnified chance of pregnancy in the subsequent cycle.[11]

Therefore, the goal of this work was to evaluate the effect of ovarian stimulation and local injury on endometrial VEGF levels and NK cells during implantation window.

Materials and methods

Patients

This work was conducted according to the Declaration of Helsinki for Medical Research, with appropriate ethical approval by CECIC (Ethics Committee of Cer Institute, Buenos Aires, Argentina). Endometrial biopsies were obtained from fertile oocyte donors with regular menstrual cycle who voluntarily participated in this study after signing informed consent. The inclusion criteria were age 21–32 years, proven fertility (at least one live birth), BMI ≤ 26, normal hormone levels on day 2–3 of the cycle (FSH-LH-E2), previous stimulation cycles with mature oocytes (MII) ≥ 12, and at least two good-quality embryos.

Exclusion criteria were endometritis, endometriosis, anatomical uterine pathologies.

Study Groups

Fertile oocyte donors were studied during one cycle randomized using a sealed envelope system into four different groups: natural or stimulated (controlled ovarian stimulation), with or without local injury induction by an endometrial scratching. Ten oocyte donors were included in each group.

Ovarian Stimulation Protocol

A conventional long-term GnRH agonist protocol (Lupron, 0.5 mL/day, Abbott, Buenos Aires, Argentina) was used in all cases. Administration of recombinant follicle-stimulating hormone (FSH; Puregon, Organon, Buenos Aires, Argentina) was started at day 2 of the menstrual cycle with 250 IU/day, maintained until day 6, and continued with 150 IU/day in combination with 75 IU/day of human menopausal gonadotropin (hMG; Menopur, Ferring, Buenos Aires, Argentina) until pre-ovulatory administration of human Choriogonadotropin (hCG; Pregnyl, Organon, Buenos Aires, Argentina). In the following sections of this manuscript, for simplification, this stimulation procedure will be called 'ovarian stimulation'.

Endometrial Injury

Endometrial scratching has been performed by using standard biopsy catheters (Cornier Pipelle, CCD Laboratories, Paris, France). This procedure has been carried out prior to implantation window (day 13 of the cycle) to induce inflammatory process based on the previous study.[11]

Progesterone and Estradiol Measurement

Serum progesterone and estradiol were quantified by radioimmunoassays (RIA Coat a Count; Siemens, LA, USA). Analytical sensitivity was 0.02 ng/mL and 15 pg/mL, respectively.

Tissue Samples

Endometrial tissue samples were obtained by performing biopsies using Cornier Pipelle catheters during the implantation window of the menstrual cycle (5–8 days after ovulation determined by the presence of at least one follicle > 18 mm observed by ultrasound scan). Samples were placed into sterile RPMI 1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% Fetal Calf Serum (FCS, GIBCO) and 1% of penicillin/streptomycin (GIBCO).

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Endometrial Cell Suspension

Endometrial cells were prepared for subsequent analyses. Briefly, samples were disrupted mechanically with a round pestle tissue grinder. The cell suspension was filtered through a 100-μm-pore-size sieve and incubated for 5 min with NH_4Cl/trisHCl buffer to deplete red blood cells.

Peripheral Leukocyte Separation

Blood samples have been taken from all patients the same day as endometrial biopsy. Lymphocytes have been isolated by a Histopaque®-1119 density gradient according to the protocol provided by the manufacturer (SIGMA-ALDRICH, St Louis, MO, USA).

Flow Cytometry

The following monoclonal antibodies (mAbs) conjugated with FITC, PE or PECy5 were used to analyze the phenotype of leukocytes: anti-CD3 (PECy5), anti-CD16 (FITC), and anti-CD56 (PE; all antibodies from Immunotools, Friesoythe, Germany). A total of 1×10^6 cells were incubated with 2.5 μL antibody solution for 30 min at room temperature. Cells were washed and fixed with 4% paraformaldehyde. Immunofluorescence reactivity was assessed at an Ortho Cytron Absolute Cytometer (Ortho Diagnostic, Raritan, NJ, USA).

For isotype controls, the respective murine immunoglobulin isoforms conjugated with FITC, PE (both Immunotools), and PECy5 (BD, NJ, USA) were used.

