Ulcova-Gallova Z, et al., J Clin Immunol Immunother 2019, 5: 013
DOI: 10.24966/CIIT-8844/1000013

HSOA Journal of Clinical Immunology and Immunotherapy

Case Report

Recurrent Pregnancy Loss in Two Patients with High Density of NK Cells CD56+ and CD16+ in Ovulatory Cervical Mucus and in Endometrium (Case Report)

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Abstract

We describe two patients who suffered from multiple repeated pregnancy losses and with normal hormonal status, humoral autoimmune responses to the panel of phospholipids, and normal karyotyping, without pathological trombophilia. Patients were without any clinical and laboratory symptoms of vaginitis at the time of ovulatory cervical mucus (OCM) sampling and endometrium study. Blood NK cells were studied by flow cytometry, uterine NK cells CD56+ , CD16+ and NK cellsin OCM were identified by immunocytochemistry, aPls by ELISA, indirect immunofluorescent method for detection of serumand OCM IgM, IgG antibodies against HHV-6 levels at the time of ovulation. All findings were normal except to high density of NK cells in OCM and in endometrium. Intramuscular immunoglobulins in special scheme was used for the treatment. Both patients became successfully pregnant, and delivered healthy babies.

Keywords: Missed abortion; NK cells; Endometrium; Ovulatory cervical mucus; Immunoglobulins

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Citation: Ulcova-Gallova Z, Mukensnabl P, Losan P (2019) Recurrent Pregnancy Loss in Two Patients with High Density of NK Cells CD56+ and CD16+ in Ovulatory Cervical Mucus and in Endometrium (Case Report). J Clin Immunol Immunother 5: 013.

Received: August 16, 2019; **Accepted:** August 27, 2019; **Published:** Sepetember 04, 2019

Introduction

Recurrent pregnancy loss (RPL) is associated with chromosomal, hormonal, uterine abnormalities, immunological disorders but also with infectious agents, and various enviromental factors that could play a serious problem in fertility failure. Autoimmune dysfunction representing antiphospholipid antibodies or natural killer cells dysbalance between mother and early embryo are involved in the pathogenesis of recurrent RPL too.

NK cells represent 15% of peripheral blood lymphocytes. The uterine natural killer cells (uNK) play the major immune role during implantation. The major leucocyte concentration is during the first trimester of pregnancy. The NK cells are localized to the place of embryo implantation. Decidual NK CD56+ produce the cytokines involved in trophoblast invasion, growth and angiogenesis. However, thealtered numbers of endometrial NK cells lead to pregnancy pathogenesis such as pregnancy loss owing to the increased of embryocytotoxic cytokines reducing angiogenesis, as well as trophoblast and vascular growths [1-3].

We refer two infertile women with 4-6 missed abortions with only high density of NK cells in ovulatory cervical mucus (OCM) and endometrial tissue. The investigation of NK cellsin OCM have never been studied before. We begin with our detailed diagnostic possibilities, continue with the treatment, and with the final results.

Methods

Routine examination of our two infertile women confirmed normal anatomy of their uterus, regular menstrual cycle and ovulation. Any evidence of vaginal and cervical cultures for mycoplasma, chlamydia, trichomoniasis, yeast, and HHV-6 infection was not found. Serum antisperm antibodies, thyroid, anti-zona pellucida , and panel of antiphospholipid antibodies were also negative. We also excluded clinical and laboratory symptoms of vaginitis, antiphospholipid syndrome, and congenital trombophylic factors. We focused on their anamnestical basic data (e.g. nicotinism, the length of their hormonal contraception before decision to conceive, stress). Stress factor can not be completely eliminated. Their male partners are healthy men with normal spermiogrammes. Our two patients were completely healthy at the time of OCM sampling, and endometrium examination.

Our two refered women, ZB and SP gave a written informed concent before publication oftheir cases. They had normal findings of blood NK cells investigated by flow cytometry [4,5]. We focused on the density of NK cells CD56+ and CD16+ in OCM (never been studied before), and then in secretory endometrium. We also took blood and OCM samples for immunofluorescent identification of the IgM, IgG antibodies against HHV-6 to exclude viral infection.

Collection of OCM

The ovulatory phaseis routinely established by pelvic ultrasonography

The conditions for OCM taking indays 12-14 of menstrual cycle are: Protective intercourse by condom to avoid semen contamination

from the last day of menstruation to patient´s examination. During routine gynecological examination without lubricant, the ectocervix is gently wiped and cleaned of vaginal debris with a sterile swab. Endocervical mucus is carefully aspirated with a special syringe (Fortuna Optima, Denmark) directly into sterile glass capillaries, immediately spread on to glass slides and fixed for the next examination. Blood collection was performed at thetime of ovulation, both sera were screened for the aPLs (classical ELISA).

Antibodies against HHV-6

The indirect immunofluorescent method was used for identification of IgM or IgG HHV-6 infection in serum, and in OCM by IF Viditest (fy Vidia) using FITS conjugat anti IgG and FITS conjugat anti IgM. Antibodies IgG are evaluated in titre 1:10 and 1:40, for IgM in 1:10.

The ELISA method was used for detection of sperm, zona pellucida, panel of antiphospholipid antibodies.

Examination of NKcells in endometrium and OCM

The endometrium was obtained by hysteroscopy performed on the 22nd-24th day of the menstrual cycle. We use formol fixed paraffin embedded biopsies to do a conventional and routine anti-CD56+ and anti CD16+ staining (Human CD56, Anti Human CD16, DAKO, Leica Microsystems GmbH, UK) for determination of lymphocytes CD56+ and CD16+. Then we use a microscope (Olympus, magnification 10x20) which allows us to count and calculate cells per mm^2 surface for OCM and endometrial tissue. We consider more than 20% of positively labelled cells into OCM, and more than 50% of labelled cellsin endometrium to bepositive, such as pathological indicator.

Treatment with intramuscular immunoglobulins

We developed a special scheme of immunoglobulin treatment for patients with pathological findings of NK cells in endometrium (Scheme 1). During this treatment, patients take low-molecular-weight-heparins (the duration of this treatment depends on the current hematological outcomes). We also add gestagens, and vitamins group B.

Patients

ZB

A 36-year-old patient(ZB), gravida IV, para 0 was sent to the Division of Infertility and Immunology of Reproduction at Gennetics Institute, Pilsen in 2017 because of her recurrent pregnancy losses. The patient's childhood development was normal, with common pediatric infectious disease. She works as a teacher, she does not smoke. She has a slight pollen alergy. Sheused 15 years hormonal contraception (HC) before conception. ZB has been pregnant four times (four RPL). Anomalies of her uterus was excluded.

Laboratory findings showed the absence of antibody to sperm, and zona pellucida, to panel of eighteen various phospholipids. The levels of ANA, C3, C4, ANCA were normal. Thyroid and celiac disease, genetic factors (factor V 1691 G ≥ A (Leiden mutation), FII 20210 G ≥ A mutation, and MTHFR 1298 A ≥ C variants) were not present. OCM was normal, with the absence of sperm and HHV-6 antibodies.

Her husband displayed a normal spermiogram with no sperm antibodies, and with normal intra-acrosomal enzymes examined by monoclonal antibodies. No chromosomal abnormalities were detected in either partner. Both partners have normal karyotypes: 46, XX and 46, XY.

Density of NK CD 56+ and CD 16+ was high in OCM, and in endometrium.

Due to the high density of local NK cells, ZB was treated by intramuscular immunoglobulins according to the scheme (Table 1), and the combined treatment of LWMH, folic acid, pyridoxine, and gestagens immediately after positivity of HCG of her fifth pregnancy which was all the time normal.

	ZB	SP
Age (years)	36	34
Menstrual cycle	regular	regular
Number of missed abortion	4	6
Stress	none	sometimes
Allergy	polen	none
HC (years)	15	5
Genetics examination	normal	normal
Vaginal infection	none	none
NK cells-blood	normal number	normal number
NK cells-OCM	high density CD56+	high density CD56+
	lower density CD16+	lower density CD16+
NK cells-endometrium	high density CD 56+	high density CD56+
	lower density CD16+	high density CD16+
Treatment	IMIG, gestagens, vitamin B	IMIG, gestagens, vitamin B
Delivery	son, 3050g/52 cm	daughter, 3280g/50 cm

Table 1: Summarises all data about our two selected patients.

Abbreviations: RPL-repeated pregnancy loss, NK-nautral killer cells, HC hormonal contraception, IMIG-intramuscular immunoglobulin.

Result

She gave a spontaneous birth to her son (3050 g/52 cm).

SP

A 34-year-old patient(SP), gravida VI, para 0 was also sent to the Division of Infertility and Immunology of Reproduction at Genetics Institute, Pilsen in 2018/1 because of her recurrent pregnancy losses. Her family history and childhood were uneventful. She is secretary, with secondary education, with regular menstrual cycle, her lengt h of HC was 5 years, non-smoker. Her genetics, hematological, and immunological profiles were completely normal. The levels of ANA, ANCA and C3 and C4, were normal.

Blood, OCM and secretory endometrium were examined on the density of NK cells CD56+, and CD16+. We found normal NK in the blood, however, the cervical and endometrial density was high, primarily NK cells CD 56+. When she successfully conceived, she was also treated by low doses of gestagens, vitamins B, LMWH, andintramuscular immunoglobulins.

She gave a birth spontaneously her daughter (3280/50) atweek 40.

Discussion

Recurrent pregnancy loss effects up to 5% of couples. RPL includes genetic, endocrinological, immunological, anatomical, and enviromental causes. We start with an extesive history, gynecological, and immunological examination to identify the cause of pregnancy failure.