Analysis of NK Cell Subsets

Percentage of total circulating CD56 + CD3−, $CD56^{dim}CD16+$, and $CD56^{bright}CD16-$ cells were obtained after flow cytometry analysis of a fixed total count of 3×10^5 total peripheral blood mononuclear cells (PBMC). A tight gate was drawn in the lymphocyte region of PBMC, and this was sent to a two-parameter density plot graph of CD56/CD3, in which the percentage of CD56 + CD3− was obtained in the upper left quadrant. In the same way, this quadrant was sent to a two-parameter graph of CD56/CD16, in which the percentage of $CD56^{dim}CD16+$ and $CD56^{bright}CD16-$ cells was calculated.

Percentages of respective endometrial cell populations were obtained from analysis of a fixed total count of 1×10^6 endometrial cells per sample as described earlier.

Intracellular VEGF Staining

Intracellular VEGF levels were determined after stimulation of endometrial and peripheral cell suspension with phorbol 12-myristate 13-acetate (PMA; 10 ng/mL), ionomycin (1 μM) and Brefeldin A (10 μg/mL; all Sigma, St Louis, MO, USA) during 4 hr. Cells were washed with phosphate buffer solution (PBS) and fixed in 4% paraformaldehyde (in PBS supplemented with 2% FCS), for 20 min at room temperature. Fixed cells were permeabilized with 0.5% (w/v) saponin (Sigma) in PBS for 30 min at room temperature. Permeabilized cells were incubated with 2.5 μL mouse-anti-human VEGF (IgM) and 2.5 μL goat-anti-mouse IgM-FITC (both from Immunotools) for 30 min at room temperature.

Cell Culture Supernatants

Endometrial and peripheral cells (4×10^5 cells/150 μL) were cultivated in 96-well tissue culture plates using Roswell Park Memorial Institute medium (RPMI 1640, GIBCO, Carlsbad, CA, USA), supplemented with 10% of fetal bovine serum (SFB,GIBCO) and 1% of penicillin/streptomycin (GIBCO). Incubation was performed at 5% CO_2 humid atmosphere at 37°C. After 48 hr, supernatants were collected, centrifuged first at $2000 \times g$ and then at $8000 \times g$, and stored at −70°C for cytokine analysis.

VEGF Quantification by ELISA

Levels of soluble total VEGF produced by endometrial and PMBC cells were determined by a double antibody-sandwich ELISA method[12] by using a commercially available kit (R&D, Minneápolis, MN, USA). Briefly, 96-well plates were coated with anti-cytokine monoclonal antibody and kept overnight at room temperature (RT). After successive washes in phosphate buffer solution (PBS) (0.01 M PO_4H-Na_2/PO_4HNa y 0.15 M ClNa) pH 7.4, free sites were blocked with 1% bovine serum albumin (BSA, SIGMA, St Louis, MO, USA) in PBS and incubated at RT for 1 hr. Washes were repeated with PBS supplemented with 0.05% Tween-20 (tween, Anedra, San Fernando, Argentina). Samples were cultured

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for 2 hr at RT. After successive washes, the biotinylated detection antibody was cultured at RT for 1 hr. A new wash cycle was followed by the addition of horseradish peroxidase (HRP) with streptavidine for 20 min at RT. Color reaction was developed by 3, 3′, 5,5′-tetramethylbenzidine (TMB, MP Biomedicals, OH, USA) and stopped with 1 M solution H_2SO_4. Optical density was measured at 450 nm using an automatic plate reader (Metertech, Austria.). Levels of VEGF (pg/mL) were calculated by linear regression using a standard curve constructed with recombinant human VEGF provided by the manufacturer.

Ultrasound Assessment

Endometrial images of the endometrium were obtained immediately before endometrial biopsy using ultrasound equipment Voluson Expert (General Electric Healthcare, USA) and a 7.5–10 MHz transvaginal probe. The uterus was examined systematically in sagittal direction. Endometrial thickness was defined as the maximum distance between the echogenic interfaces of myometrium and endometrium, measured in the plane through the central longitudinal axis of the uterus body. Endometrial thickness of more than 7 mm was considered normal. Endometrial vascularization was assessed by using Voluson Expert in combination with 3D Power-Doppler software with VOCAL method (General Electric Healthcare). Three vascular indices were calculated: the vascularization index (VI) is the ratio of color voxels to all voxels in the region of interest, expressed as a percentage, it reflects the blood vessel density within the tissue; the flow index (FI) is the mean intensity of all the power Doppler voxels in the volume analyzed, and it represents the energy reflected from the blood corpuscules in the vessels of the volume, it reflex blood flow density; and the vascular flow index (VFI) is the sum of the weighted color voxels divided by all voxels in the region of interest, which indicates both blood flow and vascularization.[13,14]

Statistical Analysis

One-way analysis of variance (ANOVA) and Tukey post-test were applied to test intergroup differences in total and regulatory NK cells. Student's *t*-test was used for all of the rest parameters.