Recent studies [1-10] show an immunological association between mother and her foetus in a normal and pathological pregnancy, where the mother is exposed to a great number of autoimmune and alloimmune antigens which modulate her immune activities.

We examined NK cells CD56+ and CD16+ together in endometrium and OCM in patients with ovulation, and with RPL [11], but until now, we have found pathological density of NK cells only in two of them, excluding the influence of HHV-6 infection.

Previously we investigated [4] association between density of NK cells CD56+ and NK cells CD16+ in the blood and secretory endometrium in patients with unexplained recurrent miscarriage. We monitored their blood NK cells by flow cytometry and NK cells in endometrium by immunohistochemistry. The cellular immunity was focused on the identification of lymphocytes CD56+ and CD16+. NK cells CD56+ and NK cells CD16+ in the blood were in normal number in contrast with high density of above mentioned cells in endometrium. Only two refered patients had present NK cells in OCM, and in endometrium together,we excluded influence of HHV-6 infection.

We know that NK cells in the blood, decidua, and also in secretory endometrium are among the most important cells of our immune defense system [1,6,8,9]. Generally, NK cells are basic heterogenous representatives of cellular immunity, belonging to the lymphocyte family. They are characterized by natural cytotoxicity participating in anti-infectious immunity, producing cytotoxic cytokines or/- are activated by these cytokines. NK cells also participate in the destruction of tumorous tissue. In the immunology of reproduction, those cells carrying CD56+ and CD16+ are very important . The extent of cytotoxicity depends on the expression of these signs. NK cells have also a regulatory function, T-regulatory lymphocytes and dendritic cells are increased at the time of implantation and , their cytotoxicity can be increased incase of Th1 prevalence. According to the density of NK cells we could determine a high or small degree of their possible cytotoxicity. NK cells have numerous characteristics. We have selected those expressing CD56+ and CD16+, very important in reproduction. We detect them routinely in endometrium and in OCM.

Repeated miscarriage has multifactorial causes. We reported incidence of high density of NK cells in endometrium and OCM in our two infertile women. Immuno-histological findings comprise increased density of CD56+ and CD16+ . We demonstrated that application of lower dosages of immunoglobulin treatment was in both cases successfull. Detailed diagnosis improves the chances for a live birth.

It has been shown [1-3,5-7,10] that the imbalance in local cellular immunity can lead to an impairement of immunological tolerance, to anincrease of phagocytic activity, and Th1/Th17 rearrangement with increasing levels of embryocytotoxic cytokines, particulary of TNF-alpha and INF-gamma especially in women with repeated implant failure or early fetal loss.

If the dysregulation between NK cells and T-lymphocytes in endometrium is present, a cytotoxic environment occurs directly in the uterus, and this condition leads to impairement of proliferation and differentiation of the trophoblast, angiogenesis, and increased oxidative stress due to local ischemic changes. Repeated abnormalities of T-cells (increasing Th1 and Th17 responses and decreased Th2) are frequent in patients with pregnancy loss [10], who easily become pregnant, but their pregnancy terminates with RPL. We did not find a correlation between local and peripheral NK cells [4].

Little is known about NK cells CD56+ and CD16+ in OCM. High density of NK cells CD56+ and/or CD16+ in cervicalovulatory mucus and in endometrium in only two infertile women with RPL was found. It seems, that NK cells in OCM are very rare, but it is not clear if NK cells in OCM are fixed among secretory cells of the cervical glands and cervical crypts of the uterine cervix, or whether these cells migrate from endometrium in the OCM. Evidence of NK cells in the OCM suggests that the uterine cervix belongs to the active immunological organs of the female genital tract involvied in the local immune responses. The NK cells findings in this localization are very interesting for the future research. However, according to immunohistological examinations, NK cells also are present in cervical area but the question remains: are cervical NK cells fixed or migrating from the endometrium? Or, are cervical NK cells involved inlatent infection such as HHV-6 [10]? which was not proven in this work.

Conclusion

We presented two cases who suffered from RPL due to underlying local autoimmune cell reaction. Treatment of both patients was mainly based on immunomodulation (immunoglobulins), and anti-coagulation (LMWH), and has lend to successful births of healthy babies.

Scheme 1: Scheme of the treatment with intramuscular immunoglobulins (IMIG)

Igamplia (5ml, 800mg) devided in two doses is administered intramusculary to two different muscle sites(data, and medical doctor' s signature)

On the day of positive HCG

6th week of pregnancy

10th week of pregnancy

12th week of pregnancy

16th week of pregnancy

22th week of pregnancy

28th week of pregnancy

32th week of pregnancy-it is usually the last application of the Igamplia

After application of IMIG, our patients remain in the waiting room at least an hour and are monitored by medical personnel because of possible side effects.

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