Results

Progesterone and Estradiol Serum Levels

Serum levels of progesterone and estradiol were compared in fertile women during natural and stimulated cycles. The ovarian stimulation significantly induces elevated serum levels of both estradiol and progesterone (Fig. 1).

Effect of Ovarian Stimulation and Local Injury on the Balance of NK Cells

Ovarian stimulation significantly decreases the number of both total and regulatory NK cells in the endometrium during the implantation window (Fig. 2). It does not affect the peripheral levels of NK cells ($P > 0.05$, data not shown).

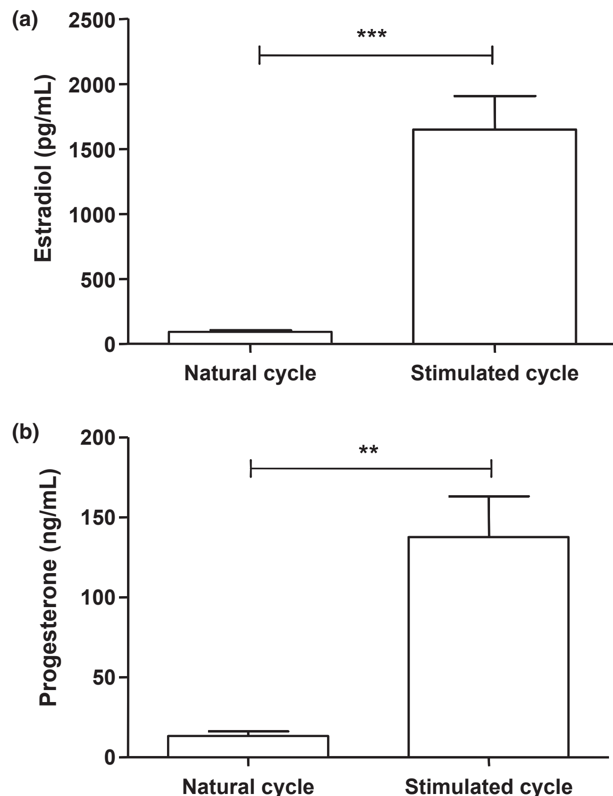

Fig. 1 Estradiol (a) and progesterone (b) levels measured by radioimmunoassays in endometrial cells from fertile oocyte donors in natural and ovarian stimulated cycles. Error bars indicate standard error. $^{**}P < 0.005$; $^{***}P < 0.001$ (*t* test); $n = 10$ patients per group.

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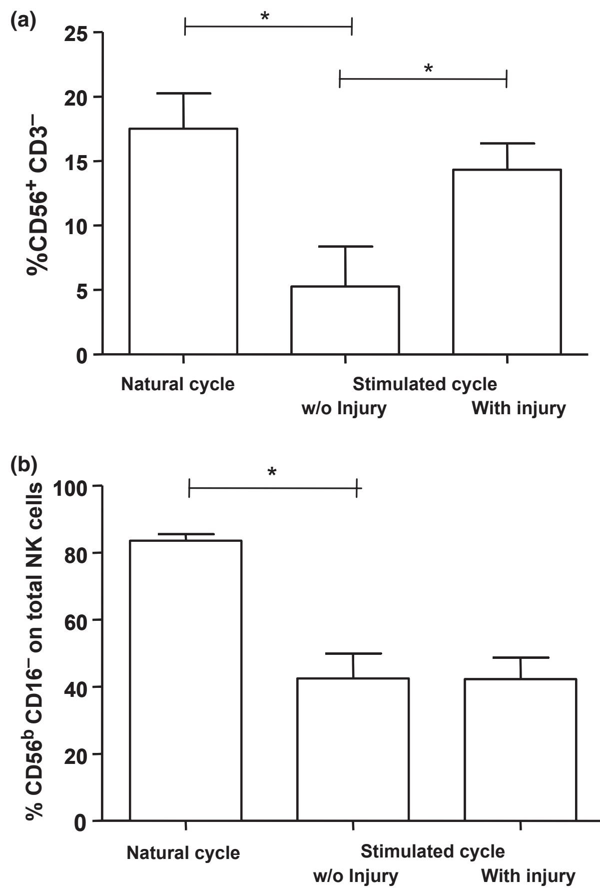

Fig. 2 Percentage of total NK cells (CD56 + CD3−) on all endometrial cells (a) and percentage of CD56^{bright} CD16^{-} on total NK cells (b) as assessed by flow cytometry in endometrial biopsies from fertile oocyte donors in natural and ovarian stimulated cycles with and without local injury treatment. *$P < 0.05$ (ANOVA and Tukey); $n = 10$ patients per group.

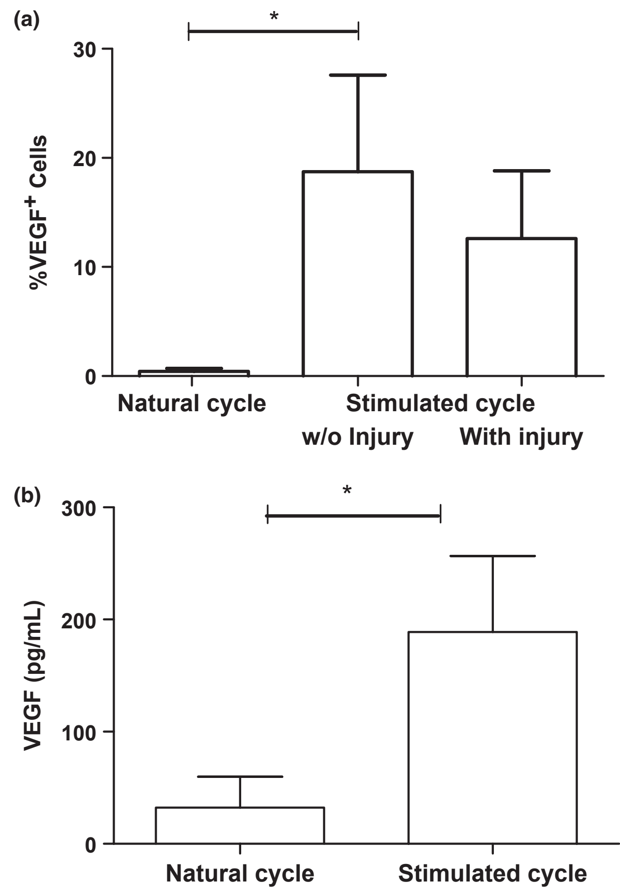

Fig. 3 (a) Percentage of VEGF positive cells from total endometrial cells derived from biopsies from fertile oocyte donors during natural or stimulated cycles, with or without local injury. They have been stimulated with PMA, ionomycin and brefeldin A. After permeabilization of membranes, intracellular VEGF has been analyzed by flow cytometry. *$P < 0.05$ (ANOVA and Tukey); $n = 10$ patients per group. (b) Expression of VEGF (pg/mL) determined by ELISA in culture supernatants from total endometrial cells derived from biopsies from fertile oocyte donors during natural or stimulated cycles.

Local injury treatment did not affect the peripheral or endometrial NK cell counts in natural cycles ($P > 0.05$, data not shown).

However, it recuperates the total NK cell number after stimulation (Fig. 2).

Effect of Ovarian Stimulation and Local Injury on VEGF Levels

The production of intracellular and soluble VEGF by total endometrial cells significantly increases after ovarian stimulation. Local injury treatment reduced this effect without statistical significance (Fig. 3). This treatment does not affect VEGF expression in natural cycles ($P = 0.75$, data not shown).

On the other hand, VEGF expression by PBMCs has not been affected by ovarian stimulation or local injury (data not shown).

Effect of Ovarian Stimulation and Local Injury on Endometrial Ultrasound-Doppler Parameters

All analyzed uteri show normal thickness (data not shown). Ovarian stimulation significantly reduces the vascularization index assessed by spiral artery Doppler during the implantation window (Fig. 4a). Local injury was not able to compensate for this deleterious effect (Fig. 4b). On the other hand, flow

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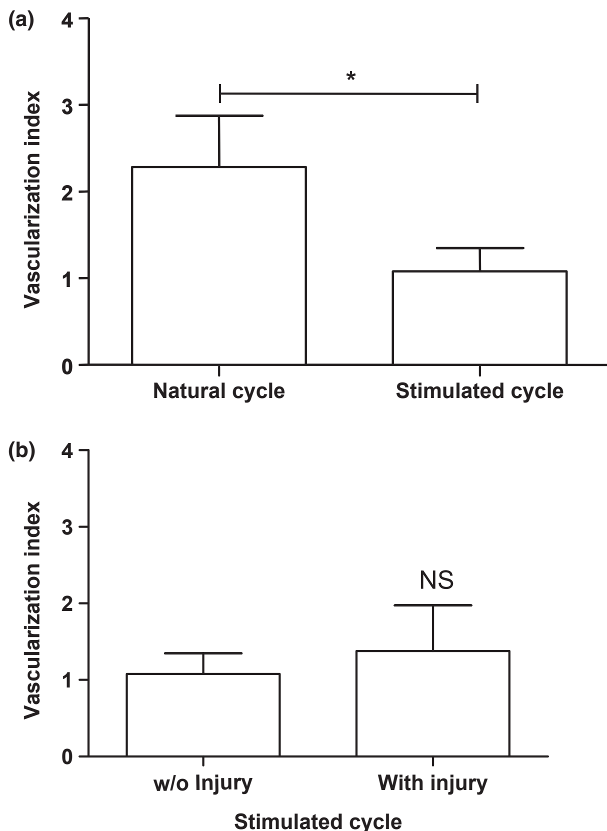

Fig. 4 Vascularization index of the endometrium measured by Doppler ultrasound in the uteri of fertile oocyte donors in (a) natural versus ovarian stimulated cycles and (b) stimulated cycle with or without local injury. $^{*}P < 0.05$ (*t* test); $n = 10$ patients per group. $^{ns}P > 0.05$(t test); $n = 10$ patients per group.

index and vascular flow index remain stable after treatments (data not shown).

Discussion

A pre-condition for successful establishment of implantation is the coordinated process of endometrial decidualization. Human endometrium has varying angiogenic potential throughout the stages of menstrual cycle. Elongation of existing vessels during the early proliferative phase as well as growth and coiling of the spiral vessels during the secretory phase demand far higher angiogenic activity than outgrowth and maintenance of vessels during the late proliferative phase. Despite this important angiogenic activity, the mechanism exerted by angiogenic factors such us VEGF in this process is still unclear.[15]

Taking into account that regulatory NK cells ($CD56^{bright}CD16-$) surround spiral arteries and glands during the late secretory phase,[16] we postulate that this perivascular distribution may be associated with angiogenic regulation. Moreover, some authors hypothesize that regulatory NK cells are the principal source of VEGF in the endometrium.[1]

Normal pregnancy levels of progesterone decrease the number of peripheral (cytotoxic) NK cells but facilitate NK cell homing into the endometrium.[1] VEGF production by endometrial cells during menstrual cycle depends on estradiol levels.[17] Because ovarian stimulation characteristics observed in this study show a significant increase in progesterone and estradiol serum levels (Fig. 1), we decided to study the effect of ovarian stimulation on the proportion of total and regulatory endometrial NK cells, VEGF levels, and endometrial angiogenic parameters obtained by Doppler.

The deficient levels of total and regulatory endometrial NK cells observed in this study during implantation window of ovarian stimulated cycles may be related to the implantation failure as previously described in ovarian stimulated patients with high estradiol serum levels.[18] Moreover, ovarian stimulation showed an inhibiting effect on the endometrial vascularization index during the implantation window, although the VEGF expression was found vigorously elevated in these women. This effect could be explained by the concept that estradiol and progesterone may modulate vascular permeability necessary for implantation by its induction of VEGF expression.[17,19] It is necessary to consider that NK cells seem not to be the only source of endometrial VEGF. Actually, it has been published that endometrial NK cells produce mainly VEGF-C.[20] Therefore, the expression of this isoform should be included in further studies conducted in different reproductive pathologies such us implantation failure.

This study is focused in the maternal side; however, the dialog with embryonic cytokines should also be considered. In this sense, we recently published that mRNA–VEGF silencing decreases the invasion capacity of trophoblastic cells.[21]

Some authors consider that local injuries induced by biopsy catheters increase the chances of implantation at the following cycle.[11,22] This effect may be related to inflammatory cytokine secretion that magnifies endometrial cell growth as well as the chance of pregnancy after the succeeding cycle.[11]

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Encouraged through these reports, we studied the effect of a preceding endometrial biopsy (injury) during the ovulation period on different angiogenic parameters. We observed that this procedure reverses the decreased number of total uterine NK cells after ovarian stimulation, even there remains an imbalance of the ratio between regulatory and cytotoxic endometrial NK cells.

We conclude that hormonal therapy for ovarian stimulation may be associated with poor endometrial vascularization. This effect might be provoked by decreased NK cell homing, by inhibition of switching from cytotoxic to regulatory NK cells or by reduced endometrial NK cell predecessor proliferation. Setting a local injury before the implantation window seems not to influence endometrial angiogenic parameters altered by ovarian stimulation.

Further studies are being conducted to investigate these parameters in different reproductive pathologies such us implantation failure or endometriosis.

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